EMOTIONAL MODULATION OF THE SYNAPSE

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EMOTIONAL MODULATION OF THE SYNAPSE

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Highly emotional events tend to be well remembered. The adaptive value in this is clear – those events that have a bearing on survival should be stored for future use as long-term memories whereas memories of inconsequential events would not as likely contribute to future survival. Enduring changes in the structure and function of synapses, neural circuitry, and ultimately behavior, can be modulated by highly aversive or rewarding experiences. In the last decade, the convergence of cellular, molecular, and systems neuroscience has produced new insights into the biological mechanisms that determine whether a memory will be stored for the long-term or lost forever. This Research Topic brings together leading experts, who work at multiple levels of analysis, to reveal recent discoveries and concepts regarding the synaptic mechanisms of consolidation and extinction of emotionally arousing memories.

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Table of Contents

05 ***Emotional modulation of synapses, circuits and memory***
Jonathan E. Ploski and Christa K. McIntyre

08 ***CREB regulates spine density of lateral amygdala neurons: implications for memory allocation***
Derya Sargin, Valentina Mercaldo, Adelaide P. Yiu, Gemma Higgs, Jin-Hee Han, Paul W. Frankland and Sheena A. Josselyn

17 ***Differential activation of amygdale* Arc *expression by positive and negatively valenced emotional learning conditions***
Erica J. Young and Cedric L. Williams

30 ***In sync: gamma oscillations and emotional memory***
Drew B. Headley and Denis Paré

42 ***Sleep and protein synthesis-dependent synaptic plasticity: impacts of sleep loss and stress***
Janne Grønli, Jonathan Soulé and Clive R. Bramham

60 ***Vagus nerve stimulation enhances extinction of conditioned fear and modulates plasticity in the pathway from the ventromedial prefrontal cortex to the amygdala***
David Frausto Peña, Jessica E. Childs, Shawn Willett, Analicia Vital, Christa K. McIntyre and Sven Kroener

68 ***Deep brain stimulation of the ventral striatum increases BDNF in the fear extinction circuit***
Fabricio H. Do-Monte, Jose Rodriguez-Romaguera, Luis E. Rosas-Vidal and Gregory J. Quirk

77 ***Appetitive behavioral traits and stimulus intensity influence maintenance of conditioned fear***
Megan E. Olshavsky, Carolyn E. Jones, Hongjoo J. Lee and Marie-H. Monfils

84 ***Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus***
Megan E. Olshavsky, Bryan J. Song, Daniel J. Powell, Carolyn E. Jones, Marie-H. Monfils and Hongjoo J. Lee

98 ***Mechanisms governing the reactivation-dependent destabilization of memories and their role in extinction***
Charlotte R. Flavell, Elliot A. Lambert, Boyer D. Winters and Timothy W. Bredy

109 ***A phosphodiesterase 4-controlled switch between memory extinction and strengthening in the hippocampus***
Rafael Roesler, Gustavo K. Reolon, Natasha Maurmann, Gilberto Schwartsmann, Nadja Schröder, Olavo B. Amaral, Samira Valvassori and João Quevedo

117 ***Memory formation for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex***
David S. Reis, Timothy J. Jarome and Fred J. Helmstetter

124 ***An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD)***
Andrea Berardi, Viviana Trezza, Maura Palmery, Luigia Trabace, Vincenzo Cuomo and Patrizia Campolongo

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Emotional modulation of synapses, circuits and memory

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Keywords: amygdala, fear conditioning, extinction, reconsolidation, oscillations, synapse allocation

Emotion is a powerful tool for change in the central nervous system. Whereas, most long-term memories are stored after practice or rehearsal, an emotionally arousing memory can be consolidated after a single experience. Research examining the influence of emotion on synaptic function provides a window of opportunity for exploring the mechanisms of memory consolidation during the minutes to hours after a single, well-remembered experience. It can also shed light on numerous psychiatric conditions, bringing the field closer to preventing or treating those conditions that stem from traumatic memories. This research topic brings together leading experts who share their recent findings and perspectives on how emotion may influence brain function.

In this issue, four articles review or describe evidence of a specialized influence of emotion on synaptic function. Sheena Josselyn's laboratory previously found that neurons with high levels of cAMP Responsive Element Binding Protein (CREB) at the time of training are preferentially allocated to the memory trace (Han et al., 2007). In this issue, Sargin et al. (2013) provide a cellular mechanism as to why this may be. They report that overexpression of CREB within neurons of the lateral nucleus of the amygdala (LA), leads to an increase in dendritic spine density, whereas LA neurons with low CREB activity exhibit a decrease in dendritic spine density. These data support the hypothesis that CREB may increase a neuron's propensity for being included in a memory trace by increasing dendritic spine density.

Young and Williams addressed the issue of lateralization of amygdala recruitment during memory consolidation. Imaging studies in humans indicate that activity in the right amygdala is associated with aversive memories (Morris et al., 1999). Others indicate that the left amygdala is preferentially involved in encoding memories that have a positive valence (Zalla et al., 2000; Hamann et al., 2002). In this issue, Young and Williams (2013) examine expression of a marker of synaptic plasticity in the rat amygdala. They report that the synaptic plasticity-related protein Arc is specifically elevated in the right amygdala following training on an aversive task, and in the left amygdala following training on an appetitive task. Considering that Arc expression in the amygdala is necessary for consolidation of conditioned fear (Ploski et al., 2008), these findings indicate that, memory-related synaptic plasticity in the amygdala is lateralized and valence-dependent.

Headley and Paré (2013) add a temporal dimension to this research topic. They review literature on temporal synchrony of firing across brain regions involved in emotional memory. They describe gamma oscillations and emotional memory, citing recent reports of enhanced gamma oscillations in the neocortex and amygdala during emotional situations, and evidence that gamma oscillations have predictive value for synaptic plasticity and emotional memory.

Grønli et al. (2013) take a different view on the effects of emotion on memory and plasticity. These authors review the cellular and molecular evidence that stress may impair cognition by interfering with sleep. They describe the importance of sleep for the cellular and molecular processes that contribute to memory consolidation, and suggest that stress that interferes with sleep is associated with deficient synaptic plasticity and impaired cognitive performance.

Several of the contributions to this issue examined synaptic changes related to extinction of conditioned fear in rats. Both post-traumatic stress disorder (PTSD) and obsessive compulsive disorder (OCD) are characterized by avoidance of stimuli that are perceived as threatening, even in the absence of real danger. Impaired extinction of conditioned fear could contribute to the persistence of maladaptive behaviors seen in these disorders (Milad et al., 2008, 2013). It was recently demonstrated that vagus nerve stimulation (VNS) enhances extinction of conditioned fear in rats (Peña et al., 2013). In this issue, (Peña et al., 2014) report that extinction-enhancing VNS reverses synaptic depression in the infralimbic prefrontal cortex basolateral amygdala pathway, a circuit that is implicated in extinction memory (Sierra-Mercado et al., 2011). Collectively, these findings suggest that VNS could be paired with exposure therapy to facilitate extinction of conditioned fear and reverse pathological synaptic function seen in anxiety disorders. Another contribution investigated the effects of deep brain stimulation (DBS) on the extinction circuitry. In 2010, several groups reported that stimulation of the ventral capsule/ventral striatum reduced symptoms of OCD in refractory patients (Denys et al., 2010; Goodman et al., 2010; Greenberg et al., 2010). In rats, stimulation of the ventral striatum enhances extinction of conditioned fear (Rodriguez-Romaguera et al., 2012). In this issue, research from Greg Quirk's lab demonstrates that extinction-enhancing stimulation of the ventral striatum increases expression of the brain-derived neurotrophic factor protein (BDNF) in the medial prefrontal cortex, indicating that the clinical benefits of DBS may be mediated by BDNF-associated synaptic changes in the extinction pathway (Do-Monte et al., 2013).

One of the limitations of exposure therapy is that conditioned fear can return even after successful extinction learning. In

2009, Monfils and colleagues reported evidence that pairing of a brief retrieval trial with extinction training produced a persistent reduction of conditioned fear that was not susceptible to spontaneous recovery, reinstatement, or renewal of fear (Monfils et al., 2009). This effect is now referred to as "reconsolidation updating" because the brief retrieval trial is thought to destabilize the memory trace. The destabilized conditioned fear memory trace is thought to be modified by the extinction training. However, because some laboratories do not observe persistent reductions in fear using the reconsolidation-extinction approach, researchers in the laboratories of Marie Monfils and Hongjo Lee collaborated to determine whether orienting phenotype affects the persistence of the fear reduction. These authors contributed two articles to this issue. Both of these studies distinguished rats that direct attention to a conditioned stimulus during appetitive conditioning (orienters) from rats that do not (non-orienters). Expression of conditioned fear 24 h after training is greater in non-orienters. However, pairing a brief retrieval trial with an extinction session prevents spontaneous recovery of fear in both phenotypes, indicating that orienting phenotype is not a boundary condition that would interfere with the permanence of the effect (Olshavsky et al., 2013a). In contrast, orienting phenotype has a significant effect on the persistence of reconsolidation updating when the conditioned stimulus is appetitive. Conditioned responding spontaneously recovers only in the non-orienting phenotype (Olshavsky et al., 2013b).

Historically, reconsolidation updating and extinction learning have been considered separate processes. However, with the discovery and development of the reconsolidation-extinction paradigm (Monfils et al., 2009), it has become apparent that there is overlap between reconsolidation and extinction processes. In this issue Flavell and colleagues review what is known about the cellular and molecular mechanisms that govern the reactivation-dependent destabilization of memory and how these processes influence the permanent weakening of memory. Considering not all memories become destabilized upon retrieval, and therefore are resistant to being modified, this area of research holds great promise for the development of treatments targeting pathological memory (Flavell et al., 2013).

Roesler and colleagues sought to enhance extinction of conditioned fear in rats but instead found that intra-dorsal hippocampus infusions of the phosphodiesterase type 4 (PDE4) inhibitor, rolipram, during extinction training, can switch the behavioral outcome from extinction to enhanced fear (Roesler et al., 2014). These intriguing findings indicate that inhibition of PDE4 during extinction training may promote reconsolidation update mechanisms and/or inhibit extinction learning.

Extensive evidence supports the role of *de novo* protein synthesis in the consolidation and reconsolidation of memory. In addition, there is accumulating evidence indicating that coordinated protein degradation via the ubiquitin-proteasome system (UPS) is required for these processes. For example, inhibition of the UPS disrupts the consolidation of memory and the destabilization phase of memory updating (Jarome and Helmstetter, 2013). Recently Kwapis et al. (2014) demonstrated that protein degradation via the UPS, within the LA was critical for the consolidation of delay fear conditioning. In this issue, Reis et al. (2013) extend these findings by demonstrating that pharmacologically inhibiting the UPS, with the proteasome inhibitor clasto-lactacystin-β-lactone (β-lac) within the prefrontal cortex, selectively disrupts trace fear conditioning, while leaving delay fear conditioning intact. These findings further support the role of the PFC in trace, but not delay conditioning and underscore the wide spread importance of UPS mediated protein degradation in learning and memory phenomena.

Animal models of trauma and anxiety disorders are helpful in translating these discoveries to therapies. Berardi et al. (2014) present a rodent model of PTSD that meets cognitive and emotional criteria for diagnosis according to the most recent version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). They found that rats exposed to inescapable footshocks and housed in isolation exhibited fear of the context that was associated with the shock for 56 days. These rats also exhibited long-lasting alterations in social interactions and exploration on the elevated plus maze. Such advancements in animal models of PTSD may provide new avenues for exploring the effects of traumatic experiences on the brain as well as opportunities for testing possible therapies.

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CREB regulates spine density of lateral amygdala neurons: implications for memory allocation

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Neurons may compete against one another for integration into a memory trace. Specifically, neurons in the lateral nucleus of the amygdala with relatively higher levels of cAMP Responsive Element Binding Protein (CREB) seem to be preferentially allocated to a fear memory trace, while neurons with relatively decreased CREB function seem to be excluded from a fear memory trace. CREB is a ubiquitous transcription factor that modulates many diverse cellular processes, raising the question as to which of these CREB-mediated processes underlie memory allocation. CREB is implicated in modulating dendritic spine number and morphology. As dendritic spines are intimately involved in memory formation, we investigated whether manipulations of CREB function alter spine number or morphology of neurons at the time of fear conditioning. We used viral vectors to manipulate CREB function in the lateral amygdala (LA) principal neurons in mice maintained in their homecages. At the time that fear conditioning normally occurs, we observed that neurons with high levels of CREB had more dendritic spines, while neurons with low CREB function had relatively fewer spines compared to control neurons. These results suggest that the modulation of spine density provides a potential mechanism for preferential allocation of a subset of neurons to the memory trace.

Keywords: CREB, amygdala, fear memory, dendritic spines, viral vector

INTRODUCTION

The cAMP Responsive Element Binding Protein (CREB) is an activity regulated transcription factor that modulates the transcription of genes with cAMP responsive elements (CRE) located in their promoter regions. Early research in *Aplysia* (Dash et al., 1990; Kaang et al., 1993; Bartsch et al., 1995) and *D. melanogaster* (Yin et al., 1994, 1995; Perazzona et al., 2004) first implicated CREB in memory formation. Since that time, the important role of CREB in memory has been shown across a variety of species from *C. elegans* (Kauffman et al., 2010; Lau et al., 2013) to rats (Guzowski and McGaugh, 1997; Josselyn et al., 2001), mice (Bourtchuladze et al., 1994; Kida et al., 2002; Pittenger et al., 2002; Gruart et al., 2012) and humans (Harum et al., 2001) (for review, see Josselyn and Nguyen, 2005) but see Balschun et al. (2003). For instance, we (Han et al., 2007), and others (Zhou et al., 2009; Rexach et al., 2012) previously showed that increasing CREB function in a small portion of lateral amygdala (LA) neurons (roughly 8–10% of LA principal neurons) was sufficient to enhance auditory fear memory. Moreover, we observed that LA neurons with relatively higher CREB function at the time of training were preferentially included, whereas neurons with lower CREB function were excluded, from the subsequent LA fear memory trace (Han et al., 2007, 2009). Conversely, disrupting CREB function by expressing a dominant negative version of CREB ($CREB^{S133A}$)in a similar small percentage of LA neurons did not affect auditory fear memory, likely because the neurons expressing $CREB^{S133A}$ were largely excluded from the memory trace. Furthermore, post-training ablation (Han et al., 2009) or silencing (Zhou et al., 2009) of neurons overexpressing CREB disrupted subsequent expression of the fear memory, confirming the importance of these neurons. Together, these data suggest that neurons with high levels of CREB at the time of training are preferentially allocated to the memory trace because they somehow outcompete their neighbors (Won and Silva, 2008).

CREB is a ubiquitous transcription factor implicated in many diverse cellular processes in addition to memory formation, including regulation of proliferation, survival, apoptosis, differentiation, metabolism, glucose homeostasis, spine density, and morphology (Bourtchuladze et al., 1994; Murphy and Segal, 1997; Silva et al., 1998; Mayr and Montminy, 2001; Lonze et al., 2002; Wayman et al., 2006; Aguado et al., 2009; Altarejos and Montminy, 2011). Which of these CREB-mediated processes is/are important for memory allocation? Here we investigated one CREB-mediated process, the regulation of spine density and morphology.

Dendritic spines are small, highly motile structures on dendritic shafts which provide flexibility to neuronal networks. As an increase in the synaptic strength between neurons is thought

to underlie memory formation (Bailey and Kandel, 1993; Bailey et al., 1996) and the majority of excitatory synapses occur on dendritic spines (Harris and Stevens, 1988, 1989; Farb et al., 1992), it has long been thought that dendritic spines serve as storage sites for synaptic strength, an idea first proposed by Santiago Ramón y Cajal over 100 years ago (Cajal, 1995). In this way, the growth and re-structuring of dendritic spines is thought to be crucial for memory formation.

A role for CREB in spine formation was first reported by Murphy and Segal (1997) who showed that estradiol treatment increased both levels of activated (phosphorylated) CREB and spine density in cultured hippocampal neurons. CREB was subsequently shown to regulate spine morphology in hippocampal neurons both in organotypic culture (Impey et al., 2010) and *in vivo* (Marie et al., 2005), as well as in visual cortex principal neurons (Suzuki et al., 2007). The new spines formed following overexpression of CREB may contain silent synapses (NMDA receptors only), suggesting that they may be "primed" for incorporation into future memory circuits (Marie et al., 2005). Consistent with this, increasing CREB function in hippocampal CA1 principal neurons was sufficient to restore both the decrease in spine density and spatial memory in a mouse model of Alzheimer's disease (Yiu et al., 2011).

We previously reported that neurons with increased CREB at the time of training are selectively allocated to a fear memory trace and a variety of evidence shows that increasing CREB function increases spine density. Therefore, we investigated whether neurons with increased CREB at the time of training also have an increase in dendritic spine density, thereby providing a potential mechanism of the preferential allocation of these neurons to the memory trace.

MATERIALS AND METHODS

MICE

Adult male F1 hybrid (C57 BL/6NTac × 129S6/SvEvTac) mice were used for all experiments. This genetic background has been used extensively in behavioral studies and are well characterized (Silva et al., 1997). Mice were group housed (2–5 mice per cage) on a 12 h light/dark cycle and provided with food and water *ad libitum*. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) and the National Institutes of Health (NIH) and approved by the Animal Care Committee at the Hospital for Sick Children.

HSV VECTORS

Neurotropic replication-defective herpes simplex viral (HSV) vectors were used to manipulate CREB function in individual LA principal neurons. Wild-type or dominant negative CREB^{S133A} cDNAs were cloned into the HSV amplicon under the control of the constitutive promoter for the HSV immediate early gene IE4/5. These vectors co-expressed GFP which was driven by CMV promoter [HSV-p1005; Russo et al., 2009]. In this vector therefore, the GFP protein is not fused to CREB and may thus fill the infected cell. As a control, we used HSV vector expressing GFP alone. HSV virus was packaged using a replication-defective helper virus as previously described (Josselyn et al., 2001; Barrot et al., 2002; Carlezon and Neve, 2003; Han et al., 2008; Vetere et al., 2011; Cole et al., 2012). Virus was purified on a sucrose gradient, pelleted and resuspended in 10% sucrose. The average titer of the virus stocks was typically 4.0×10^7 infectious units/ml.

SURGERY

Mice were pretreated with atropine sulfate (0.1 mg/kg, i.p.), anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame. Skin was retracted and holes were drilled in the skull above the LA (anteroposterior $= -1.4$, mediolateral $= \pm 3.4$, ventral $= -5.0$ mm from bregma) according to (Paxinos and Franklin, 2001). Viral vector was microinjected through glass micropipettes connected via polyethylene tubing to a microsyringe (Hamilton, Reno, NV) at a rate of 0.1 µl/min. Micropipettes were left in place an additional 10 min following microinjection to ensure diffusion of vector. For behavior analysis, a volume of 1.5 µl and for spine analysis, a volume of 1.0 µl was microinjected bilaterally at a rate of 0.1 µl/min. Micropipettes were slowly retracted, the incision site closed and mice were treated with analgesic (ketoprofen, 5 mg/kg, s.c.). Three d following surgery, at a maximal transgene expression for HSV vector system (Josselyn et al., 2001; Barrot et al., 2002; Vetere et al., 2011; Cole et al., 2012), mice were either fear conditioned or perfused for dendritic spine analysis.

AUDITORY (TONE) FEAR CONDITIONING

During training, mice were placed in a Med Associates (St. Albans, VT) Plexiglas and metal chamber (24 × 30 × 21 cm, context A; Cxt A) located in a soundproof room. After 2 min, a tone (2800 Hz, 30 s, 85 dB) that co-terminated with a footshock (2 s, 0.4 mA) was presented. Mice remained in the chamber for an additional 30 s and then returned to the homecage. Testing for auditory fear memory occurred 24 h later by placing mice in a novel context (context B; Cxt B) and 2 min later, presenting the tone previously paired with footshock for 3 min. The percentage of time mice spent freezing (the cessation of all movement except respiration) before and during the tone was measured using an automated system (Actimetrics) and was used as our index of memory. Immediately after testing, mice were deeply anesthetized and perfused.

IMMUNOHISTOCHEMISTRY

To visualize the number and morphology of dendritic spines in the neurons we infected, we took advantage of the GFP expressed by all viral vectors. We amplified the GFP signal using an antibody directed against GFP. 72 h after surgery, mice were deeply anesthetized using chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA). Brains were post-fixed overnight in 4% PFA and transferred to 30% sucrose for cryoprotection. Coronal (50 µm) sections were prepared and immunohistochemistry for GFP was performed. Free-floating sections were incubated in blocking solution (0.1% BSA, 5% NGS, 0.2% Triton-X-100 in 0.1 M PBS) for 1 h and labeled with anti-GFP rabbit polyclonal antibody (1:500, Invitrogen) overnight at 4°C. Following PBS washes, sections were incubated with goat anti-rabbit Alexa 488 (1:500, Invitrogen) for 2 h at room temperature. Sections were washed

with PBS, mounted on gelatin-coated slides and coverslipped using Vectashield Hardmount with DAPI (Vector Laboratories).

CONFOCAL ANALYSIS

GFP-positive LA neurons (neurons infected by viral vectors) were first identified using a 10x objective (LSM 710, Zeiss). Infected neurons were included in the subsequent spine analysis if (i) cell body was not damaged; (ii) dendritic projections remained within the LA; (iii) neurons could clearly be identified without interference from neighboring infected cells; (iv) neurons had first, second, and third order branches. Fourth order branches were not included in the analysis as they often appeared truncated in our 50 μm sections. Selected GFP-positive neurons were imaged using a 100x oil-immersion objective. Z series were obtained by imaging serial confocal planes at 0.25 μm intervals. Dendrites and spines were traced manually from the image stacks using Neurolucida software and analyzed with Neurolucida Explorer (MBF version 9).

Dendritic morphology

Image analysis was performed by two researchers unaware of the treatment condition of the mouse. Dendrites were traced. The first dendritic process emanating from the cell body was defined as the primary (first order) branch. Subsequent branches that bifurcated from the first branch order were designated as second order branches, and so forth. Truncated branches or those that did not remain within the image window were excluded from subsequent analysis.

Spine morphology

Dendritic spines were defined as small protrusions connected to the dendritic shaft (Feldman and Dowd, 1975). Spines show a distinct morphology and vary in length from 0.5–4 μm (Peters and Kaiserman-Abramof, 1970; Horner and Arbuthnott, 1991). Therefore, we analyzed all dendritic protrusions that were less than or equal to 4 μm in length (Horner and Arbuthnott, 1991). Because this method has been shown to produce reliable results (Horner and Arbuthnott, 1991), no attempt was made to introduce a correction factor for hidden spines. Spines were counted and spine density was calculated as the number of spines on a branch divided by the length of the branch. Spine length was defined as the distance between the spine tip and the base of the spine. Spine head diameter was identified as the maximum width of the spine head (see **Figure 2B**).

STATISTICAL ANALYSIS

Data were analyzed with 1 or 2-Way analyses of variance (ANOVAs) using Statistica (Statsoft) software. For the auditory fear conditioning data, we analyzed the percentage of time spent freezing to before (2 min) and during (3 min) the tone. For dendritic and spine morphological analysis, data were first averaged by branch order per cell, then by animal and finally by vector group (GFP, CREB, or $CREB^{S133A}$). Newman-Keuls *post-hoc* tests were performed where appropriate. To protect against potential type 1 errors resulting from multiple comparisons of 5 different measures of neuronal morphology (i.e., spine density, spine length, spine head diameter, dendrite length, and dendrite volume), we also performed a Bonferroni correction (corrected $\alpha = 0.01$). All significant main effects remained significant after correction.

RESULTS

INCREASING CREB IN A SMALL PORTION OF LA NEURONS ENHANCES MEMORY FORMATION WHILE EXPRESSING THE DOMINANT NEGATIVE VERSION OF CREB HAS NO EFFECT

We first confirmed the effects of manipulating CREB function in a small portion (∼8–10%) of LA neurons on the formation of tone fear memory by microinjecting HSV vectors encoding GFP, CREB or dominant-negative CREB ($CREB^{S133A}$) into the LA of adult mice 3 d before fear conditioning (see **Figure 1A**). During training (Cxt A), mice received a single tone (conditioned stimulus, CS) footshock (0.4 mA) (US) pairing that did not induce ceiling levels of freezing. Tone fear memory was assessed 24 h after training. Mice were placed in a novel context (Cxt B) and 2 min later the tone was presented for 3 min (**Figure 1B**). Consistent with our earlier findings (Han et al., 2007, 2009) and those of other research groups (Zhou et al., 2009; Rexach et al., 2012), increasing CREB levels in a small portion of LA neurons enhanced tone fear memory, while disrupting CREB function by microinjecting $CREB^{S133A}$ vector had no effect on fear memory (**Figures 1C,D**). These results were supported by a *Vector* (GFP, CREB, $CREB^{S133A}$ vector) × Time (5 min) ANOVA showing significant main effects of *Vector* [$F_{(2,\ 28)} = 6.8$, $p = 0.004$] and *Time* [$F_{(4,112)} = 16.5$, $p \leq 0.0001$] but no *Vector* × *Time* interaction [$F_{(8,112)} = 6.8$, $p = 0.32$]. A subsequent One-Way ANOVA performed on freezing during the entire CS presentation showed a significant effect of Vector [$F_{(2,28)} = 7.1$, $p = 0.003$], as mice microinjected with CREB vector froze significantly more than mice with GFP ($p = 0.006$) or $CREB^{S133A}$ vector ($p = 0.004$), which did not differ from each other ($p = 0.83$) (Newman-Keuls *post-hoc*) (**Figure 1D**). Importantly, when first placed in Cxt B, mice generally showed little freezing before the tone was presented and baseline levels of freezing in CREB or $CREB^{S133A}$ groups did not differ from the GFP group ($p = 0.18$, $p = 0.19$ respectively). We next examined a possible mechanism underlying this preferential recruitment to the memory trace.

CREB MODULATES DENDRITIC SPINE DENSITY OF LA NEURONS

CREB is a ubiquitous transcription factor that has been implicated in many cellular processes, including regulating dendritic spine density. We hypothesized that neurons may be recruited to the memory trace based on their relative spine density, and examined whether neurons infected with CREB vector show greater dendritic spine density *at the time of training* than neurons infected with $CREB^{S133A}$ or Control GFP vector. We microinjected a separate co-hort of mice with GFP, CREB, or $CREB^{S133A}$ vector as above but did not train these mice. Instead, 72 h following surgery (at a time when they would have received auditory fear conditioning) we removed their brains and examined spine density (**Figure 2A**).

There are two major neuronal cell populations in LA: pyramidal glutamatergic projection neurons and local circuit γ-aminobutyric acid (GABA)-ergic interneurons (McDonald, 1984). Glutamatergic pyramidal-like principal neurons comprise the majority (85–90%) (McDonald, 1992; Sah et al., 2003) and

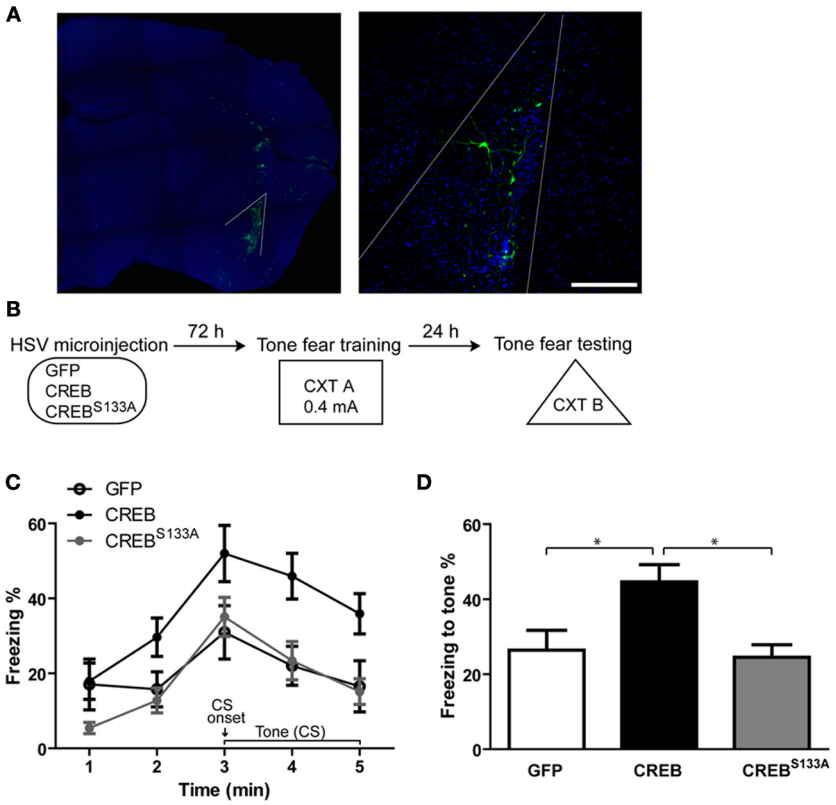

FIGURE 1 | Overexpressing CREB in LA neurons enhances, while dominant negative CREB^{S133A} does not affect, auditory fear memory. **(A)** Visualizing neurons infected with viral vectors. Left: Outline of the LA. Maximum intensity projection is shown. Right: LA principal neurons expressing GFP 72 h following viral vector microinjection (nuclei stained with DAPI, infected neurons visualized with anti-GFP antibody). 0.25 μm optical section is shown. Scale bar, 100 μm. **(B)** Experimental design. Auditory (tone) fear conditioning was conducted 72 h after HSV microinjection. Mice were placed in CXT A and presented with a tone (30 s) that co-terminated with a footshock (0.4 mA). Memory was assessed 24 h later in CXT B. **(C)** Mice microinjected with CREB vector ($n = 10$) showed increased freezing during (but not before) subsequent presentation of the tone compared to mice microinjected with GFP ($n = 12$) or CREB^{S133A} ($n = 11$) vectors. **(D)** Mice overexpressing CREB in LA neurons showed enhanced fear memory for the tone indicated by higher freezing levels during the 3–min tone, compared to mice with GFP or CREB^{S133A}vectors. Data represent mean ± s.e.m. $^*p < 0.05$.

can be visually identified according to the shape of their somata. Thus, we identified infected neurons as LA principal neurons based on their pyramidal shaped somata. In mice microinjected with CREB vector, infected neurons showed higher spine density compared to infected neurons in mice microinjected with Control (GFP-only) vector. In contrast, CREB^{S133A}-infected neurons showed lower spine density than control neurons. This pattern of results was observed across branch order (**Figures 2C,D**). A *Vector* (GFP, CREB, CREB^{S133A}) by *Branch order* (3) repeated-measures ANOVA showed significant main effects of *Vector* [$F_{(2,15)} = 16.8$, $p \leq 0.0001$] and *Branch order* [$F_{(2,30)} = 37.2$, $p \leq 0.0001$] but no significant interaction between *Vector*× *Branch order* [$F_{(4,30)} = 0.2$, $p = 0.90$]. *Post-hoc* Newman-Keuls analysis on the significant main effects revealed that neurons with CREB vector had significantly greater spine density across branch orders compared to neurons infected with GFP ($p = 0.02$) or CREB^{S133A}($p = 0.0002$) vectors (**Figures 2C,E**), while neurons with CREB^{S133A} vector had lower spine density across branches relative to those expressing GFP only ($p = 0.004$) (**Figures 2C,E**). It is important to note that these changes in spine density occurred even though all mice were maintained in the homecage (and therefore, these changes in spine density cannot be attributed to fear conditioning).

Importantly, dendritic length per branch (**Figure 3A**) or total dendritic length did not appear to differ between vectors (**Figure 3B**). This observation was supported by repeated measures ANOVA showing no significant effect of *Vector* [$F_{(2,15)} = 2.8$, $p = 0.09$] or interaction of *Vector* × *Branch order* [$F_{(4,30)} = 0.7$, $p = 0.60$], but a significant main effect of *Branch order* [$F_{(2,30)} = 10.9$, $p = 0.0003$]. Therefore, dendritic length increased with increasing branch order, but this was not changed by CREB manipulation (**Figures 3A,B**). We also observed no difference in dendritic volume between neurons infected with the various vectors (**Figures 3C,D**). An ANOVA revealed no significant effects of *Vector* [$F_{(2,15)} = 1.3$, $p = 0.29$], *Branch order* [$F_{(2,\ 30)} = 1.0$, $p = 0.37$] or *Vector*× *Branch order* interaction [$F_{(4,30)} = 1.9$, $p = 0.13$]. Therefore, manipulations of CREB function changed dendritic spine density without changing dendritic morphology.

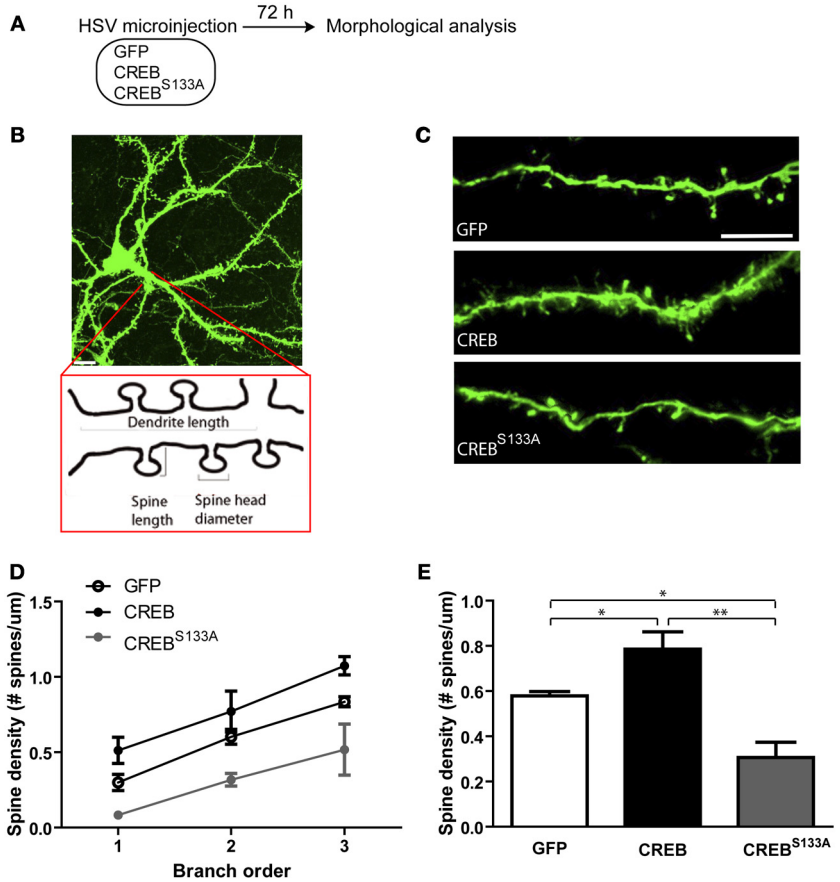

FIGURE 2 | CREB modulates dendritic spine density in LA neurons. (A) Experimental design. The morphology of infected LA neurons was analyzed 72 h after mice were microinjected with GFP, CREB or, $CREB^{S133A}$vectors (at the same time-point as training occurred in **Figure 1**). Mice remained in the homecage after microinjection and were not fear conditioned. **(B)** Schematic representation of a dendritic segment showing the parameters analyzed (dendrite length, spine length, spine head diameter and spine density). Scale bar, 10 μm. **(C)** Representative dendritic segments of LA neurons from mice microinjected with GFP, CREB, or $CREB^{S133A}$ vector. Scale bar, 5 μm. **(D)** Dendritic spine density shown at each branch order and **(E)** across all branches is increased in neurons with CREB vector ($n = 6$) and decreased in neurons with $CREB^{S133A}$ vector ($n = 5$) when compared to neurons with GFP vector ($n = 7$). Data represent mean ± s.e.m. $^{*}p < 0.05$. $^{**}p < 0.001$.

SPINE MORPHOLOGY IS NOT ALTERED BY CREB OR $CREB^{S133A}$ EXPRESSION

Alterations in spine morphology have been correlated with changes in spine function (Matsuzaki et al., 2001, 2004) and increasing CREB may induce formation of silent synapses (Marie et al., 2005). Spines with large bulbous heads are thought to contain large post-synaptic densities (PSD) (Harris et al., 1992) whereas spines with small heads and long necks may contain silent synapses (Matsuzaki et al., 2001, 2004). Prompted by these observations, we analyzed whether manipulations of CREB function altered spine morphology in mice maintained in the homecage by measuring spine length and head diameter (see **Figure 2B**). Interestingly, spine length, regardless of vector, increased slightly with increasing branch order {**Figures 4A,B**; ANOVA showing no significant main effect of *Vector* [$F_{(2,15)} = 0.8$, $p = 0.45$] or *Vector* × *Branch order* interaction [$F_{(4,30)} = 0.6$, $p = 0.63$], but a significant main effect of *Branch order* [$F_{(2,30)} = 8.9$, $p = 0.0009$]}. We next examined whether CREB manipulation influenced spine head diameter (widest distance of the spine head, see **Figure 2B**). We observed no difference between spine head diameter between vectors, but a small change per branch order across all vectors {**Figures 4C,D** no significant effect of *Vector* [$F_{(2,15)} = 0.7$, $p = 0.50$] or *Vector* × *Branch order* interaction [$F_{(4,30)} = 1.0$, $p = 0.41$] but a significant main effect of *Branch order* [$F_{(2,30)} = 4.2$, $p = 0.02$]}. Therefore, although CREB manipulations produced changes in dendritic spine density, these were not accompanied by changes in dendrite or overall spine morphology.

DISCUSSION

Previously, we and others observed that neurons with relatively increased CREB function at the time of training seem to be competitively advantaged over neighboring neurons for allocation to a fear memory trace. Here we examined whether an increase in spine density at the time of training might mediate this competitive advantage. To this end, we examined the effects of manipulating CREB function on dendritic spine density at the time of training. We found that in mice taken directly from the homecage,

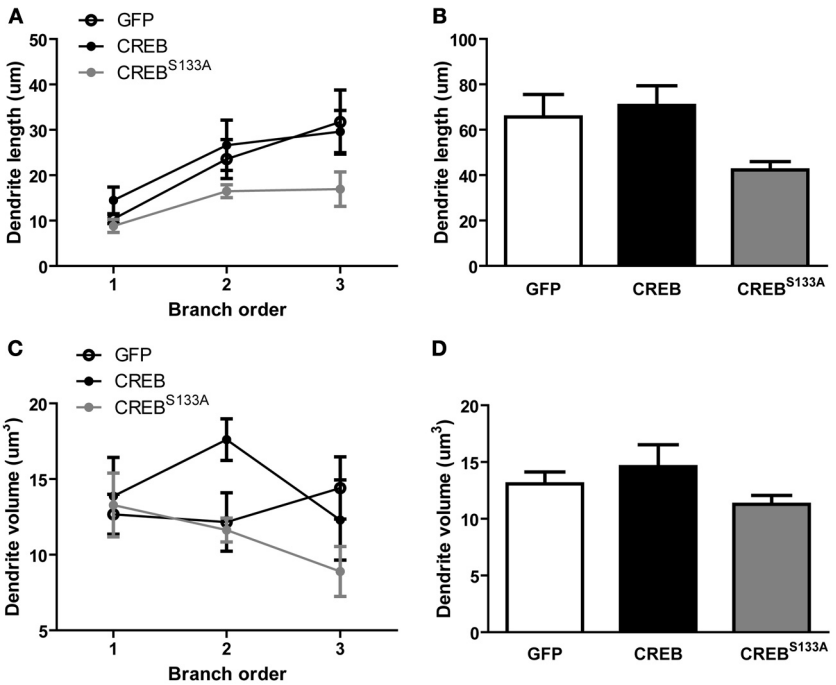

FIGURE 3 | CREB does not affect dendritic length and volume. (A) Dendritic length per branch order and **(B)** across all branches was comparable between LA neurons overexpressing GFP ($n = 7$), CREB ($n = 6$), or $CREB^{S133A}$ ($n = 5$) vectors. **(C)** Dendrite volume per branch order and **(D)** across all branches did not differ between LA neurons overexpressing GFP ($n = 7$), CREB ($n = 6$), or $CREB^{S133A}$ ($n = 5$) vectors.

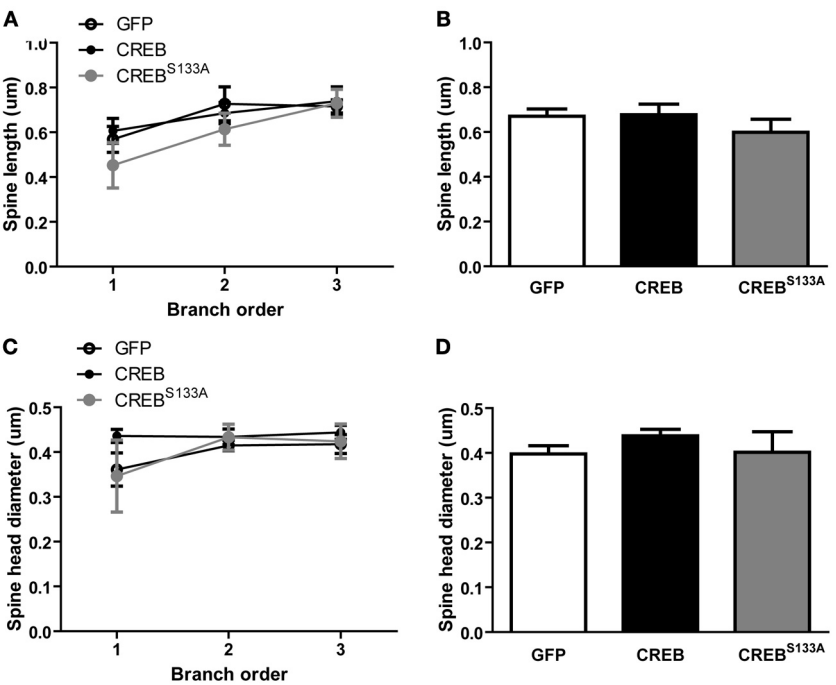

FIGURE 4 | CREB does not affect spine morphology. (A) Spine length at each branch order and **(B)** across all branches was similar between LA neurons with GFP ($n = 7$), CREB ($n = 6$), or $CREB^{S133A}$($n = 5$) vectors. **(C)** Spine head diameter at each branch order and **(D)** across all branches did not differ between LA neurons with GFP ($n = 7$), CREB ($n = 6$), or $CREB^{S133A}$ ($n = 5$) vectors.

neurons with CREB overexpression showed higher, while neurons with CREB^{S133A} showed lower spine density, than control infected neurons. These data are consistent with the notion that one factor that may determine neuronal allocation for memory formation is relative spine density.

The LA is a key brain region important in mediating fear and anxiety (Davis, 1992) and some studies implicate CREB in "emotional" behavior (Barrot et al., 2002; Pandey et al., 2003). It is possible, therefore, that CREB overexpression in a small population of LA neurons leads to a general increase in fear and/or anxiety. Disruption of CREB function either generally in the brain (Valverde et al., 2004) or specifically in the amygdala (Pandey et al., 2005) has been reported to increase anxiety-like behavior in mice. On the other hand, local CREB overexpression has been shown to enhance excitability of LA neurons without causing alterations in anxiety or locomotor activity (Viosca et al., 2009). In our experiments, before the presentation of the tone (pre-CS), freezing levels of mice overexpressing CREB or CREB^{S133A} did not differ from those overexpressing GFP. This ruled out the possibility that CREB or CREB^{S133A}might lead to alterations in general fear and anxiety.

Studies in the 1990s first implicated CREB in the formation of long-term memory (LTM) (Dash et al., 1990; Yin et al., 1994, 1995). Building on these important findings, we (Josselyn and Nguyen, 2005; Han et al., 2007, 2009; Cole et al., 2012), and others (Bourtchuladze et al., 1994; Kida et al., 2002) (Viosca et al., 2009; Zhou et al., 2009; Rexach et al., 2012), showed that decreasing CREB function disrupts, while increasing CREB function enhances the formation of many types of memory in mammals [but see Balschun et al. (2003)]. The role of CREB in fear memory has been extensively studied in rodents. Mice lacking α and δ isoforms of CREB (CREB$^{\alpha\delta}$) showed impaired in LTM for both context and tone fear memories (Bourtchuladze et al., 1994). Similarly, CREB^{comp} mice, carrying one allele for the β isoform of CREB, showed deficits in LTM for context and tone fear memories (Gass et al., 1998). CREB^{IR} mice which express CREB^{S133A} in a temporally regulated manner have impaired context and tone fear memory following repression of CREB activity before training (Kida et al., 2002). Viral delivery of CREB into the amygdala using HSV enhanced LTM induced by massed training protocol in the fear potentiated startle paradigm in rats (Josselyn et al., 2001). CREB is thought to activate the transcription of target genes which ultimately serve as the building blocks for increasing the synaptic connections between neurons important for memory formation (Bartsch et al., 1998). It is interesting to note that CREB has also been implicated in human memory (Harum et al., 2001) and several human cognitive/memory disorders are linked to disruptions in the CREB signaling pathway (Josselyn and Nguyen, 2005). Together these data converge to indicate that CREB is critical for memory formation.

Previous studies have also established a possible role of CREB in maintaining spine number and morphology. Estradiol treatment in cultured hippocampal neurons led to increased phosphorylation of CREB which correlated with spinogenesis (Murphy and Segal, 1997). Enhancing CREB function upon expression of a constitutively active form of CREB (caCREB) in the CA1 region of hippocampus increased spine density in hippocampal neurons *in vivo* (Marie et al., 2005). CREB was also shown to regulate spine morphology in pyramidal neurons of the visual cortex (Suzuki et al., 2007). Expression of caCREB in organotypic hippocampal neurons increased spine density while decreasing CREB function by expression of a dominant negative CREB or a CREB-targeted shRNA inhibited spine formation (Impey et al., 2010). Consistent with this, increasing CREB function in hippocampal CA1 principal neurons restored the decrease in spine density and improved spatial memory in a mouse model of Alzheimer's disease (Yiu et al., 2011). Recent work has shown that CREB-induced excitability of LA neurons may be a potential mechanism for preferential recruitment of these neurons to the fear memory trace (Zhou et al., 2009).

Based on the previous work, we hypothesized that CREB's role in allocation of tone fear memory may be caused by its effect on regulation of spine density of LA neurons. The LA receives sensory (both tone and footshock) information directly from auditory cortex and thalamus (LeDoux et al., 1990; Campeau and Davis, 1995) and is thought to be the critical site for convergence of US and CS inputs in auditory fear conditioning experiments. Therefore, neurons with more dendritic spines may be preferentially activated by the CS and US convergence and become part of the memory trace.

Here, we observed that changes in CREB function alone were sufficient to change dendritic spine density, and that neurons with increased CREB function showed higher dendritic spine density. Furthermore we observed that neurons with higher CREB function were preferentially allocated to the memory trace. Because synapses and spines play a key role in neuronal information processing, changes in dendritic spine density or morphology of a neuron may affect synaptic function and local circuit organization. Along with other factors, such as changes in neuronal excitability (Zhou et al., 2009), changes in the synapse and spine number and morphology may influence neuronal spiking activity and play important roles in neuronal memory allocation.

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Differential activation of amygdala *Arc* expression by positive and negatively valenced emotional learning conditions

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Norepinephrine is released in the amygdala following negatively arousing learning conditions. This event initiates a cascade of changes including the transcription of activity-regulated cytoskeleton-associated protein (*Arc*) expression, an early-immediate gene associated with memory encoding. Recent evidence suggests that the valence of emotionally laden encounters may generate lateralized, as opposed to symmetric release of this transmitter in the right or left amygdala. It is currently not clear if valence-induced patterns of selective norepinephrine output across hemispheres are also reproduced in downstream pathways of cellular signaling necessary for memory formation. This question was addressed by determining if *Arc* expression is differentially distributed across the right and left amygdala following exposure to positively or negatively valenced learning conditions respectively. Male Sprague Dawley rats were randomly assigned to groups exposed to the Homecage only, five auditory tones only, or five auditory tones paired with footshock (0.35 mA) during Pavlovian fear conditioning. Western blot analysis revealed that *Arc* expression in the right amygdala was elevated significantly above that observed in the left amygdala 60 and 90 min following fear conditioning. Similarly, subjects exposed to a negatively valenced outcome consisting of an unexpected reduction in food rewards showed a greater level of *Arc* expression in only the right, but not left basolateral amygdala. Presenting a positively valenced event involving an unexpected increase in food reward magnitude following bar pressing, resulted in significantly greater *Arc* expression in the left, but not right basolateral amygdala ($p < 0.01$). These findings indicate that the valence of emotionally arousing learning conditions is reflected at later stages of synaptic plasticity involving the transcription of immediate early genes such as *Arc*.

Keywords: emotional arousal, memory modulation, amygdala, *Arc* expression, brain asymmetry, amygdala lateralization

INTRODUCTION

Converging evidence suggests that the left and right amygdala are preferentially activated during the encoding of emotional events containing positive or negatively valenced stimuli. Molecular markers and immediate-early gene expression induced by new types of learning conditions reflect similar asymmetric patterns of activation. For example, elevated levels of protein kinase C (PKC) or cyclic AMP response element binding protein (CREB) are integral components of the molecular cascades that convert new information from short, to long term memory (Zhou et al., 2009; de Oliveira Coelho et al., 2013; Pinho et al., 2013). Surprisingly both proteins are upregulated in the right, but not left amygdala after exposure to a tone previously paired with an aversive experience such as footshock during fear conditioning training or in response to exposure to a threatening predator (Blundell and Adamec, 2007; Orman and Stewart, 2007). Another molecular marker required for regulating neural plasticity in response to inflammatory pain is extracellular signal-regulated kinase (ERK) signaling in the amygdala. Inflammation-induced mechanical sensitivity is reduced by blocking right amygdala ERK activation, regardless of the side of the peripheral injury (Carrasquillo and Gereau, 2008). Furthermore, expression of the immediate-early gene *c-fos* is elevated in the right amygdala following reexposure to the fear conditioning context. This finding demonstrates that the responsiveness of amygdala neurons are lateralized even when the organism re-experiences stimuli with a negative valence (Sciclli et al., 2004) and provides additional evidence that unpleasant or aversive events produce asymmetric changes within the amygdala that are expressed at a molecular level.

Norepinephrine released following experimental manipulations facilitates new learning and improves later retention or recall by upregulating signaling cascades leading to *Arc* dependent actin rearrangement necessary for long-term potentiation (LTP) in the hippocampus (Hou et al., 2009) as well as important cellular changes within the amygdala (Liu et al., 2012) and cingulate cortex (Holloway-Erickson et al., 2012). These findings illustrate the important contribution of norepinephrine release in the basolateral amygdala during encoding by influence on intracellular events associated with memory formation. When

norepinephrine binds to post synaptic β-noradrenergic receptors it initiates cAMP formation. Elevating cAMP levels leads to the activation of cAMP dependent kinases, such as MAPK, PKA and PKC, which in turn phosphorylates CREB. Phosphorylated CREB is a transcription factor that upregulates gene transcription, including immediate-early genes such as *c-fos* and *Arc* (Davies et al., 2004; Blundell and Adamec, 2007; Orman and Stewart, 2007; Canal et al., 2008). Expression of *Arc* protein is induced by strong synaptic activation and is quickly transported to the active dendrites to participate in synaptic remodeling during associative learning and LTP (Bramham et al., 2008; Miyashita et al., 2008). Stabilization of actin molecules by *Arc* protein allows active synapses that represent features of the new event in the amygdala or hippocampus to be bound and associated together (Bramham et al., 2010). If valence dependent asymmetric release of norepinephrine is a catalyst for stimulating cellular activity, then immediate-early gene expression of activity-regulated cytoskeleton-associated (*Arc*) protein should also be disproportionally upregulated in either the right or left basolateral amygdala depending on the valence of the learning condition.

In light of these findings, these studies investigated whether exposure to stimuli of opposing valence differentially influences *Arc* expression in the right or left basolateral amygdala. Experiment 1 utilized western blots to assess *Arc* expression in the right and left amygdala at different time points following presentation of a negatively valenced stimulus consisting of footshock during Pavlovian fear conditioning. Experiments 2 and 3 expanded these finding by assessing whether training with appetitive stimuli in an operant learning task produces different patterns of *Arc* expression across the left or right basolateral amygdala following unexpected positive or negative consequences to barpressing.

METHODS

SUBJECTS

Sixty-two male Sprague-Dawley rats (275–300 g) obtained from Charles River Laboratories (Wilmington, MA) were used in Experiments 1 ($n = 25$), 2 ($n = 15$) and 3 ($n = 22$). Rats were individually housed in plastic cages and maintained on a standard 12:12 h light-dark cycle with lights on at 7:00 am. Food and water were available *ad libitum* during the 7 days undisturbed adaptation period to the vivarium. All experiments were conducted in accordance to the policies and guidelines of the University of Virginia's Animal Care and Use Committee.

BEHAVIORAL PROCEDURES

Behavioral apparatus

The apparatus used for Pavlovian Fear conditioning and the Operant task consisted of a Coulbourn (Allentown, PA) behavioral chamber (12″W × 10″D × 12″H, Model #: H13–16) that was enclosed in a larger sound-attenuating box (28″W × 16″D × 16″H). The walls of the chamber were constructed of clear plastic with stainless steel sides and a removable stainless steel grid floor. The conditioning chambers were cleaned with a 10% alcohol solution after training.

FEAR CONDITIONING

Rats were transported from the vivarium to the lab 4 h prior to training. The rats were first habituated to the conditioning chamber with 5 min of free exploration. Twenty-four hours later, they were placed in the training context for a 3 min baseline followed by a 30 s tone (5 kHz, 75 db) conditioned stimulus (CS) that co-terminated with a 1 s, 0.35 mA foot shock unconditioned stimulus (US). A 60 s inter-trial interval (ITI) separated a foot-shock from the presentation of the next tone. Conditioning consisted of five (CS) tone- (US) shock pairings. Freezing behavior was defined as an absence of movement except respiratory function (Blanchard and Blanchard, 1972) and was recorded with a Coulbourn (Allentown, PA), infrared activity monitor (Model #: H24–61) that automatically samples movement every 400 ms. A customized program developed by Coulbourn was used to convert recordings of the absence of movement into a Microsoft Excel macro that calculated the percent of this measure during each of the five, 30 s periods of tone presentation.

OPERANT TASK

Training

All subjects were placed on a weight maintenance schedule 7 days prior to training (i.e., a 15% reduction in body weight) and remained on this schedule throughout the experiment. The animals were initially shaped to lever press for food rewards consisting of 45 mg sucrose pellets. They were then trained on a Fixed Ratio 5 schedule (FR5) over the course of 10 days. A light signaled the start of each trial and the animals were required to make five lever presses to initiate delivery of sucrose pellets to the food cup. Head pokes into the food cup interrupted an infra-red beam that turned the light off and signaled the end of each trial. There was an ITI of 30 s between each of the 10 daily trials.

Shift in reward expectations

Subjects were divided into two groups during the initial 10 days of training. One group received (10) sucrose pellets after each FR5 schedule as opposed to the second group that was reinforced with only (1) pellet after the five lever presses. After 10 days of training with the (1) or (10) pellet food reward, half of the animals from these two groups were randomly assigned to a *Down-shift* or *Up-shift* group that experienced an unexpected reduction or increase in food rewards respectively.

For instance, one-half of the rats originally rewarded with (10) pellets were *Down-shifted* on Day 11 and given only (1) pellet for each FR5 trial. The *Down-shift* in reward quantity elicits a negative psychological state that is reflected by a reduction in approach behavior toward the food cup and a level of frustration manifested by increased responding on the lever (Crespi, 1942; Goldman et al., 1973). Subjects in this condition are referred to as (Group 10-1) to denote the difference in reward quantity prior to and after the shift. Accordingly, subjects that continued to receive the same level of food rewards are labeled as (i.e., Group 10-10).

One-half of the remaining subjects that initially received (1) pellet following each FR5 trial were *Up-shifted* on Day 11 and rewarded with (10) sucrose pellets per trial to create a positively valenced outcome. Induction of positive affect was evidenced by increased approach behavior toward the food cup and this

change was quantified by recording differences in number of "nose pokes" between Day 10 and 11 to interrupt the infra-red beam in the recessed food cup. Subjects in the *Up-shift* group are referred to as (Group 1-10) while the remaining one-half of subjects that did not experience a change in reward quantity after the shift are labeled as (i.e., Group 1-1).

STATISTICAL ANALYSIS

Behavioral measures from the fear-conditioning task were expressed as the mean percentage of time ± standard errors (SE) rats spend immobile during the presentation of the three retention test tones. Between-group comparisons for freezing behavior measured during retention testing was made with a factorial and repeated measures analysis of variance (ANOVAs) followed by Fisher's *post-hoc* tests. Differences less than $p = 0.05$ were considered statistically significant.

In the operant task, between-group comparisons for total number of lever presses and nose pokes made during the session on day 10 and *shift* day were made with a factorial two-way analysis of variance (ANOVA) followed by Fisher's *post-hoc* tests. Between-group and within-group comparisons for changes in lever presses and nose poking across day 10 and *shift* day were made with a repeated measures two-way analysis of variance followed by Fisher's *post-hoc* tests. A repeated measures ANOVA was also used for between-group comparisons of trial-by-trial changes in lever pressing and nose poking on each day. Differences less than $p = 0.05$ were considered statistically significant.

WESTERN BLOTTING

Thirty minutes, 1 h or 90 min following completion of Pavlovian fear conditioning animals were briefly sedated with isoflorine gas and decapitated. The brains were quickly removed and placed in ice cold 0.9% saline for a few minutes. Three 2 μm slices were taken per animals and the amygdala in each hemisphere was punched with 1 μm diameter punch (Fine Tools). Punches from the right and the left amygdala for each animal were placed in separate vials containing 100 μl of RIPA buffer and 1 μl of protease inhibitor. Samples were vortexed, placed on ice for 30 min and then centrifuged at 21000 rcf for 10 min at 4°C. Total protein concentration contained in the right and left amygdala homogenate of each animal was determined using a spectrophotometer (absorbance set at 562 mm) and Micro BCA protein assay kit (Thermo scientific). Approximately 5.5 μg of protein from the left and right amygdala of each animal was combined with *Laemlli* 2 × and 5% β-mercaptoethanol and boiled at 95°C for 4 min. The protein solution was loaded into a separate well and then ran on 10% Tris-HCl gel (Bio-Rad). MagicMark (Invitrogen) was run on all of the gels to determine molecular weight for each immunoreactive band. Proteins were transferred from the gel to a nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad). Membranes were blocked overnight at 4°C in a blocking buffer composed of 5% membrane blocking agent (GE Healthcare) in Tris buffered saline (TBS) and 0.1% 20-Tween (TBS-Tween). Prior to antibody incubation, membranes underwent several washes in TBS-Tween for a total of 30 min. During the first round of probing, the primary antibody was anti-Arc diluted in TBS-Tween (rabbit polyclonal; 1:3000, Synaptic Systems) for 1 h followed by an additional 30 min of washes. The membrane was then incubated for an hour with the secondary HRP-linked antibody (goat anti-rabbit; 1:3000, Millipore). Following an additional 30 min wash, chemiluminescence (ECL Plus Western Blotting detection system; GE Healthcare) was used to detect immunoreactivity. A stripping buffer (Thermo Scientific) was then applied to the membranes for 15 min. Membranes underwent five, 10-min washes in TBS-Tween. The membranes were then blocked for 1 h at room temperature in the blocking buffer mentioned previously. The second round of probing consisted of incubation in the primary antibody anti-β actin diluted in the blocking buffer (mouse; 1:10,000, Sigma) for an hour and in secondary HRP-linked antibody (goat anti-mouse; 1:80,000, Sigma). Washes and chemiluminescence were conducted in the same manner as during the first round of probing. Densitometric quantification was conducted by scanning the films and analyzed band density with Nikon Imaging Software.

IMMUNOFLUORESCENCE

Extraction procedures

One hour after completion of Pavlovian fear conditioning or the operant task, animals were anesthetized with pentobarbital. Homecage animals were brought to the lab and anesthetized with pentobarbital. All animals were then perfused transcardially with a 0.9% saline solution followed by a mixture of 4% paraformaldehyde for 5–10 min. The brains were removed and submerged in a 4% paraformaldehyde solution for 24 h, placed in a 30% sucrose solution for 48 h, dissected on a vibratome at a thickness of 50 μm and stored in phosphate buffered saline (PBS) containing 0.1 M sodium azide at 4°C.

Immunofluorescence procedures

Tissue samples were treated with two different fluorescence dyes to first, visualize *Arc* expression and second, to visualize cell bodies within the amygdala. The samples were incubated in an ice cold 1:1 ratio of acetone: methanol mixture for 10 min. After gentle rinsing in 2 × SSC for 5 min, the tissue was incubated for 15 min to reduce nonspecific interactions. The tissue was rinsed again in 2 × SSC for 5 min and then quenched in enogenous peroxidase for 15 min. Following thorough washes in 2 × SSC, the tissue was incubated in TSA blocking buffer for an hour and then with the primary anti-*Arc* antibody (1:1,000 dilution, SySy, Gottingen, Germany), for 24 h at 4°C. The next day after more thorough washes in 2 × SSC, the tissue was incubated in biotinilated secondary anti-rabbit biotin antibody (1:500 dilution, Vector Laboratories, Burlingame, CA) for 24 h at 4°C. The tissue was then rinsed in 2 × SSC, incubated in ABC Elite Reagent for 5 min, washed an incubated in Cyanine 3 tyramide (Cy3) reagent for 45 min (1:50 dilution, Perkin-Elmer, Waltham, MA) and dapi (1:500 dilution, Invitrogen, Carlsbad, CA) for 15 min. The samples were then washed several times in 2 × SSC tween and TBS in between the Cy3 reagent and dapi staining. Prior to mounting, the tissue was gently rinsed in TBS for 10 min.

Quantification procedures

The tissue was imaged using Nikon imaging software at a magnification of 40×. Three slices per animal ranging from −2.6

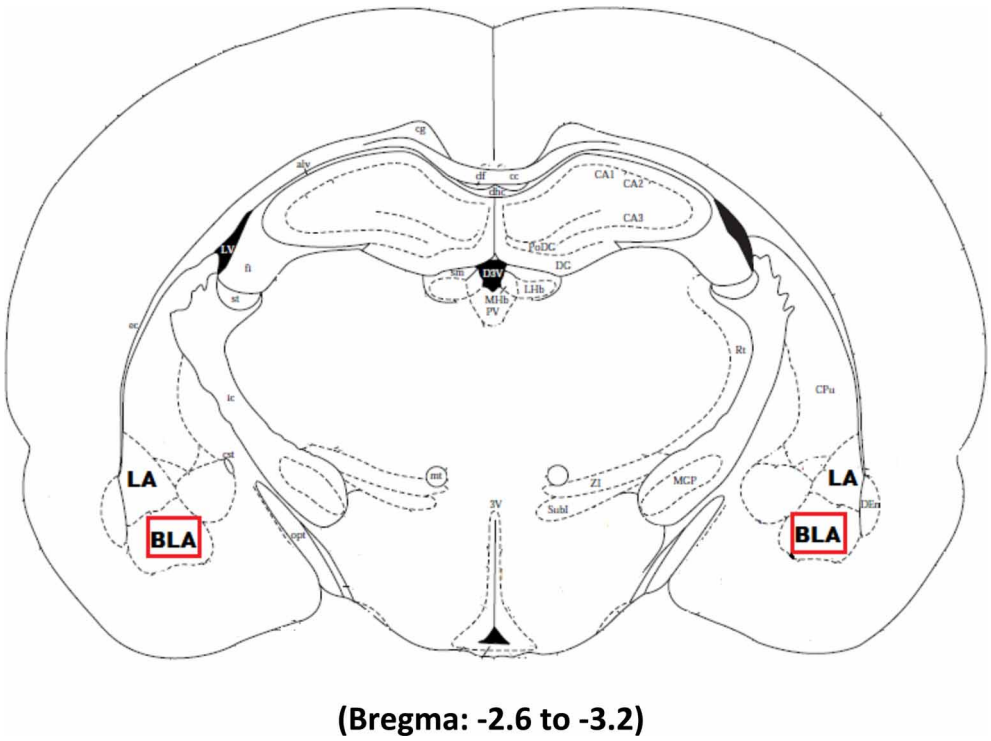

FIGURE 1 | The boxed square represents the area of interest between −2.6 and −3.2 from Bregma selected for quantification of *Arc* signal in the right and left basolateral amygdala after Pavlovian Fear Conditioning (Experiments 1 and 2) or Appetitive Training with positive or negative reinforcement contingencies (Experiment 3).

to −3.2 from bregma were chosen for imaging. **Figure 1** illustrates the area of interest in the most medial aspect of the right and left basolateral amygdala selected for imaging in Experiments 2 and 3 whereas the whole amygdala was selected for imaging in Experiment 1. *Arc* expression was analyzed using 1 μm *z* stacks with 30 steps at an exposure time set to 30 ms. The median and 2 standard deviations above the mean was determined for each image and then averaged together for each animal. The intensity threshold was individually set for each animal to be 2 standard deviations above their mean signal intensity. The size threshold was set to be the same size as a dapi cell. A macro was written to count every signal above the intensity and size threshold. Expression was quantified as the number of Cy3 labeled cells in the area of interest and a marco counted every dapi cell contained within this region to determine the percentage of cells expressing *Arc*.

RESULTS

EXPERIMENT 1

Behavioral data

Western blots were run to analyze *Arc* protein levels in animals that had previously undergone Pavlovian fear conditioning. *Arc* protein levels associated with fear conditioning were examined 30, 60, and 90 min following training. Protein levels of animals subjected to negatively valenced stimuli during Pavlovian conditioning was compared to controls that experienced tone presentations in the absence of footshock or controls that remained within their homecage. As depicted in **Figure 2**, a two-way ANOVA on the behavioral data indicated that animals trained in the fear conditioning task froze significantly more than controls that only experienced five tone presentations [$F_{(3,\ 19)} = 32.0$, $p < 0.01$]. Comparison of freezing levels during the fifth tone presentation using a Factorial analysis revealed that animals that underwent fear conditioning froze significantly more than animals that only experienced the tone presentations (*Tone Only* vs. *Fear Conditioning 30 min*, $p < 0.01$; *Tone Only* vs. *Fear Conditioning 60 min*, $p < 0.01$; *Tone Only* vs. *Fear Conditioning 90 min*, $p < 0.01$).

Western blot data

As illustrated in **Figure 3A**, fear conditioning significantly affected *Arc* expression in the right but not left amygdala 60 and 90 min following training [$F_{(1,\ 24)} = 4.3$, $p < 0.05$]. Further analysis (**Figure 3B**) revealed that experiencing either tone presentations or fear conditioning elicited greater *Arc* expression compared to levels measured in homecage control animals (Tone Only vs. Homecage, $p < 0.05$; Fear Conditioning 30 min vs. Homecage, $p < 0.01$; Fear Conditioning 60 min vs. Homecage, $p < 0.01$; Fear Conditioning 90 min vs. Homecage, $p < 0.01$). Furthermore, levels of *Arc* expression in fear conditioned animals was greater than levels sampled from animals presented with only the tone (Fear Conditioning 30 min vs. Tone Only, $p < 0.01$; Fear Conditioning 60 min vs. Tone Only, $p < 0.01$; Fear Conditioning 90 min vs. Tone Only, $p < 0.01$). Factorial analysis showed that

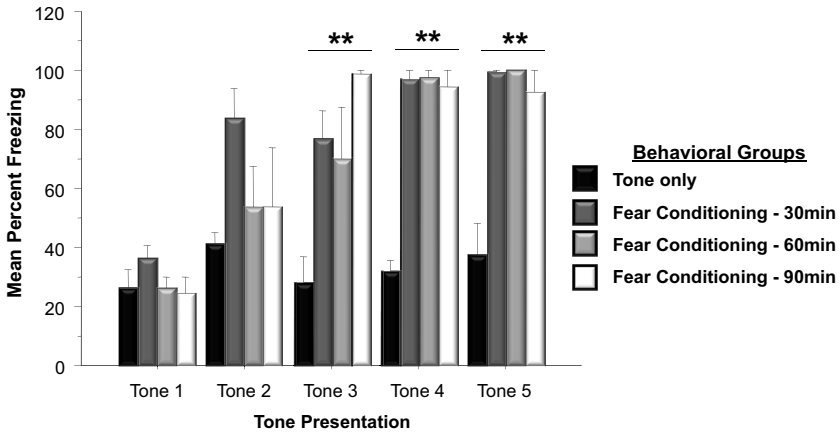

FIGURE 2 | Mean (+SE) percentage of time freezing during Pavlovian conditioning with a 30 s tone CS that coterminated with a 1 s 35 mA footshock. Subjects in the Fear Conditioning group displayed significantly more freezing during the final three tone presentations than controls in the Tone Only Group (Fear Conditioning 30 min vs. Tone Only, $p < 0.01$; Fear Conditioning 60 min vs. Tone Only, $p < 0.01$; Fear Conditioning 90 min vs. Tone Only, $p < 0.01$). $^{**}p < 0.01$.

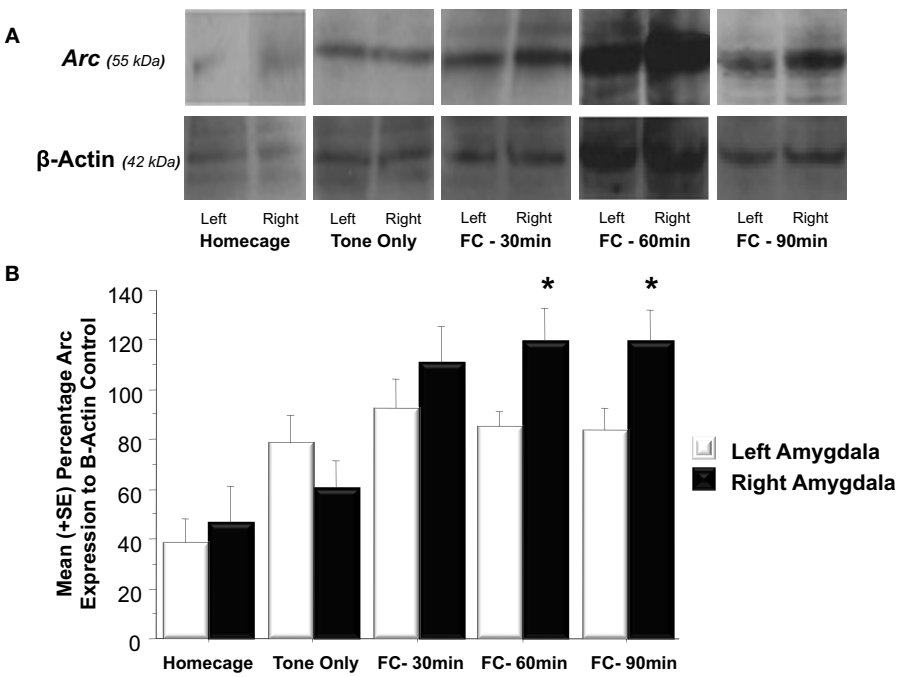

FIGURE 3 | (A) Western blot analysis of left and right amygdala homogenates of Homecage controls, Tone only controls and animals that received Pavlovian fear conditioning with five tone-shock pairings 30, 60, or 90 min prior to sacrifice and brain extraction. **(B)** Western blot quantification of *Arc* expression in the left and right amygdala of the three groups described in **(A)**. Fear conditioning produced a significant increase in *Arc* expression relative to the change produced by resting in the homecage or following tone presentation (FC 30 min vs. Tone Only, $p < 0.01$, FC 60 min vs. Tone Only, $p < 0.01$, FC 90 min vs. Tone Only, $p < 0.01$). *Arc* levels measured in the left and right amygdala did not differ in Homecage controls, Tone Only or the Fear Conditioning 30 min group. *Arc* expression was significantly elevated in the right amygdala compared to the left at 60 and 90 min following fear conditioning (FC 60 min: right vs. left, $p < 0.05$; FC 90 min: right vs. left, $p < 0.05$). $^{*}p < 0.05$.

Arc is not expressed at similar levels in the left and right amygdala following training. Thirty minutes following training, *Arc* is expressed at similar levels in both the right and left amygdala ($p = ns$). However, 60 min following training there was significantly more *Arc* protein in the right amygdala compared to the left ($p < 0.05$). *Arc* expression in the right amygdala was still elevated compared to the left 90 min following training ($p < 0.05$). These findings indicate that Pavlovian fear conditioning elicits asymmetric expression of *Arc* 60 min following training which remains elevated for an additional 30 min.

EXPERIMENT 2

Behavioral data

The western blots conducted in Experiment 1 examined *Arc* protein levels in the whole amygdala whereas Experiment 2 examined *Arc* protein expression in the basolateral amygdala. The right and left basolateral amygdala was imaged using immunohistochemistry to determine *Arc* expression following a physically unpleasant training task, Pavlovian fear conditioning. A two-way ANOVA indicated that animals that received footshocks during training froze significantly more than animals that only experienced tone presentations [$F_{(1,\ 10)} = 39.8, p < 0.01$]. As shown in **Figure 4**, animals assigned to the fear conditioning group froze significantly more than animals in the Tone Only group during Tone 2 ($p < 0.01$), Tone 3 ($p < 0.01$), Tone 4 ($p < 0.01$), and Tone ($p < 0.01$).

Immunohistochemistry data

Tissue samples extracted from the right and left basolateral were processed to determine if *Arc* expression was asymmetrically distributed in response to Pavlovian fear conditioning (see **Figure 5A**). As shown in **Figure 5B**, a factorial analysis demonstrated no significant differences in *Arc* expression in the right and left basolateral amygdala of homecage controls ($p = ns$) or those presented with the tone only. However, right basolateral amygdala *Arc* expression in fear conditioning animals was significantly higher than levels found in the right or left amygdala of homecage controls ($^{*}p < 0.04$ for both comparisons) as well as both hemispheres in subjects exposed to only the CS-tone ($^{**}p < 0.01$ vs. right; $^{*}p < 0.05$ vs. left amygdala).

To further test *Arc* expression levels in the basolateral amygdala following a negatively valenced learning experience, the percentage of cells that displayed *Arc* signaling was determined (see **Figure 5B**). There was a significant overall effect of hemisphere and group on the percent of *Arc* activation [$F_{(5,\ 244)} = 2.91$, $p < 0.03$]. The percent of cells that displayed *Arc* signaling in animals assigned to the homecage group was not significantly different between hemispheres ($p = ns$). In animals that experienced fear conditioning there was a significantly higher percent of cells that displayed *Arc* signal in the right basolateral compared to the left ($p < 0.05$). As shown in **Figure 5C**, there were no differences in *Arc* expression between the right and left basolateral amygdala in animals assigned to the Tone Only group ($p = ns$). The percent of cells expressing *Arc* in the right basolateral amygdala of Fear Conditioning animals was significantly higher than levels measured in Homecage or Tone Only animals ($p < 0.05$). These finding indicate that exposure to emotionally aversive learning experiences such as Pavlovian fear conditioning induces an asymmetric increase of *Arc* expression in the right basolateral amygdala.

EXPERIMENT 3

Behavioral data

Experiment 1 and 2 utilized two different molecular approaches to examine *Arc* expression following exposure to negatively valenced noxious stimuli associated with Pavlovian fear conditioning. Experiment 3 extends these findings by examining whether induction of positive affect by increasing the magnitude of expected rewards or alternatively, generating frustration by violating a subject's expectations of reinforcement may be registered by more robust patterns of *Arc* expression across the left and right amygdala respectively. Each of four groups received 10 consecutive days of training. Two of these groups were rewarded with one pellet after completing each FR schedule and the other pair was given the higher quantity of 10 pellets. Factorial analysis on mean bar presses per group revealed a significant overall effect [$F_{(3,\ 21)} = 37.5, p < 0.01$]. Fisher's *Post-hoc* analysis revealed that the magnitude of reward (1 or 10 pellets) influenced the number of lever presses. As illustrated in **Figure 6A**, subjects given one pellet made significantly more lever presses than those in the 10 pellet reward group (1-1 vs. 10-10, $p < 0.01$; 1-1 vs. 10-1, $p < 0.05$; 1-10 vs. 10-10, $p < 0.01$; 1-10 vs. 10-1, $p < 0.01$). However, there were no differences in lever press performance between paired

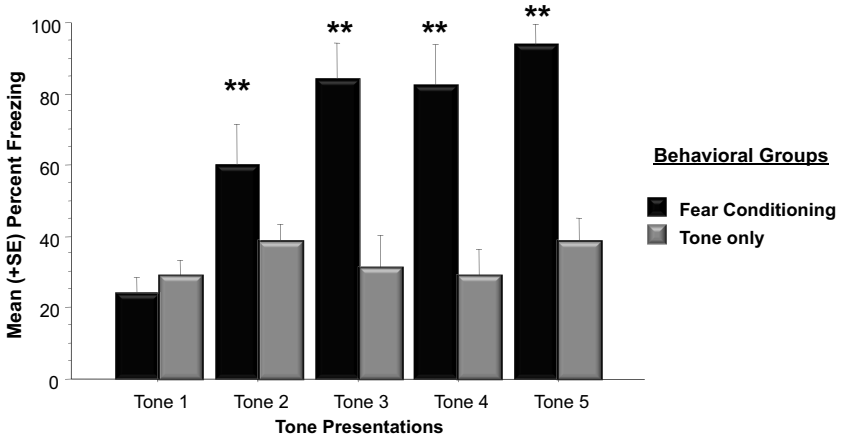

FIGURE 4 | Mean (+SE) percentage of time spent freezing during training to each tone presentation. Animals utilized for immunohistochemistry underwent either five tone presentations or fear conditioning consisting of five tone-shock pairs. Animals that received a footshock coterminating with the tone presentation froze significantly more during Tones 2–5 (Tone 2: Fear Conditioning vs. Tone Only, $p < 0.01$; Tone 3: Fear Conditioning vs. Tone Only, $p < 0.01$; Tone 4: Fear Conditioning vs. Tone Only, $p < 0.01$; Tone 5: Fear Conditioning vs. Tone Only, $p < 0.01$). $^{**}p < 0.01$.

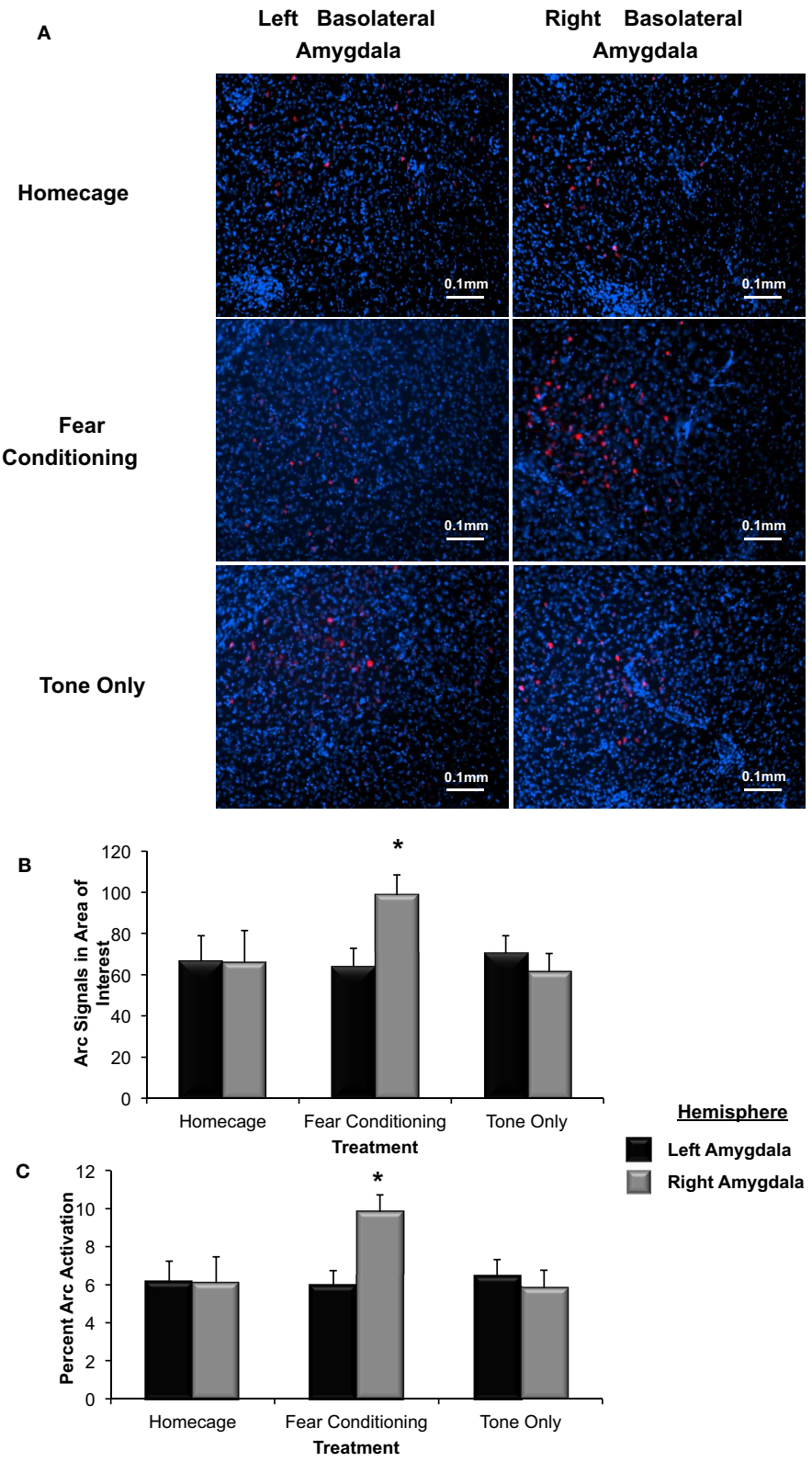

FIGURE 5 | (A) Immunohistochemical analysis of the effect of fear conditioning on *Arc* expression in the right and left basolateral amygdala. Fear conditioning elevates *Arc* immunoreactivity in the right, but not left basolateral amygdala. **(B)** Mean (+SE) number of *Arc* signals measured in the basolateral amygdala. Tone presentation alone did not alter *Arc* expression in the basolateral amygdala compared to the signals measured in Homecage controls. Fear conditioning with five tone-shock pairings produced a

(Continued)

FIGURE 5 | Continued
significant increase in *Arc* expression that was statistically greater in the right, relative to left basolateral amygdala (*$p < 0.05$). The level of *Arc* expression in the right amygdala of the Fear Conditioning group was also significantly greater than *Arc* expression measured in the right or left amygdala of homecage controls or the Tone only controls (*$p < 0.05$). **(C)** Quantification of *Arc* expression in the amygdala reveals that the percentage of cells expressing *Arc* was only elevated in the right basolateral amygdala of animals that experienced fear conditioning when compared to Homecage controls or Tone only controls (*$p < 0.05$).

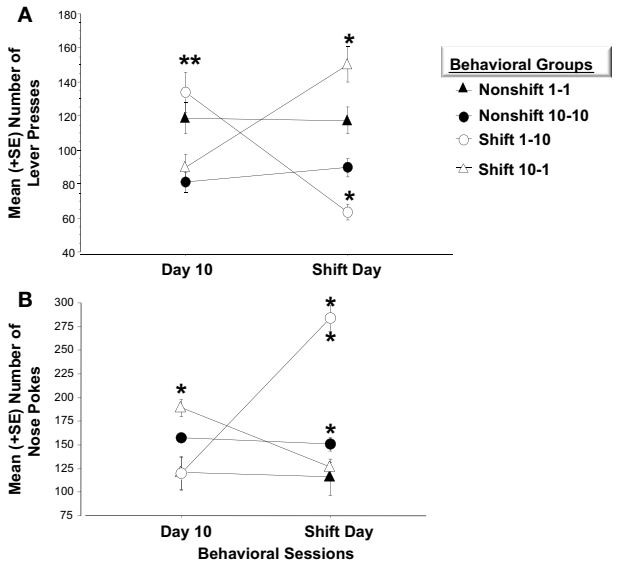

FIGURE 6 | (A) Mean (+SE) number of lever presses on the final day of initial training and in response to the unexpected change in reward magnitude on the *Shift Day*. On Day 10, animals accustomed to only (1) sucrose pellet following each FR-5 schedule (i.e., *open circle and closed triangle*), make significantly more lever presses than those given (10) sucrose pellets for each FR-5 during the first 10 days of training, (**$p < 0.01$). Animals assigned to either of the *Nonshift* groups (i.e., *closed triangle and circle*) did not alter lever pressing behavior on the *Shift* day. A decrease in reward quantity (i.e., *Shift* 10-1*; open triangle*) caused a significant increase in lever press responses relative to the number recorded on Day 10 (**$p < 0.01$) before the decrease in reward magnitude. Subjects in the *Down-shift* group displayed a significantly higher level of lever presses on the *Shift* day compared to animals that received (1) sucrose reward throughout the experiment (**$p < 0.01$; *open triangle* vs. *closed triangle*). Conversely, animals that experienced the *Up-shift* in reward from (1) to (10) sucrose pellets (i.e., *open circle*) decreased lever pressing behavior on the *Shift* day compared to Day 10 (**$p < 0.01$). **(B)** Mean (+*SE*) number of nose pokes into the food hopper on the last day of training and on the *Shift* day. On Day 10, the last day of training, animals that received (10) sucrose pellet following each FR 5, nose poked into the food hopper significantly more than animals that received (1) sucrose pellets ($p < 0.05$). *Non-shift* groups received the same reward magnitude throughout the experiment and did not altered nose poke behavior on Day 11. Animals that experienced an *Up-shift* in reward magnitude from (1) to (10) sucrose pellets increased nose poke behavior following the shift compared to Day 10 ($p < 0.01$). Additionally, these animals made more nose pokes on the *Shift* day compared to animals assigned to the *Non-shift* 10-10 group ($p < 0.01$). Conversely, animals that received a *Down-shift* from (10) to (1) sucrose pellets decreased nose poke behavior compared to Day 10 behavior ($p < 0.05$). *$p < 0.05$; **$p < 0.01$.

groups given the same quantity of reward before the experimental shift (i.e., 10-10 vs. 10-1; $p = ns$; 1-1 vs. 1-10; $p = ns$). Reward magnitude also influenced the number of nose pokes into the food dispenser. Factorial ANOVA's run on the mean number of nose pokes during Day 10 revealed a significant overall effect [$F_{(3,\ 21)} = 58.2$, $p < 0.01$]. *Post-hoc* analysis revealed inverse trends to bar pressing performance. Mainly, subjects rewarded with 10 sucrose pellets made more pokes than those in the one pellet groups (Non-shift 10-10 vs. Non-shift 1-1, $p < 0.05$; Non-shift 10-10 vs. Up-shift 1-10, $p < 0.01$; Down-shift 10-1 vs. Non-shift 1-1, $p < 0.05$; Down-shift 10-1 vs. Up-shift 1-10, $p < 0.01$) (**Figure 6B**).

Animals in the non-shifted groups continued to receive the same quantity of rewards on the shift day as during training (i.e., 1-1 and 10-10). Thus, no changes in lever pressing were expected on the shift day. Repeated measures ANOVA verified that these groups did not differ on any of the behavior measures. However, the unexpected increases or decreases in reward quantity caused by the shift on Day 11, significantly affected lever press and nose poke behavior. A repeated measure ANOVA indicated that bar pressing performance in subjects experiencing the downshift (i.e., from 10 to 1 pellet) increased significantly on the day of the shift ($p < 0.01$) while nose poke responses decreased ($p < 0.05$). Conversely, animals assigned to the upshift group and therefore given 10 as opposed to 1 sucrose pellet displayed a significant decrease in bar pressing ($p < 0.01$) while significantly increasing nose poke behavior ($p < 0.01$). The changes in lever press performance observed in the "Frustrated" downshift group (10-1) and in the "Elated" upshift group (1-10) conformed to Crespi's (1942) finding that previous reward history and expectations of reward quantity impacts later performance when these expectations are violated.

Factorial analysis on behavior of Day 11 revealed a significant overall effect of changes in reward quantity on bar press [$F_{(3,\ 21)} = 24.8$, $p < 0.01$] and nose poke behavior [$F_{(3,\ 21)} = 38.3$, $p < 0.01$]. As shown in **Figure 6A**, animals assigned to the Frustrated, downshift group made more lever presses after the decrease in reward quantity than the non-shifted subjects that continued to receive 10 pellets ($p < 0.01$). They also bar pressed more than subjects assigned to receive one pellet for the duration of the study (1-1) ($p < 0.01$). Conversely, animals assigned to the upshift group made significantly fewer lever presses following the increase in reward quantity than animals assigned to the nonshift groups (1-10 vs. 10-10, $p < 0.05$; 1-10 vs. 1-1, $p < 0.01$). Additionally, as shown in **Figure 6B** these animals made significantly more nose pokes than animals that were rewarded with 10 sucrose pellets throughout training ($p < 0.01$).

Immunohistochemistry data

Arc expression was measured following a violation of the organism's expectation of reward quantity to determine whether these emotional reactions are reflected by asymmetric patterns of *Arc* expression in the left or right basolateral amygdala. As illustrated in **Figure 7A**, *Arc* expression in area of interest was elevated in the right but not left basolateral amygdala following a downshift in

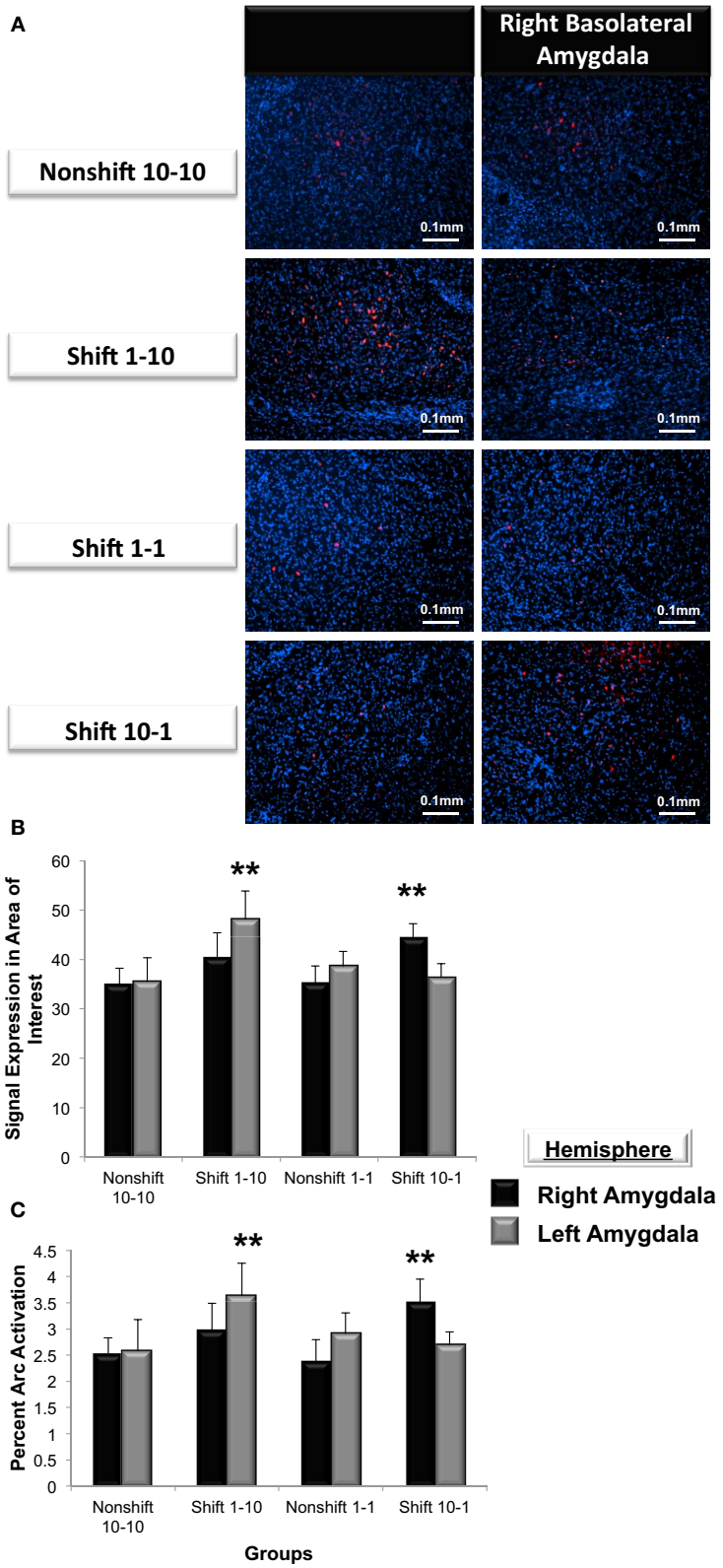

FIGURE 7 | (A) Immunohistochemical analysis of *Arc* expression in the basolateral amygdala after an unexpected increase (*Shift* 1-10), decrease (*Shift* 10-1) or no change in reward magnitude following FR-5 lever pressing. The behavioral manipulations produced a significant change in left basolateral *Arc* expression only following an increase in reward quantity (*Shift* 1-10), whereas right basolateral *Arc* expression was elevated only by the unexpected reduction in reward quantity after the FR-5 (*Shift* 10-1). **(B)** Mean

(Continued)

FIGURE 7 | Continued
(+SE) number of *Arc* signals measured following the Shift in reward expectancies. There were no changes in *Arc* signal sampled from the amygdala of Non-shifted animals. *Arc* signals in the left basolateral amygdala were significantly greater than levels in the right (**$p < 0.01$) in subjects assigned to receive an *up-shift* in reward quantity (*Shift* 1-10). Conversely, *Arc* signals in the right basolateral amygdala were elevated compared levels sampled from left, in the group that experienced an unexpected *Down-shift* (*Shift* 10-1) in reward magnitude (**$p < 0.01$).

(C) Quantification of the mean percentage of basolateral cells that express *Arc* following presentation of stimuli of opposing valence. Approximately 2.5% of cells in the basolateral amygdala express *Arc* in animals that received an expected reward quantity of either 10 or 1 sucrose pellets. Significantly more cells in the left, but not right basolateral amygdala expressed *Arc* following an expected *Up-shift* in reward quantity (**$p < 0.01$). Conversely, following a *Down-shift* more cells in the right basolateral amygdala expressed *Arc* compared to levels sampled in the left basolateral amygdala (**$p < 0.01$).

reward quantity (i.e., *from* 10-1 *food pellets*) and elevated in the left but not right following an upshift (i.e., *from* 1-10 *food pellets*) in reward quantity [$F_{(3,\ 21)} = 3.9$, $p < 0.01$]. Factorial analysis revealed that *Arc* expression measured in the right or the left basolateral amygdala in nonshift groups was not significantly different (1-1: right vs. left, $p = ns$; 10-10: right vs. left, $p = ns$). As shown in **Figure 7B**, animals that expected 10 sucrose pellets and were downshifted to receive only 1 pellet exhibited a greater level of *Arc* expression in the right but not left basolateral amygdala (right vs. left, $p < 0.01$). Conversely, subjects that experienced an upshift from 1 to 10 sucrose pellets there was greater *Arc* expression in the left but nor right basolateral amygdala (right vs. left, $p < 0.01$).

To validate these findings, *Arc* expression was quantified as the percentage of cells located in the area of interest expressing *Arc* (**Figure 7C**). The valence of a learning task selectively increased the percentage of cells expressing *Arc* in one amygdala vs. the contralateral side ($F_{(3,\ 21)} = 5.2, p < 0.01$). Factorial analysis revealed that the percent *Arc* expression measured in the right or the left basolateral amygdala in nonshift groups was not significantly different (1-1: right vs. left, $p = ns$; 10-10: right vs. left, $p = ns$). Consistent with the findings described above, the downshift in reward quantity produced a greater percent change in *Arc* expression in the right but not left basolateral amygdala (right vs. left, $p < 0.05$) whereas the positively valenced upshift led to greater levels of Arc expression in the left but not right basolateral amygdala (right vs. left, $p < 0.01$).

DISCUSSION

Previous reports from functional scans (Morris et al., 1999; Zalla et al., 2000; Hamann et al., 2002; Etkin et al., 2004), lesions (Blundell and Adamec, 2007; Orman and Stewart, 2007; Carrasquillo and Gereau, 2008), and electrophysiology studies (Lanteaume et al., 2007; Ji and Neugebauer, 2009) indicate that the amygdala encodes emotionally arousing events differentially, depending on the valence of the stimuli Additionally, a novel finding to emerge from our lab is that norepinephrine released in the basolateral amygdala encodes negatively or positively arousing learning tasks through asymmetric norepinephrine release in right or left basolateral amygdala, respectively (Young and Williams, 2010). Furthermore, there is evidence that enhancing asymmetric norepinephrine activity in the right, but not left basolateral amygdala potentiates memory formation for negatively arousing events (LaLumiere and McGaugh, 2005). However, whether asymmetric neurotransmitter activity in the amygdala influences molecular events associated with memory formation has not been investigated. The current studies were developed to address this shortcoming in the literature by assessing whether *Arc* is selectively expressed in either the right and left basolateral amygdala following exposure to either negatively or positively arousing learning conditions.

To assess valence dependent expression of *Arc* in the amygdala, it was first necessary to determine if the valence of a learning condition produces differential patterns of *Arc* across the right or left amygdala and then examine whether the expression fluctuates during the time period this gene plays an active role in memory formation. Experiment 1 was instrumental in achieving this objective by utilizing western blot analysis of *Arc* expression in the right and left amygdala of naïve, non-aroused and negatively aroused animals. For example, experiencing either the non-arousing five tone presentations or the negatively arousing fear conditioning increased overall *Arc* expression by 162 and 237%, respectively, compared to levels measured in naïve Homecage animals. Furthermore, experiencing fear conditioning elicited significantly greater overall *Arc* expression in the amygdala compared to animals that were only presented with the auditory tones. Further analysis comparing levels of *Arc* in the right and left amygdala revealed that there was no significant different between *Arc* levels sampled from the right and left amygdala in Homecage and Tone Only animals. Interestingly asymmetric differences were only found in animals that underwent Pavlovian fear conditioning.

The second objective of Experiment 1 was to ascertain whether selective expression of *Arc* fluctuated across the three sampling periods selected at 30, 60, and 90 min following Pavlovian fear learning. For example, *Arc* levels in the right and left amygdala did not differ significantly at 30 min following Pavlovian fear learning but were elevated in the right amygdala compared to the left at 60 and 90 min following training. This result is consistent with previous findings showing that *c-fos* expression, a different immediate-early gene, is asymmetrically elevated in the right amygdala following reexposure to a negatively arousing context (Sciclli et al., 2004). Furthermore, the current findings indicate that *Arc* expression in the right amygdala is downstream of norepinephrine release in the basolateral amygdala. Fear conditioning was previously shown to significantly elevate norepinephrine release in the right basolateral amygdala compared to the left 20 min following training and to remain elevated for 40 min (Young and Williams, 2010). The findings that emerged from this study indicates that *Arc* expression in the right amygdala is significantly elevated from levels sampled from the left at 60 and 90 min following training by which time norepinephrine levels are returning to baseline amounts.

Experiment 2 extended these findings by assessing whether *Arc* expression in the basolateral amygdala is selectively elicited in

the right basolateral amygdala following fear learning since norepinephrine concentrations are elevated in the right but not left basolateral following this type of training (Young and Williams, 2010) and treatments that increase activity of this transmitter in only the right basolateral are is sufficient to enhance memory (Coleman-Mesches et al., 1996; Baker and Kim, 2004; LaLumiere and McGaugh, 2005). Based on findings garnered from Experiment 1, *Arc* expression was measured in naïve animals or 60 min following either five tone presentations or fear conditioning. Fear conditioning with five tone-shock pairings produced significantly more freezing during presentation of the last four tones than that observed in subjects given tone-only presentations without footshock. The differences in freezing reflect the level of associative CS-US learning in the former group whereas the Tone Only subjects displayed minimal levels of fear to the tone since this auditory cue was not accompanied with an aversive consequence like footshock. Immunohistochemical findings from this experiment differ from finding in Experiment 1 in that tone only presentations did not significantly alter *Arc* levels in the basolateral amygdala compared to Homecage animals. This finding is likely due to the fact that the majority of tonal information is processed by the lateral, and not the basolateral amygdala. Therefore, overall *Arc* expression in the whole amygdala would reflect tone presentation whereas *Arc* expression in the basolateral amygdala would not.

Findings emerging from Experiment 2 reveal that exposure to negatively arousing learning conditions are accompanied by a lateralized increase in *Arc* expression in the right basolateral amygdala. Naïve Homecage and Tone Only animals did not demonstrate hemispheric differences in *Arc* expression whereas those subjected to tone-shock pairings during fear conditioning displayed a significantly higher level of *Arc* signals and percentage of cells expressing *Arc* in the right, but not left basolateral amygdala. Approximately, 3.6% of cells, 45 cells total, in the right basolateral amygdala expressed *Arc* following fear conditioning. The level of *Arc* expression sampled from the amygdala corresponds to levels reported previously in the dentate gyrus of the hippocampus and the basolateral amygdala. For example, approximately 2% of cells in the basolateral amygdala of Homecage controls expressed *Arc*, a number that is slightly higher than the 1.6% of cells previously reported to express *Arc* in the dentate gyrus (Small et al., 2004). While there the majority of studies examine *Arc* expression following negative arousal there is paucity of studies that examine the mechanisms that permit positively arousing stimuli to produce lateralized changes in neuronal functioning in the basolateral amygdala.

To verify if *Arc* expression in the basolateral amygdala is affected by positively or negatively valenced outcomes, an appetitive behavioral task with reinforcement contingencies of opposing valance was utilized to elicit either positive or negative arousal. Experiment 3 was instrumental in meeting this objective by including an *Up-shift* or *Down-shift* in expected reward magnitude to represent positive and negative changes in the subjects learned expectations respectively. Accordingly, these experimental manipulations produce patterns of responding characteristic of those observed following exposure to positive or negatively valenced environmental events. For example, animals subjected to the *Up-shift* in reward quantity from (1) to (10) sucrose pellets displayed a 48% reduction in lever press behavior relative to their performance on the previous non-shifted day of training. *Up-shift* animals also made significantly fewer level presses than animals that received 10 sucrose pellet throughout training. Interestingly, animals assigned to the *Up-shift* displayed a 237% increase in nose poke behavior compared to Day 10. This increase in behavior is not the result of receiving 10 sucrose pellets because *Upshift* animals made on average 133 more nose pokes on Day 11 than animals that received 10 sucrose pellets throughout training. Rather, this form of increased behavioral responding is most likely due to the induction of positive affect associated with the organism's perception of the large contrast in the magnitude of expected vs. actual rewards (Crespi, 1942). Increases in reward magnitude has been used previously to amplify positive affect as laboratory rats increase consumption through lick rate during an *Up-shift* produced by higher levels of sucrose concentration (Ainge et al., 2006; Liang et al., 2008). However, licking behavior is decreased at the beginning of a trial when animals anticipate an *Up-shift* in sucrose concentrations to occur during the latter half of the trial (Weatherly et al., 2005).

An important finding to emerge from this study is that a positively arousing learning conditioning consisting of an unexpected increase in reward quantity elicited a significant elevation in *Arc* expression in the left but not right basolateral amygdala. For example, the brains of animals surprised with an unexpected increase in reward quantity to 10 vs. 1 food reward displayed a higher percentage of cells in the left basolateral amygdala that expressed *Arc* compared to the right. This increases in *Arc* expression was also significantly greater than that observed in subjects that received the expected reward of 10 sucrose pellets throughout training. Additionally there was no difference between *Arc* levels measured in the right and left basolateral amygdala of these animals. These findings indicate there are significant asymmetric changes in the levels of *Arc* expressed in the left basolateral amygdala following exposure to a positively arousing learning event. A number of studies have examined *Arc* expression following stressful events, such as fear conditioning, conditioned place aversion or novelty (Ploski et al., 2008; Barot et al., 2009; Hou et al., 2009; Panja et al., 2009). This is the first study to investigate *Arc* expression following a rewarding experience and it indicates that a similar number of cells encode rewarding learning conditions in the left compared to *Arc* expressing cells in the right basolateral amygdala following a negatively arousing learning condition.

In contrast to the behavioral *Up-shift* findings, a reduction in the expected number of rewarding sucrose pellets produced by a *Down-shift* in expected reward quantity led to dramatic increases in lever press responding. After the *Down-shift* in reward quantity, animals increased lever pressing behavior by 169%. An indication of increased arousal following the decrease in reward quantity is that *Down-shifted* subjects made significantly more lever presses than animals that received the (1) sucrose pellet reward throughout the 10 days of training as well as on the 11 th day of the shift. This type of negatively valenced event in the face of reduced reward has been associated with the onset of frustration and is replicated in several behavioral conditions that involve a discrepancy between an organism's expectation and the actual

amount of reward that it receives (Crespi, 1942; Levine et al., 1972; Goldman et al., 1973; Hatfield et al., 1996; Salinas et al., 1997). Frustration-induced increases in responding are produced by similar manipulations that violate an organism's expectations by reducing the expected magnitude of a reward (Amsel and Roussel, 1952; Corr, 2002).

The present findings expand previous reports by revealing that *Arc* expression is also elevated in the right basolateral amygdala following a behavioral condition that produces frustration. Sixty minutes following unexpected decrease from 10 to 1 sucrose reward pellets there was significantly more signals in the right basolateral amygdala compared to signals measured in the left. Additionally, there were a greater percentage of cells in the right basolateral amygdala that expressed *Arc* compared to the percentage of cells expressing *Arc* in the left basolateral amygdala. Approximately 45 cells in the area of interest in the right basolateral amygdala express *Arc* which is reasonable if 80 cells in the whole basolateral amygdala were labeled for *Arc* expression following fear conditioning (Ploski et al., 2008). Additionally, the level of *Arc* expression in the right basolateral amygdala following the *Down-shift* was similar to levels measured in the right basolateral amygdala following fear conditioning. These findings are not the result of reward magnitude because animals that received the expected 1 sucrose reward displayed significantly lower bilateral levels of *Arc* expression. Taken together, the present findings expand upon the current knowledge of *Arc* activity by indicating that stimuli of different valences are encoded asymmetrically in either the right or left basolateral amygdala for negatively or positively arousing learning conditions.

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In sync: gamma oscillations and emotional memory

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Emotional experiences leave vivid memories that can last a lifetime. The emotional facilitation of memory has been attributed to the engagement of diffusely projecting neuromodulatory systems that enhance the consolidation of synaptic plasticity in regions activated by the experience. This process requires the propagation of signals between brain regions, and for those signals to induce long-lasting synaptic plasticity. Both of these demands are met by gamma oscillations, which reflect synchronous population activity on a fast timescale (35–120 Hz). Regions known to participate in the formation of emotional memories, such as the basolateral amygdala, also promote gamma-band activation throughout cortical and subcortical circuits. Recent studies have demonstrated that gamma oscillations are enhanced during emotional situations, coherent between regions engaged by salient stimuli, and predict subsequent memory for cues associated with aversive stimuli. Furthermore, neutral stimuli that come to predict emotional events develop enhanced gamma oscillations, reflecting altered processing in the brain, which may underpin how past emotional experiences color future learning and memory.

Keywords: gamma oscillations, emotional memory, associative memory, arousal, amygdala

INTRODUCTION

Emotions are layered upon the stream of sensations that we experience throughout our lives. Beyond being merely another event in an experience, they shape the quality, vividness, and persistence of what is remembered. The mechanisms underlying this likely encompasses processes during encoding of the experience, its subsequent consolidation, and eventually retrieval.

In principle, the neural processes that support emotional memory should exhibit similar properties to emotional memory itself. Such an isomorphism underpins virtually every attempt to construct neural explanations for psychological processes (Boring, 1936). This review will argue that gamma oscillations contribute to the persistence and vividness of emotional memories. Suggestively, and independent of any observed linkage with emotion, gamma oscillations allow for efficient transmission of information between neural ensembles, and in doing so may produce lasting changes in their connectivity. More directly, gamma oscillations are observed in numerous studies of emotional memory and processing. As reviewed below, they are generated during circumstances that incite emotional arousal and are enhanced by stimuli that predict affective events. Furthermore, much evidence indicates that gamma oscillations predict subsequent memory for emotional experiences, and the accompanying neuroplasticity. This is not to say that gamma oscillations in particular support emotional memory, but that understanding why emotional events have such a profound impact on memory requires an appreciation of how they engage gamma oscillations.

BACKGROUND

EMOTIONAL MEMORIES: WHAT ARE THEY?

Before addressing the neural basis of emotional memories, we must outline their properties and how they are studied. Readers familiar with these concepts can skip to the subsequent sections.

Emotional memories are typified by their endurance, vividness, and ease of recall (Rapaport, 1971; Christianson and Loftus, 1987; Bradley et al., 1992; Cahill and McGaugh, 1995; Kensinger and Schacter, 2008). Operationally defined, emotional memories are long-lasting changes in behavior arising from affective experiences. Packed into this definition are two essential concepts. The first is a definition of memory itself, an experience-dependent change in behavior. In the behaviorist tradition, which is the *de facto* psychological paradigm in neuroscience, memory, like all psychological processes, is inferred by observing the actions of an organism. The other defining characteristic is the presence of affective events. What constitutes affective is deceptively simple. Affective stimuli often have a direct impact on an organism's homeostasis, either by providing nourishment or inflicting physical distress. But for many situations, the emotional stressor is just perceived or anticipated. For instance, emotional memory can arise from viewing a gruesome picture, or the delivery of bad news. Thus, an emotional experience does not require an overt biologically salient stimulus. And even if the stimulus is overtly affective, its salience is influenced by a subject's goals and circumstances; a food reward will not be arousing to a satiated subject.

The distinction between perceived and actual affective stimuli is most stark when comparing studies of emotional memory in humans and other animals. Affective stimuli in human memory studies are often images of violence or nudity (e.g., Bradley et al., 1992), at worst electric shocks, while in non-human animal studies they range from electric shocks to copulation (e.g., Pfaus et al., 2001). This systematic difference in the intensity of experiences used to study emotional memory complicates the search for a general cross-species framework. Nevertheless, we will assume that all subjects in the studies we review underwent emotional experiences.

Studies of emotional memory typically include three phases, and these will organize our coverage of the literature (**Table 1**). The first phase is *acquisition*, when subjects are exposed to the circumstances to be remembered. In most human studies, subjects are exposed to multiple stimuli, some neutral and others emotional, and they must remember which stimuli they were exposed to. For other species, a subject learns that particular neutral stimuli precede emotional ones. Pervading the description of these training paradigms is Pavlovian terminology, which will be used throughout this review (Pavlov, 1927). Neutral stimuli lacking inherent motivational properties are referred to as conditioned stimuli (CS). If multiple CSs are present, then a stimulus that predicts an affective event it is called a CS+, while one that does not is a CS−. Inherently affective stimuli are referred to as unconditioned stimuli (US).

Table 1 | Summary of cited literature on gamma oscillations and emotional stimuli.

Citation	Species	Emotional valence	Effect on gamma
PASSIVE VIEWING			
Freeman, 1960	Cat	Positive	↑
Müller et al., 1999	Human	Positive/negative	↑
Keil et al., 2001	Human	Positive/negative	↑
Oya et al., 2002	Human	Positive/negative	–/↑
Matsumoto et al., 2006	Human	Negative	↑
Balconi and Pozzoli, 2007	Human	Negative	↑
Luo et al., 2007	Human	Negative	↑
Basar et al., 2008	Human	Positive	–
Glauser and Scherer, 2008	Human	Positive/negative	↓
Oathes et al., 2008	Human	Negative	↑
Luo et al., 2009	Human	Negative	↑
Siegle et al., 2010	Human	Negative	↑
Jung et al., 2011	Human	Positive/negative	↑/↓
Sato et al., 2011	Human	Positive/negative	↑/–
Senkowski et al., 2011	Human	Positive/negative	↑
Martini et al., 2012	Human	Negative	↑
ACQUISITION			
Miltner et al., 1999	Human	Negative	↑
Keil et al., 2007	Human	Negative	↑
Jeschke et al., 2008	Gerbil	Negative	↑
Headley and Weinberger, 2011	Rat	Negative	↑
CONSOLIDATION			
Popa et al., 2010	Rat	Negative	–
RETRIEVAL AND EXPRESSION			
Dumenko, 1995	Dog	Positive	↑
Bauer et al., 2007	Cat	Positive	↑
Popescu et al., 2009	Cat	Positive	↑
Headley and Weinberger, 2013	Rat	Negative	↑

This list only covers the studies mentioned in this review, and thus should not be considered exhaustive.

The second phase is *consolidation*, wherein the memory for an experience strengthens with time, becoming less sensitive to interference and disruption (Müller and Pilzecker, 1900). Consolidation begins during the experience and persists for hours, days, perhaps even years (Squire and Cohen, 1979). Emotional states that occur during or shortly after an experience influence its consolidation through a cascade of physiological processes, often enhancing memory duration and strength (McGaugh, 2004; Izquierdo et al., 2006).

The final phase is *retrieval* or *expression*. Here subjects either explicitly express their recollection of an emotional stimulus, or behave in a way that anticipates it. For instance, hungry subjects approach cues that predict food. During this period, the memory and any associated changes in the brain are assessed.

CORTICAL ACTIVATION: AN EARLY NEURAL CORRELATE OF AROUSAL

Before discussing gamma oscillations and their role in emotional memory, it is worth revisiting a related phenomenon, electroencephalographic (EEG) activation, that received extensive investigation as a signature of emotional processing. Since at least the 1940s, it has been known that emotionally salient stimuli drive the nervous system differently. EEG studies were the first to identify the stimulus-driven neural correlates of arousal. Cortical EEG activation was one such response, referred to variously as "activation," "desynchronization," "low-voltage fast," or "alpha-blocking," because it replaced the slow EEG rhythms that accompany a relaxed state with a fast low amplitude chatter. What made the activation response compelling was that it increased with the arousing properties of the triggering stimuli and could be acquired by neutral stimuli that gained behavioral relevance (Magoun, 1958). The activation response could also diminish for stimuli that were repeatedly presented without overt reinforcement, initially encompassing the entire neocortex, but across trials retreating to the cortical region corresponding to a stimulus' particular modality (Rheinberger and Jasper, 1937; For review see: Morrell, 1961). Of relevance to emotional memory, pairing a tone with shock strengthened the cortical activation to the tone (Gluck and Rowland, 1959).

Soon, it was found that cortical activation and behavioral arousal reactions could be elicited by electrical stimulation of brain stem nuclei (Moruzzi and Magoun, 1949), which released acetylcholine throughout the cortex (Kanai and Szerb, 1965). Later studies revealed that this cholinergic component of cortical activation was produced by the nucleus basalis (NB), the dominant source of cholinergic innervation to the cortex (Mesulam et al., 1983). Electrical stimulation of the NB produces neocortical activation that is blocked by muscarinic cholinergic receptor antagonists (Metherate et al., 1992). The NB also receives projections from the amygdala, a structure long implicated in emotion (Kluver and Bucy, 1939). This pathway likely mediates the cortical activation evoked by amygdala stimulation (Dringenberg and Vanderwolf, 1996).

What does cortical activation have to do with gamma? Early investigators recognized that cortical activation is associated with the enhancement of high frequency oscillations (Jasper and Andrews, 1938). More modern work has thoroughly demonstrated that gamma oscillations suffuse the activated cortical state

(Steriade et al., 1996). Therefore, studies of cortical activation between the thirties and sixties were likely the first systematic, albeit unintended, investigations of gamma oscillations during emotional experiences.

GENERATION OF GAMMA OSCILLATIONS

By virtue of their intrinsic properties and connectivity, neuronal networks throughout the brain are prone to oscillating. Oscillations in the gamma-band, from 35 up to 120 Hz, of the local field potential (LFP) and EEG, have recently received particular attention because they co-occur with numerous psychological processes, and are attractive candidates for coordinating activity within local circuits and between distant brain regions (Fries, 2005; Wang, 2010). Their relevance to emotional behavior arises from these properties.

Stimulus-driven gamma oscillations in the cortex can be classified into two types based on a scheme devised by Galambos (1992). The first are *evoked*, defined by their consistent phase-locking with stimulus onset. This consistency leads to the evoked gamma oscillation being readily observable by averaging the neural response (EEG or LFP) across repeated stimulus presentations. They usually occur at a relatively short-latency from stimulus onset, with a laminar profile similar to potentials evoked by thalamic stimulation (Sukov and Barth, 1998). The other form of stimulus–driven gamma is *induced*, which is not time-locked with stimulus onset and typically emerges a few hundred milliseconds after evoked gamma has subsided. Induced gamma oscillations can be distributed across all cortical layers without reversing polarity (Steriade and Amzica, 1996), and their longer latency suggests a top-down origin or modulatory influence (e.g., Parikh et al., 2007).

Similar circuits produce gamma oscillations in the neocortex (Whittington et al., 2011), hippocampus (Csicsvari et al., 2003), and amygdala (Randall et al., 2011). In each case, gamma oscillations arise from the interaction between reciprocally connected groups of excitatory principal neurons and inhibitory interneurons. Given the similarities, we will focus on the neocortical circuit to describe the emergence of gamma.

Principal excitatory neurons in the neocortex are sparsely interconnected, with a connection probability of around 10% for pairs that are within 150 μm of each other (Brown and Hestrin, 2009; Oswald et al., 2009; Levy and Reyes, 2012). These connections are highly specific, with reciprocally connected pairs occurring in excess of chance. Moreover, the average connection strength in a neocortical ensemble increases with ensemble size (Perin et al., 2011). Taken together, these findings suggest that subsets of principal neurons form highly specific cellular ensembles that may be fully activated by exciting only a few of their members.

In contrast, principal neurons form extensive connections with inhibitory interneurons, peaking at 50% connection probability with their nearest neighbors (Levy and Reyes, 2012). While there is a profusion of interneuron types in the neocortex (For review see: Markram et al., 2004), we will focus on the parvalbumin positive subtype, because of its established role in neocortical gamma oscillations (Sohal et al., 2009; Buzsaki and Wang, 2012). These interneurons are highly interconnected, both with chemical and electrical synapses (gap junctions), promoting synchronization of their activity (Gibson et al., 1999). In keeping with the diffuse connectivity into and within the inhibitory network, inhibitory interneurons project back onto excitatory principal neurons with exceptionally high probability, peaking between 50 and 100% connectivity with their immediately adjacent neighbors (Packer and Yuste, 2011; Levy and Reyes, 2012). Furthermore, these return projections do not discriminate between principal neuron ensembles (Fino and Yuste, 2011; Packer and Yuste, 2011), which suggests that the inhibitory network behaves as a powerful suppressive force in the cortical circuit; it is activated by any ensemble of principal neurons, and in turn inhibits all other ensembles.

Modeling studies have revealed that this recurrent network of excitatory and inhibitory interneurons is sufficient for generating gamma oscillations (Borgers et al., 2005; Oswald et al., 2009). *In vivo*, neocortical excitatory and inhibitory neurons fire at specific phases of the gamma oscillation (Hasenstaub et al., 2005). As the gamma oscillation approaches its negative trough (intracellular depolarization), principal neurons begin to fire, followed shortly after by inhibitory interneurons. In line with the sparse connectivity between principal neurons, they only fire sporadically during gamma oscillations, typically once every five to seven cycles. In contrast, inhibitory interneurons fire during most gamma cycles, which partly reflects their higher intrinsic excitability (McCormick et al., 1985) and innervation by principal neurons. This interplay can be initiated by punctate activation of principal neurons (Sohal et al., 2009). Indeed, Sohal and colleagues expressed channelrhodopsin (ChR2) in pyramidal neurons of the mouse prefrontal cortex, along with halorhodopsin (NpHR) in a subset of inhibitory interneurons. ChR2 activation of the pyramidal neurons elicited several cycles of gamma oscillations, which were suppressed by NpHR driven silencing of inhibitory interneurons.

The ubiquity of the network interactions described above suggests that gamma oscillations are fundamental to the telencephalon's operation. This has been borne out in numerous studies demonstrating gamma's mediation of synchrony both within and between cortical regions. For instance, Singer and colleagues demonstrated stimulus induced gamma synchronization for co-tuned populations of neurons within the primary visual cortex (Freiwald et al., 1995), across hemispheres (Engel et al., 1991a), and between primary and secondary visual cortices (Engel et al., 1991b). Underscoring the behavioral importance of gamma oscillations, attending to a stimulus enhances gamma synchrony both within (Fries et al., 2001) and between cortical regions (Gregoriou et al., 2009).

How would gamma oscillations promote coordination and integration of activity between regions? In particular, it is generally believed that the compression of ensemble activity into brief recurring epochs, in this case firing during a particular phase of gamma, facilitates postsynaptic depolarization through spatial integration (Konig et al., 1996; Volgushev et al., 1998; Salinas and Sejnowski, 2000). The temporal coordination through gamma provides a greater gain than independent increases in firing rate (Abeles, 1982; Kuhn et al., 2002).

Beyond their established role in coordinating inter-regional spiking, gamma oscillations are potentially important regulators of synaptic plasticity. However, the literature on this topic is sparse. Several forms of synaptic plasticity, such as associative long-term potentiation (Levy and Steward, 1983) and spike timing-dependent plasticity (Markram et al., 1997; Bi and Poo, 1998), depend on the coincidence of pre- and postsynaptic activity in a narrow temporal window, on the order of tens of milliseconds. Intriguingly, gamma oscillations regulate pre- and postsynaptic spiking on a similarly brief timescale, ∼20 ms. In keeping with this, the direction of synaptic plasticity in cortical neurons is determined by the relative timing of excitatory postsynaptic potentials (EPSPs) to the phase of a 40 Hz postsynaptic subthreshold oscillation (Wespatat et al., 2004). In that study, potentiation occurred when EPSPs were coincident with the depolarizing peak of the oscillation, while coincidence with the hyperpolarizing peak lead to depression. An important caveat to these studies is that they rely upon highly stereotyped and repetitive stimulation protocols, which do not occur naturally in the nervous system. Thus, whether these experimental models engage the same plasticity mechanisms used *in vivo* remains unclear (Martinez and Derrick, 1996; Martin et al., 2000).

GAMMA OSCILLATIONS AND EMOTIONAL MEMORY

EMOTIONAL STIMULI AND STATES

Returning to the topic at hand, if gamma oscillations are ubiquitous, why are they particularly relevant to emotional memory? There are several reasons immediately evident from the material reviewed thus far. First, EEG phenomena produced by arousing stimuli, such as cortical activation, likely reflect gamma oscillations. Second, gamma oscillations facilitate the coordination of signaling between brain regions, which is essential for the encoding and behavioral expression of memory. Lastly, attention, which figures prominently in several cognitive models of emotional behavior (e.g., Mather and Sutherland, 2011), enhances gamma oscillations and their coordinating abilities. These findings provide the basis for hypothesizing that the salience of emotional memory depends, in part, on the enhanced strength of gamma oscillations induced by emotional experience. Therefore, the rest of this review will explore the experimental evidence supporting this claim.

The 'simplest' forms of memory are those for individual stimuli (e.g., habituation, recognition), therefore gamma-band responses to single stimuli must be understood. After this, we will cover combinations of stimuli, where subjects learn that a neutral stimulus precedes an affective event. Each of the phases supporting this learning will be covered separately, namely acquisition, consolidation, and retrieval.

Stimuli are often dichotomized into two classes, neutral and affective. This is variously manifested across subfields; for instance, practitioners of associative learning divide stimuli into those that are *conditioned* and *unconditioned*. Unconditioned stimuli have affective qualities that typically drive behavioral responses without prior training. Depending on their intensity they can elicit emotional responses. In contrast, conditioned stimuli generally do not evoke a behavioral response, except when they are novel or out of place (Sokolov and Vinogradova, 1975).

Affective stimuli engender gamma oscillations in a variety of cortical and subcortical regions. In the human literature, numerous studies have demonstrated that the strength of gamma oscillations evoked by faces is partly determined by the emotions they express. For instance, Luo and colleagues were able to derive gamma-band activation in the amygdala from magnetoencephalographic (MEG) signals (Luo et al., 2007). During MEG recordings, subjects were required to identify the sex of faces that had a neutral, angry, or fearful expression. Fearful faces, in particular, produced enhanced gamma activation in the right amygdala within 30 ms of stimulus presentation. However, an important confound in studies of the neural correlates of image perception is that gamma oscillations have been linked to conscious awareness of visual stimuli (Tallon-Baudry and Bertrand, 1999; but see: Yuval-Greenberg et al., 2008). Thus, the gamma enhancement seen for fearful faces may arise from a potentially higher salience, independent of emotional valence. To rule this out, the conscious awareness of the stimuli and their emotional content have to be manipulated independently. If gamma-band activation reflects the emotionality of stimuli, then an effect of emotional content should be evident beyond the conscious awareness for the stimulus. To investigate this, Luo et al. (2009) presented subjects with neutral or fearful faces for either 30 (subliminal, unaware) or 100 ms (supraliminal, aware). Each face was preceded and followed by a 100 ms masking stimulus. Filler trials replaced the face with an empty oval. Subjects were asked to indicate whether a face had been present. Even for subliminal stimuli, there was a significant main effect of emotion in the gamma-band for visual, parietal, and posterior cingulate cortices, along with the right amygdala, spanning 30–140 ms post-stimulus onset. The enhancement of gamma by emotional faces has been confirmed repeatedly using MEG, scalp, and intracranial EEG (Balconi and Pozzoli, 2007; Jung et al., 2011; Sato et al., 2011; Senkowski et al., 2011; but see: Basar et al., 2008).

In addition to faces, other emotional pictorial stimuli can enhance gamma-band activity. Fortunately, many of the studies investigating this have drawn their stimuli from the International Affective Picture System (Lang et al., 1999), which facilitates comparisons between studies. For instance, Oya et al. (2002) studied patients with amygdala electrodes for presurgical mapping of intractable epilepsy. Patients passively viewed images that were either of positive, negative, or neutral emotional valence. LFP recording sites were selected for analysis only if they showed significant activation to any of the picture categories. Of these sites, only the negatively valenced stimuli elicited robust gamma activation in the amygdala. Besides the amygdala, cortical regions also exhibit enhanced gamma for pictures with affective qualities (Keil et al., 2001). In that study, subjects passively viewed pictures while EEG recordings were obtained. At posterior recording sites, negative affective stimuli produced an early onset (∼80 ms) increase in the lower portion of the gamma-band. Starting at ∼200 ms after stimulus onset, both negative and positive stimuli elicited increases in gamma-band power that lasted for several hundred milliseconds (see also: Müller et al., 1999; for a negative result see: Glauser and Scherer, 2008). Similar results were obtained with written words (Martini et al., 2012). Interestingly, gamma enhancement by emotional stimuli is reduced in patients

with alexithymia, a condition characterized by a blunted ability to recognize and experience emotional states (Matsumoto et al., 2006). Overall, these results indicate that gamma-band activation in the amygdala and cortex tracks the emotionality of stimuli, particularly for unpleasant items, and independently of training.

Appetitive stimuli also elicit gamma oscillations. In 1960, Walter Freeman demonstrated that LFPs from the prepyriform cortex of awake cats exhibited enhanced gamma oscillations upon presentation of fish odor (Freeman, 1960). What makes this study compelling, especially given that stimulus salience is a potential confound, was that the cat's state affected the gamma response. If the same fish odor was presented to a satiated cat, then the gamma oscillations were severely attenuated, suggesting that the sensory aspects of the odor stimulus were not the principal drivers of gamma. Also arguing against a purely stimulus-driven interpretation of gamma is the finding that gamma oscillations in the amygdala of the cat are enhanced during periods of alertness or arousal (Pagano and Gault, 1964).

In addition, human gamma-band activation is related to overall anxiety level. For instance, Oathes et al. (2008) reported that entering an anxious state produced a sustained increase in gamma power. This effect was most prominent in the left temporal area, but precise localization was not possible due to the spatial limitations of EEG. Furthermore, subjects with generalized anxiety disorder, who experience disproportionate worrying, exhibit greater gamma power during worrying than in normal subjects. Finally, other studies have found tonic enhancements of gamma power during other emotional states. For instance, clinically depressed patients exhibit increased baseline levels of gamma-band power (Siegle et al., 2010). This finding is especially curious because depression has been associated with memory impairment (Burt et al., 1995). While a plethora of factors likely contribute to this deficit, increased gamma power may be one of them, which raises the question: how does this fit into the relationship between gamma oscillations and emotional memory? One explanation is that factors that enhance memory, such as level of arousal, can become impairing if they exceed a certain level, creating a so-called "inverted-U" relationship between their magnitude and behavioral performance (Yerkes and Dodson, 1908).

At this juncture it is worth noting that while this review focuses on gamma oscillations in the neocortex and amygdala (due to their well-known involvement in emotional memory), they have also been extensively investigated in the hippocampus (for reviews see: Colgin and Moser, 2010; Buzsaki and Wang, 2012), a structure implicated in learning and memory. While the importance of the hippocampus for emotion has been reinvigorated (e.g., Kimura, 1958; Fanselow and Dong, 2010), the relevance of gamma oscillations to this role has only been explored explicitly in a few studies. Lu et al. (2011) trained mice on a passive avoidance task, wherein mice delay entering a compartment in which they were previously shocked. Following training, the investigators measured LFPs from the CA3 region, and then obtained *ex vivo* hippocampal slices that exhibited spontaneous gamma oscillations. The degree of acquired avoidance to the shock compartment did not correlate with *in vivo* gamma strength, nor *ex vivo* spontaneous and kainate-induced gamma oscillations. A separate study measured the strength of gamma oscillations in *ex vivo* slices from mice that had undergone tone-shock classical conditioning (Albrecht et al., 2013). Fear conditioning tended to diminish the strength of kainate-induced gamma oscillations recorded from stratum radiatum and stratum lacunosum moleculare. These null findings do not offer a clear interpretation for the role of hippocampal gamma oscillations in emotional processing, especially given that transgenic mice with disrupted hippocampal gamma exhibit altered anxiety related behaviors (Fuchs et al., 2007; Dzirasa et al., 2011).

ACQUISITION OF EMOTIONAL MEMORY

Both in the laboratory and in nature, organisms must learn that certain neutral stimuli predict aversive or rewarding events. An especially well investigated form of such learning is classical conditioning, where an animal learns that a neutral cue (CS) predicts an aversive or rewarding event (US), leading to the development of a conditioned behavioral response. Acquisition is generally defined as the time period during which subjects develop a reliable conditioned response to the CS[1].

Perhaps the first investigation focusing on gamma activation during the acquisition of classical conditioning was Miltner et al. (1999). For this study, human subjects were presented with two different colored lights, one of which terminated with an electric shock to the hand (CS+), while the other did not (CS−). Acquisition of the association was followed by an extinction phase. A series of questionnaires assessed the subject's preference for the CSs before training, after training, and after extinction. As expected, the subject's preference for the CS+ decreased following training, and this aversion diminished following extinction. EEG was recorded throughout the task and subjected to current source-density analysis (CSD) to improve the spatial resolution of the measured electrical activity. The authors sought to identify changes in CSD coherence between sites that tracked the acquisition of the contingency. Gamma power increased during presentation of either the CS+ or CS− at occipital recording sites, with a non-significant trend for greater gamma to the CS+. Gamma coherence was found to be enhanced to the CS+ between occipital, pericentral, and putative secondary somatosensory regions that were contralateral to the shocked hand. Enhanced coherence for the CS+ over the CS− was greatest near the end of stimulus, just prior to shock delivery. This temporal precision, in combination with the specificity for the CS+, and location of US delivery, suggests that this enhancement is not produced by a generalized arousal response, but instead reflects coordination between brain regions representing the CS+ and US. Moreover, coherence was

[1]Both empirically and conceptually it is difficult to define a dividing line between acquisition and expression. From a purely statistical standpoint, the statement that asymptotic performance has been reached is fundamentally probabilistic, simply stating that the chance that further enhancements reflect a continuing trend is highly unlikely, not that it is impossible. The division is also conceptually fraught, because expression obviously occurs during acquisition, since it is the increase in the responding, the expression, that defines that phase. A further complication is that subjects may acquire knowledge of the contingencies to be learned well before behavioral evidence is present for some response systems. For simplicities sake, the first training session will be treated as the acquisition phase, and the experimenter's chosen behavior will be assumed to adequately reflect memory.

strongest in the gamma frequency band, with neither the delta, theta, or alpha bands exhibiting a robust enhancement of coherence for the CS+ over the CS−. Extinction training reduced the enhanced coherence for the CS+, showing that the gamma coherence was not just determined by the physical properties of the CS+, but that it depended on the predictive relation between the CS+ and the shock.

A more recent study found that enhanced gamma power develops to a visual grating stimulus that is repeatedly paired with negative affective pictures (Keil et al., 2007). In this study, subjects were exposed to two grating stimuli oriented 90 degrees apart. These stimuli were presented without reinforcement during an initial baseline phase. On the following day, two training sessions occurred, wherein one of the gratings predicted the presentation of negative pictures, while the other did not. This was followed by an extinction session where the gratings were presented without reinforcement. Subjects acquired weak noise-induced startle responses to the grating that was paired with negative pictures, and this response was extinguished with extinction training. A majority of participants, however, could not verbally identify the grating that predicted negative pictures, and thus the modest startle response that was observed could have been driven by the subset of subjects that were aware of the contingency. Alternatively, the weak startle response may reflect unconscious processing of the cues. An unconscious mediation is possible given the evidence that circuits involved in threat processing, such as the amygdala, can operate for subliminal stimuli (see above). Gamma-band power was enhanced to the grating that predicted aversive pictures. Two gamma responses were produced, an early phase-locked evoked response, 60–90 ms after stimulus onset, and a longer latency induced response, that occurred 350–420 ms after stimulus onset. Importantly, only the evoked gamma-band response was enhanced by the grating predicting the aversive pictures. The induced response was evoked to both gratings, but despite its lack of stimulus specificity, it varied with training phase, peaking during initial acquisition, and then reducing across the second training session and extinction phase. This late response may best capture the mnemonic component of the task, because its lack of specificity comports with the lack of explicit memory for the task contingencies.

Overall, the studies reviewed above, and others (e.g., Jeschke et al., 2008), demonstrate that gamma oscillations occur during the acquisition of associations involving emotional stimuli. On the other hand, it is unclear if these same oscillations have an impact on subsequent memory. As reviewed below, a more successful paradigm for probing the impact of gamma oscillations on memory has been the subsequent memory effect (Paller and Wagner, 2002). In this analysis, stimuli or subjects are grouped by the strength of their memory following a training task. The neural signatures that were detected during training are then compared between groups, and a significant effect suggests that the neural signature in question has some relation with the encoding and storage of the memory. Gamma oscillations in the LFP and EEG exhibit subsequent memory effects for situations that lack emotional tone, a finding that has been demonstrated in humans with word (Fell et al., 2001; Sederberg et al., 2003, 2007) or pictorial stimuli (Osipova et al., 2006). Gamma-band synchrony between spiking activity and the LFPs in the macaque hippocampus also exhibits a subsequent memory effect for pictorial stimuli (Jutras et al., 2009). However, a drawback for many of these studies is that the time between encoding and retrieval was brief, at most an hour, and thus it is unclear if gamma at the time of encoding was important for long-term memory.

To overcome this, and to examine the relationship between gamma and emotional memory in particular, Headley and Weinberger (2011) recorded gamma oscillations from the auditory cortex of rats undergoing several daily sessions of tone/shock fear conditioning. In this task, a tone CS+ predicted the occurrence of a shock US, and the fear response to the tone was measured as a slowing of the heart rate during tone presentation, which reflects conditioned fear (Teyler, 1971). The auditory cortex is known to support fear memory for acoustic cues (Romanski and Ledoux, 1992; Boatman and Kim, 2006; Letzkus et al., 2011), beyond its obvious role in processing the tone CS+. During the first fear conditioning session, the degree of CS+ induced gamma-band activation to the tone had a positive relationship with the strength of conditioned responding on the subsequent day. After the first training day, gamma ceased to exhibit a subsequent memory effect, implying that gamma's relation to memory was confined to the acquisition phase of the task. Even so, gamma continued to occur during the remaining conditioning sessions, and so it might still have been able to facilitate new learning.

To test this, the same subjects underwent several sessions of discrimination conditioning; a novel tone was introduced that did not predict shock, the CS−. Subjects discriminated between the CSs; they maintained their fear responses to the CS+, and decreased responding to the CS−. However, not all subjects acquired this discrimination immediately. There was a range of performance, with some subjects still treating the CS− as if it predicted shock on the second discrimination training session. Curiously, these subjects exhibited stronger CS− induced gamma during the first discrimination training session. These results suggest that gamma serves as an *emotional tag*, perhaps in addition to its role in facilitating memory. Indeed, if gamma just supported memory for events as they happened irrespective of emotionality, then enhanced gamma to the CS− should have resulted in improved discrimination. Since this was not the case, the memory facilitation tied to gamma was related to the subjective emotional content of the experience.

This study also found that gamma-band activation modulated the magnitude of CS+ related plasticity in the auditory cortex. Previous work had established that fear conditioning with auditory stimuli shifts frequency tuning curves in auditory cortex so that the frequency of the CS+ elicits increased responses (Weinberger, 2004). This plasticity is evident soon after training, and persists for two months, the longest time point investigated so far (Weinberger et al., 1993), making it a candidate trace for emotional memory. In the Headley and Weinberger study (2011), changes in the tone frequency receptive fields were tracked across training days and correlated with CS+ induced gamma activation. It was found that gamma during the first training session predicted the subsequent change in the receptive field at the frequency of the CS+. Moreover, this predictive correlation was absent from all other conditioning sessions, suggesting that it was

specific to the acquisition of the association. Taken together, these predictive correlations support the claim that gamma oscillations modulate the induction of long-term emotional memory.

Presumably the gamma oscillations generated in auditory cortex are facilitated by the activation of the cholinergic afferents projecting from the NB. Thus, with respect to the auditory cortex, direct NB stimulation should emulate an unconditioned stimulus (McLin et al., 2002). As reviewed above, NB stimulation produces gamma oscillations in the cortex, and NB receives direct projections from the amygdala. Rats that had been implanted with a stimulating electrode in NB and a recording electrode in auditory cortex underwent either paired or unpaired presentations of a tone and NB stimulation. After fifteen daily sessions of tone/NB pairing, subjects went through a generalization test, where tones of different frequencies, including the training tone, were presented without NB stimulation. The authors found that relative to tones of other frequencies, the tone that had been paired with NB stimulation gained a greater ability to evoke gamma-band activation. Furthermore, subjects that received unpaired presentations of the tone and NB stimulation did not develop enhanced gamma to the training tone, or any of the adjacent frequencies. Lastly, these results were mirrored in the behavior: presentation of the training tone elicited greater changes in heart rate and respiration than the untrained frequencies. However, it is unclear what the corresponding mnemonic content is for these responses. The behavioral responses observed to the training tone, which were an interruption in respiration and changes in heart rate, are the same as those seen with emotional learning. A follow up study that measured activation in other EEG frequency bands found that only gamma showed significant enhancement to the training tone in the paired group; all other bands either did not change or significantly decreased (McLin et al., 2003). Importantly, stimulation of the NB by itself produced gamma-band activation in auditory cortex, independently of overt movements or changes in behavioral state (Miasnikov et al., 2008). This suggests that the salient and arousing aspects of an emotional experience may be separable from the neural processes mediating its enhanced subsequent memory.

CONSOLIDATION OF EMOTIONAL MEMORY

Despite an abundant literature on the consolidation of emotional memories (McGaugh, 2000), no study has found a correlation between post-acquisition gamma oscillations and recall of emotional memories. The studies reviewed thus far focused on stimulus-driven gamma-band activation, which is easier to detect because one can average neural responses across trials, enhancing the signal to noise ratio. In contrast, the consolidation period lacks external events, demanding a more sophisticated analytical approach.

Overcoming this, Laitman et al. (2011) analyzed changes in gamma power surrounding the onset and offset of REM epochs during sleep in rats that underwent tone/shock fear conditioning. They found that fourteen days following fear conditioning, gamma power increased more rapidly during the transition to REM sleep, compared with the pretraining baseline. These changes were not present in subjects that received only shocks. Given the long interval between training and assessment, it is unclear whether this effect is related to memory consolidation. Another approach to study the role of gamma oscillations in memory consolidation is to probe the structures that have been tied to it. The amygdala's role in emotional memory consolidation is to orchestrate the activity of regions that presumably support long-term storage. This implies that amygdala activity is causally related to activity in presumptive storage structures during the consolidation period. Both the medial prefrontal cortex and hippocampus have been implicated in emotional memory. To investigate the amygdala's role in consolidation, Popa et al. (2010) trained rats on a fear conditioning task, and then recorded from the amygdala, hippocampus, and medial prefrontal cortex during post-training sleep. To assess interactions between these, they determined whether the change in freezing behavior, from the end of training to the recall test on the following day (i.e., consolidation) was correlated with coordination between the amygdala and these downstream regions during post-training sleep. They found that coordinated activity in the gamma-band between amygdala and hippocampus or amygdala and medial prefrontal cortex was not predictive, while activity in the theta band was, but only during REM sleep. These results stand as a robust, negative, finding for the involvement of gamma in emotional memory consolidation.

Complicating this picture is a study on gamma coherence during post-training slow wave sleep and memory for neutral word pair associations (Molle et al., 2004). Subjects were trained to associate word pairs, followed by a sleep period during which EEG recordings were obtained. A non-mnemonic form of the task was presented as well, where subjects attended to orthographic features of word stimuli. The memory group exhibited enhanced gamma coherence between EEG sites during post-training slow wave sleep. This was particularly strong between parietal/temporal and frontal sites. No such relationship was found for the subjects performing the non-mnemonic orthographic task.

Instead of correlating gamma oscillations during the consolidation period with subsequent memory, an alternative is to induce them in brain regions involved in consolidation of emotional memories. A recent study has done just that, using optogenetic tools to selectively excite neurons in the amygdala immediately after training rats on an inhibitory avoidance task (Huff et al., 2013). In inhibitory avoidance, the subject is placed in an alley divided in half, one side of which is darkened. When a rat is placed in the lighted half, his tendency will be to enter the dark compartment. Upon entering, the rat receives several foot shocks. During a subsequent test session, the rat is again placed in the lighted half and the latency to enter the dark compartment is measured. Conditioned fear manifests as an increased latency to enter the dark compartment. This task has been a mainstay for assessing emotional modulation of memory, in particular the role played by the amygdala. When administered shortly after acquisition of inhibitory avoidance, intraamygdalar injections of pharmacological agents that enhance or depress activity in the amygdala exert corresponding effects on subsequent memory (McGaugh, 2004).

Following up on these early findings, Huff et al. (2013) delivered different patterns of excitation to the amygdala immediately

after training on inhibitory avoidance, with the goal of determining whether certain activity patterns are conducive to modulating the memory 24 h later. Of relevance to the role of gamma oscillations in emotional memory consolidation, they compared optogenetic stimulation of amygdala at either 20 or 40 Hz. They found significantly greater emotional memory for the 40 Hz stimulation subjects over the no stimulation condition, and a trend toward enhancement for 20 Hz stimulation. Complicating the interpretation of these findings, however, the 40 Hz group received more stimuli overall than the 20 Hz group, and thus it is unclear if the enhancement was a result of the frequency of the stimuli, and not just the number. Rectifying this would involve delivering the same number of stimuli, but at a different frequency, such as delivering the 20 Hz stimulation for twice as long as the 40 Hz. Despite this issue, the results still agree with the hypothesis that gamma oscillations modulate emotional memory consolidation.

The basolateral nucleus of the amygdala plays a critical role in the post-training consolidation of emotional memory (McGaugh, 2000). If such a role depends upon gamma oscillations, then the amygdala should be capable of generating them in the absence of training stimuli. This appears to be the case. Gamma oscillations in the amygdala occur spontaneously and exhibit coupling with neocortical activity, in the absence of overt stimuli (Collins et al., 2001), or even in the isolated slice with the application of agonists for glutamate (Randall et al., 2011) or cholinergic receptors (Sinfield and Collins, 2006). Moreover, the dependence of gamma oscillations in the amygdala on the cholinergic system (Popescu et al., 2009, reviewed below) may bridge them to the deep literature on hormonal regulation of amygdala function in consolidation. Indeed, blockade of cholinergic muscarinic receptors in the basolateral nucleus of the amygdala blocks the memory enhancing effects of glucocorticoids (Power et al., 2000).

EXPRESSION OF EMOTIONAL MEMORY

In contrast with the paucity of studies on the role of gamma on consolidation, their presence during the expression of emotional memories is well documented. An interesting observation to emerge from this work is that, just as affective stimuli enhance gamma oscillations, so do neutral stimuli that have come to signal affective events.

One of the first studies to show a relationship between gamma and performance on a task that could be construed as emotional was done in hungry dogs that acquired a cued instrumental response for food (Dumenko, 1995). It was found that once the dogs had acquired the contingency, the cue that indicated presence of the food evoked activation in the gamma-band.

However, perhaps more important than just demonstrating a correlation between gamma and emotional memory is to uncover its consequences for the circuits that support later performance and retrieval. Bauer et al. (2007) showed that the basolateral amygdala coordinates coherent gamma in the rhinal cortices. In this study, food-deprived cats were trained on a trace conditioning task with food reinforcement. Each trial was divided into three phases, a CS+ phase, indicating that food would soon be delivered, a delay period, and reward delivery. The gamma LFP coherence and spiking entrainment were assessed between the amygdala, perirhinal, and entorhinal cortices. Prior to conditioning, these regions exhibited LFP and unit gamma-band coherence. During initial conditioning, gamma coherence between regions did not change relative to baseline. However, by the late training sessions when performance had reached asymptote, LFP coherence between the three regions increased during the delay period and reward delivery. In parallel, unit activity in the rhinal cortices increasingly synchronized with amygdala gamma during the delay period, suggesting that the amygdala orchestrates cortical spiking on fast timescales during the performance of a well-learned appetitive task.

A similar pattern of results was also found when comparing activity in the amygdala and striatum (Popescu et al., 2009). Food restricted cats were trained on tone discrimination conditioning with food reinforcement, and over the course of several sessions learned that one of two tones predicted food delivery. Coherent gamma oscillations were present between the amygdala and striatum prior to training. Inactivation of the amygdala with the GABA agonist muscimol reduced gamma power in the striatum, implicating the amygdala as one of the sources of striatal gamma. Periods of high amplitude gamma in striatum also increased the strength of cross-correlations between striatal and amygdalar neurons. Importantly, training increased amygdalostriatal gamma-band coherence during the CS+, whereas reversing the CS contingencies decreased it.

Besides studies relying on appetitive training tasks, negative emotional learning has also been explored. Using four daily sessions of tone/shock classical fear conditioning, Headley and Weinberger (2013) tracked changes in the spectral content of LFPs during acquisition of conditioned fear, and the coordination of unit activity in primary auditory cortex. Recording sites were grouped based on the distance between preferred frequency and the CS+ frequency. Only gamma consistently increased with training, an effect seen irrespective of the recording sites preferred frequency. Yet, the enhancement tended to be stronger for sites with preferred frequencies close to the CS+. Where frequency tuning did matter was in the synchrony between unit activity and the phase of gamma oscillations (60 Hz band). Just as CS+ induced gamma power increased with training, so did the degree of phase-locking between unit activity and gamma. During the first training session, both the sites tuned near and away from the CS+ tended to decrease their phase-locking to gamma during CS+ presentation. By the last training session this had reversed, sites tuned near the CS+ now tended to increase their phase-locking with gamma during the CS+, while those tuned away had no net change. Does this change in phase-locking to gamma oscillations have consequences for the coordination of unit activity?

The synchrony arising from gamma oscillations is thought to support the binding of neurons into coactivated cell assemblies, allowing them to drive each other and their mutual downstream targets more effectively (Womelsdorf et al., 2007). Based on this work, modeling studies showed that both the phase and amplitude of gamma oscillations should modulate the correlation in spiking between neurons (Buehlmann and Deco, 2010). This was also found with the acquisition of conditioned fear. The strength of the cross correlation between unit activity at sites tuned near the CS+ frequency acquired enhanced modulation by both the

phase and amplitude of CS+ induced gamma oscillations, while those tuned away from the CS+ frequency were not significantly affected.

CONCLUSIONS

Bringing the reviewed studies together, several patterns emerge. The first is that affective stimuli, particularly those with negative valence, elicit enhanced gamma oscillations in both the neocortex and amygdala, the two structures that were the focus of this review. When these responses are *evoked*, having a short-latency and time-locked to stimulus onset, they exhibit modulation by emotional content that can be unconscious (Keil et al., 2007; Luo et al., 2009). Their short-latency suggests that this emotional modulation of gamma is bottom-up. *Induced* gamma on the other hand, which occurs several hundred milliseconds after stimulus onset and without phase-locking, reflects a subject's awareness of emotional content (Keil et al., 2007). In addition, induced gamma to training stimuli predicts memory one day later (Headley and Weinberger, 2011). The long latency of induced gamma is consonant with either top-down modulation or neuromodulatory influences.

Second, induced gamma is the form that appears most affected by learning. This is especially true for neutral events that have been paired with affective ones. The initially neutral stimuli acquire the ability to elicit stronger induced gamma oscillations. This increase typically occurs near the end of the stimulus, close to when the reinforcer would normally occur.

Third, the enhanced gamma that accompanies emotional learning appears to promote coordinated spiking between neurons. For example, this was the case for neurons in auditory cortex that responded to a fear conditioned stimulus (Headley and Weinberger, 2013), and those in peri- and entorhinal cortices with appetitive conditioning (Bauer et al., 2007). Gamma-driven increases in synchronized firing across a neuronal ensemble should allow representations of emotional stimuli to drive downstream targets more effectively (Fries, 2005).

Finally, there are presently four under-addressed issues in the study of gamma oscillations and emotional memory. The first is the dearth of studies investigating gamma's role in consolidation. Apart from the few reviewed above, to the best of our knowledge no other studies have probed this issue. The second issue is whether the function of gamma oscillations in emotional memory can be dissociated from their role in attention. Studies of gamma oscillations during emotional memory consolidation, when conducted during the post-training consolidation period, lack the discrete attention grabbing stimuli that are often reinforced. Thus, studies of gamma during this period may provide insights into their ability to coordinate activity without the ongoing influence of attentional processes. The third issue is the long-term consequences of the changes in gamma coordination that occur for stimuli that come to predict affective events. How does this enhanced gamma-band activation affect future learning about these stimuli, and are these changes in any way unique to emotional learning? Lastly, are gamma oscillations recruited in a qualitatively different manner during emotional experiences compared with non-emotional ones, or are these differences only quantitative? Perhaps emotional stimuli fall along a continuum that includes stimuli that are novel, goal-related, or devoid of emotional inflection, with each of these engaging circuits that mediate gamma oscillations to different degrees. Answering these questions could go a long way toward explaining the salience, vividness, and accessibility of emotional memories.

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Sleep and protein synthesis-dependent synaptic plasticity: impacts of sleep loss and stress

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Sleep has been ascribed a critical role in cognitive functioning. Several lines of evidence implicate sleep in the consolidation of synaptic plasticity and long-term memory. Stress disrupts sleep while impairing synaptic plasticity and cognitive performance. Here, we discuss evidence linking sleep to mechanisms of protein synthesis-dependent synaptic plasticity and synaptic scaling. We then consider how disruption of sleep by acute and chronic stress may impair these mechanisms and degrade sleep function.

Keywords: long-term potentiation, stress, sleep deprivation, mood disorder, gene expression, translation control, brain-derived neurotrophic factor, Arc/Arg3.1

INTRODUCTION

Daily, we devote 6–9 h of our life to sleep, a physiological state marked by muscle relaxation and reduced responsiveness to our surroundings. Sleep is characterized by well-defined changes in brain activity as seen on the electroencephalogram (EEG). Sleep alternates between non-rapid-eye movement (NREM) sleep and rapid-eye movement (REM) sleep in a characteristic pattern known as the sleep cycle. In humans, the typical duration of one sleep cycle is approximately 90 min and one night typically consists of 4–5 sleep cycles. The architecture within the sleep cycles changes as the night progresses. Deep slow-wave sleep (SWS) predominates NREM sleep in the first half of the night, while the duration of REM sleep epochs progressively increases throughout the night. By the end of the night a REM sleep epoch may last for more than 30 min (Carskadon and Dement, 2011). Rodents have many, short-lasting (10–15 min) sleep cycles with REM-sleep epochs lasting from 30 s to 2 min (Vivaldi et al., 1994; Comte et al., 2006).

According to the two-process model for sleep regulation, sleep and wakefulness are driven by an interplay between circadian and homeostatic processes (Borbely, 1982). The circadian factor promotes sleep during certain periods of the day, and largely determines the timing and duration of the sleep period (Czeisler et al., 1980; Dijk and Czeisler, 1995). The homeostatic factor represents a sleep propensity that accumulates during time spent awake and is reflected by the amount and intensity of SWS (Borbely et al., 1981; Banks and Dinges, 2007; Riedner et al., 2007; Vyazovskiy et al., 2007). This biological drive for sleep may additionally be overridden and influenced by environmental and behavioral factors (e.g., voluntary awake, shift/night work, noise, caffeine intake).

Sleep is vital for human cognitive performance and health. However, over the last decades major societal changes have occurred that may impact sleep in a negative way. Some describe our modern life style as a "24-h society". This refers primarily to an increased recourse to shift and night work and a prolonged use of electronic media that often delays bedtime, consequently altering both sleep duration and quality (Brunborg et al., 2011). Human and animal studies alike show that sleep restriction or sleep deprivation induces deficits in cognitive functions like behavioral alertness, performance, mood, and memory (Banks and Dinges, 2007; Rasch and Born, 2013). In parallel, advances have been made in elucidating sleep-dependent mechanisms at the cellular and molecular levels. Activity-dependent synaptic plasticity is considered essential for long-term adaptive changes in behavior, including learning and memory, and the regulation of mood and motivation. Understanding how synaptic efficacy and plasticity are modulated by sleep is therefore key to unlocking the specific contribution of sleep to cognition and the impact of sleep loss on cognition.

Here, we first outline and critically discuss current knowledge with regard to mechanisms and regulation of synaptic efficacy and long-term synaptic plasticity during sleep. Emphasis is placed on regulation of gene expression and protein translation important for the consolidation of persistent forms of plasticity. We then focus on the impact of acute and chronic stress on sleep quality and amount, and discuss how interactions between stress and sleep affect sleep-dependent gene expression, plasticity, and cognition.

SYNAPTIC PLASTICITY AND SLEEP

MODELS OF SYNAPTIC PLASTICITY

A few hours of wakefulness or sleep can modify the molecular composition of excitatory synapses, change their efficacy and make synapses grow or shrink. Before discussing sleep-dependent regulation of long-term synaptic plasticity, it is opportune to review the major mechanisms by which synapses are strengthened or weakened, reshaped, and eventually stabilized at the molecular level.

Synaptic plasticity is the ability of a synapse to change in strength in response to use or disuse. Diverse forms of synaptic plasticity exist at excitatory, glutamatergic synapses in the mammalian brain. Among them are long-term potentiation (LTP), long-term depression (LTD), and homeostatic scaling of synaptic strength. LTP and LTD are sustained increases and decreases, respectively, in synaptic efficacy induced by patterned synaptic activity. Homeostatic scaling refers to the ability of a neuron to modulate its firing rate by globally increasing or decreasing synaptic efficacy on all inputs to the dendrite. In contrast to LTP and LTD, which are input-specific (Hebbian plasticity), homeostatic scaling does not affect the relative difference in strength between inputs (non-Hebbian plasticity).

Importantly, LTP and LTD share a common set of mechanisms even if they represent opposite changes in synaptic strength. Both forms of long-term synaptic plasticity require actin cytoskeletal remodeling within dendritic spines and changes in spine morphology. Many studies report enlargement of spines in LTP and shrinkage or loss of spines in LTD (Okamoto et al., 2004; Bourne and Harris, 2008). Similarly, both LTP and LTD require *de novo* protein synthesis, including local regulation of protein translation in dendrites (Bramham and Wells, 2007). Below, we outline some of the canonical mechanisms linked to LTP and LTD.

LTP is divided into early (E-LTP) and late (L-LTP) phases which are mechanistically distinct. E-LTP typically lasts 1–2 h after LTP induction. This phase depends on the post-translational modification and trafficking of pre-existing proteins. It does not require new gene expression or protein synthesis. In brief, activated N-methyl-D-aspartate receptor (NMDAR)-type glutamate receptors trigger rapid entry of calcium into spines. Calcium influx impacts myriad signal transduction pathways, many of which are present within the postsynaptic spine itself. Initial signaling events include activation of numerous calcium-responsive protein kinases (calcium and calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated protein kinase (ERK), and protein kinases A (PKA) and C (PKC) see **Figure 1A**). Activation of these pathways regulates both endosomal trafficking of AMPA receptors and modulation of actin cytoskeletal dynamics in spines, leading to enhanced postsynaptic membrane expression of AMPA-type glutamate receptors and a transient enlargement of dendritic spines (see **Figure 1B**; Malinow and Malenka, 2002; Bosch and Hayashi, 2012; Lisman et al., 2012).

The formation of stable L-LTP, which lasts many hours and days, requires new gene expression and protein synthesis (Stanton et al., 1984; Matthies et al., 1990; Costa-Mattioli et al., 2009; Sossin and Lacaille, 2010; Gal-Ben-Ari et al., 2012). The first period of protein synthesis occurs within the first 2 h following LTP induction. L-LTP is associated with stable enlargement and remodeling of the postsynaptic density (a large multi-protein complex attached to the membrane), enlargement of pre-existing dendritic spines, as well as *de novo* synapse formation (see **Figure 1C**; Lisman and Raghavachari, 2006; Bourne and Harris, 2008). Inhibition of protein synthesis prevents maintenance of the change in synaptic efficacy initiated during E-LTP. Additionally, protein synthesis inhibitors prevent stable increases in actin filaments (F-actin) associated with L-LTP without affecting actin cytoskeletal formation during E-LTP (Bourne et al., 2007; Bramham, 2008; Murakoshi and Yasuda, 2012).

LTD may be induced following activation of metabotropic glutamate receptors (mGluRs) and NMDARs. Unlike LTP, LTD expression relies on activation of phosphatases (i.e., PP1 and calcineurin) and is accompanied by removal of AMPARs from the postsynaptic membrane, thus lowering synaptic efficacy. Again, stabilization of the change in synaptic efficacy requires protein synthesis (but not necessarily new mRNA expression; Malenka and Bear, 2004). This is paralleled by a net decrease in spine F-actin and a shrinkage or retraction of dendritic spines (Tada and Sheng, 2006; Bosch and Hayashi, 2012).

Finally, it should be noted that LTP mechanisms probably differ between brain regions, and different input patterns can generate distinct forms of LTP (For detailed accounts see Ho et al., 2011; Panja and Bramham, 2014).

SLEEP LOSS IMPAIRS LATE LONG-TERM POTENTIATION (LTP) AND COGNITIVE FUNCTIONING

The impact of sleep loss on long-term synaptic plasticity has been investigated in recent decades. The majority of studies have employed sleep deprivation or sleep restriction to assess the benefits of sleep. Several protocols have been used to induce sleep loss.

Methods in sleep deprivation studies

The methods used in the vast majority of animal studies aim at disturbing sleep by total sleep deprivation, sleep restriction, or specific sleep stage deprivation. Common protocols include forced locomotion by placing the animal in a rotating drum, treadmill or platform, gentle handling (e.g., tactile, acoustic stimuli) and disturbance of the animal's nesting material, or presentation of novel objects. Specific REM sleep deprivation is often achieved by placing animals on small platforms over water, the "flower-pot" technique. Differences in methodology may explain some discrepancies with regard to the effects of sleep loss on synaptic plasticity (Kopp et al., 2006; Vecsey et al., 2009; Havekes et al., 2012).

Sleep loss impairs long-term potentiation (LTP)

Several studies, mainly conducted in hippocampal tissue slices prepared from sleep-deprived rodents, have established that LTP expression depends on, or at least benefits from, a prolonged (non-disrupted) period of sleep. For instance, shorter (4–6 h) and longer (12–24 h) periods of sleep fragmentation by forced activity or total sleep deprivation by gentle handling impair LTP expression at Schaffer collateral-hippocampal subregion cornu ammonis 1 (CA1) synapses (Campbell et al., 2002; Kopp et al., 2006; Tartar et al., 2006; Vecsey et al., 2009). Importantly,

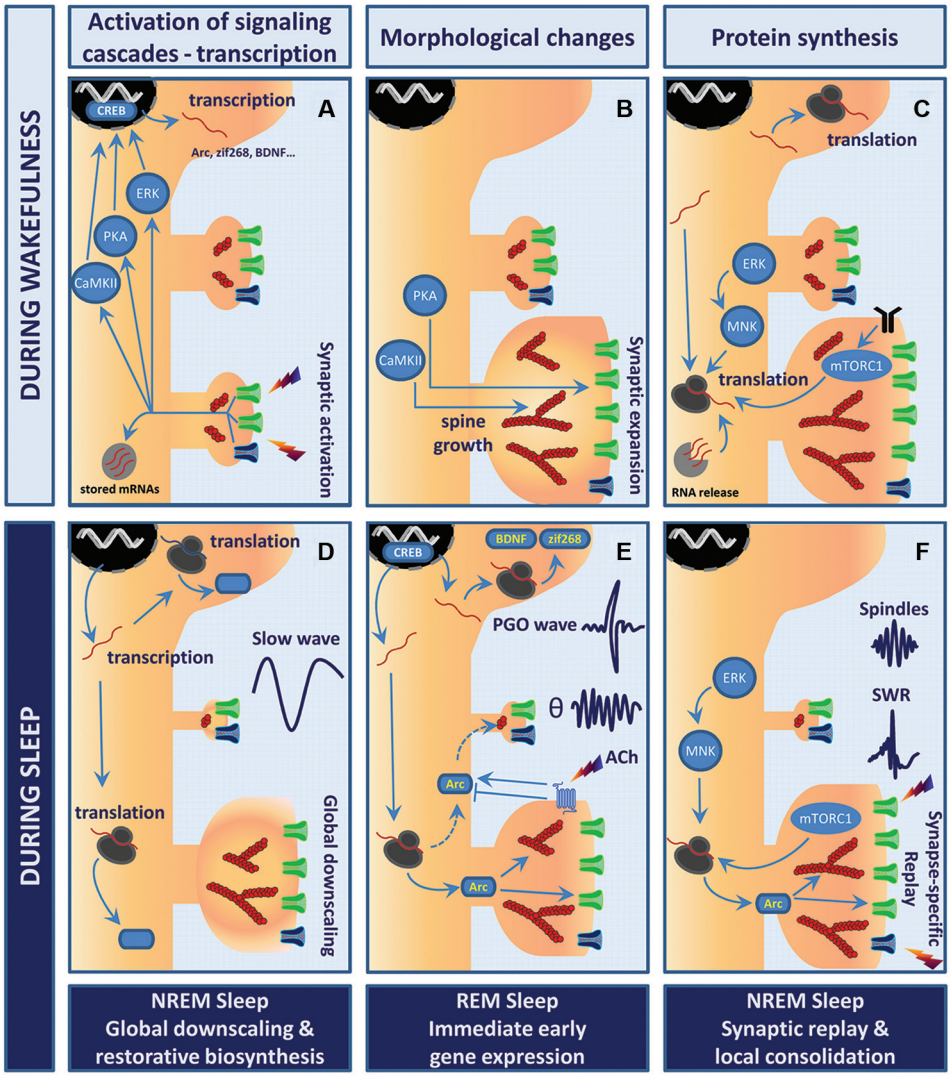

FIGURE 1 | Model of sleep stage-specific potentiation and homeostatic scaling. In this working model, waking experience (LTP-like event) is consolidated through sleep stage-specific synaptic scaling, immediate early gene expression, and protein synthesis. Wakefulness **(A–C)**. **(A)** Stimulation of glutamatergic synapses leads to rapid calcium influx into the postsynaptic compartment via NMDAR (dark blue) and AMPAR (green). Elevation of calcium levels activates multiple kinases and signaling cascades (e.g., PKA, CaMKII, ERK) which converge toward transcription factors such as cyclic AMP response element-binding protein (CREB), thus triggering rapid immediate early gene (IEG) expression. **(B)** Within minutes, polymerization of actin into filaments (red, actin filaments (F-actin)) induces remodeling of the actin cytoskeleton within spines. While CaMKII likely contributes to bundling of F-actin and expansion of the actin scaffolds, PKA promotes the insertion of AMPARs into the postsynaptic membrane. This whole process results in robust growth of synapses and enhanced synaptic efficacy. **(C)** These changes are then wake-consolidated in a *de novo* protein synthesis-dependent manner. Newly transcribed IEGs are either translated in the cell soma or trafficked further into the dendrites to be processed by the local translation machinery. Neuronal activity may release and translate dendritically stored mRNAs (light gray circle). Both mammalian target of rapamycin complex 1 (mTORC1) and mitogen-activated protein kinase-interacting kinase (MNK) signaling enhance rates of translation initiation. Postsynaptic receptors depicted are TrkB (black), AMPAR (green) and NMDAR (blue). Sleep **(D–F)**. **(D)** NREM sleep supports the homeostatic process of cellular restoration by transcription and translation of genes involved in macromolecular biosynthesis and transport. In parallel, slow-wave activity (SWA) generates global synaptic downscaling in which synapses shrink and synaptic efficacy is reduced. Synapse-specific LTD at inactive or weakly active synapses may also be involved. **(E)** REM sleep and ponto-geniculo-occipital (PGO)-waves reactivate transcription of the plasticity-related IEGs Arc, brain-derived neurotrophic factor (BDNF) and zif268. Theta (θ) activity and increased acetylcholine levels regulate Arc protein turnover at the level of translation, degradation and mRNA decay. Arc may consolidate activity-induced synaptic changes by stabilizing the actin cytoskeleton and regulating trafficking of AMPAR from and to the postsynaptic membrane. **(F)** NREM sleep events like hippocampal sharp wave-ripples (SWRs) and thalamo-cortical sleep spindles have been suggested to actively take part in memory consolidation in conjunction with replay of neuronal activity patterns representing waking experience. The precise function of SWRs at the synaptic scale is yet to be unveiled. However, sparse and synapse-specific reactivations during SWRs of NREM sleep could provide bursts of local protein synthesis that consolidate synaptic modifications and memory formation. Thus, alternations of REM sleep-associated gene expression and NREM sleep-associated synaptic replay favor protein synthesis-dependent synaptic consolidation across sleep cycles.

5–6 h of sleep deprivation specifically impairs LTP maintenance, leaving LTP induction intact (Vecsey et al., 2009; Florian et al., 2011). Investigating the bidirectionality (LTP/LTD) of synaptic modifications in CA1, Kopp et al. (2006) observed that 4 h sleep deprivation by gentle handling shifted the LTP/LTD induction threshold towards higher frequencies. Interestingly, this effect was reflected at the level of NMDAR subunit composition, suggesting that sleep deprivation modulates the function of postsynaptic membrane receptors supporting activity-dependent changes in synaptic efficacy (Kopp et al., 2006). However, a later study reported no effect on NMDAR function or LTP induction (Vecsey et al., 2009). The reasons for this discrepancy are unclear but could be related to differences in the sleep deprivation protocols. Kopp et al. (2006) employed 4 h of novel environment exposure, gentle knocking at the cage, and *ad libitum* access to nesting material, while Vecsey et al. (2009) used 5 h of gentle handling. Importantly, 2–3 min of daily acclimation handling does not disturb sleep or affect CA1 LTP (Vecsey et al., 2013).

The effect of sleep deprivation on LTD has not been studied in detail. One study indicates that 12 h of total sleep deprivation enhances expression of LTD induced by 20 Hz stimulation of the Schaffer collateral-CA1 region (Tadavarty et al., 2009). This particular stimulus paradigm evokes a generalized depression of synaptic inputs onto CA1 pyramidal cells (both activated and non-activated inputs are depressed; Sastry et al., 1984). There are no studies examining the effects of sleep deprivation on homosynaptic NMDAR-dependent LTD and mGluR-dependent LTD.

Loss of rapid-eye movement (REM) sleep impairs long-term potentiation (LTP)

Selective REM sleep deprivation produces deficits in hippocampal LTP similar to what has been observed after total sleep deprivation (both NREM and REM sleep). Prolonged REM sleep deprivation (24–72 h) impairs LTP in the hippocampus *in vitro* and *in vivo* (Davis et al., 2003b; McDermott et al., 2003, 2006; Ravassard et al., 2009; Alhaider et al., 2011). To assess whether REM sleep loss specifically impairs LTP maintenance in the dentate gyrus, rats were REM sleep-deprived for 4 h starting 1 h after LTP induction in wakefulness. LTP maintenance was reduced in REM sleep-deprived animals relative to control at 48 h (but not at 5 or 24 h). Total sleep deprivation similarly impaired L-LTP (Romcy-Pereira and Pavlides, 2004). Ishikawa et al. (2006) performed REM sleep deprivation for 24 h immediately after induction of LTP in the dentate gyrus of awake rats. LTP of the evoked population spike (which reflects synchronous neuronal firing) was strongly reduced compared to a non-sleep deprived group and yoked controls awoken in NREM sleep.

Importantly, REM sleep deprivation has opposite effects on LTP in different brain regions. In the study of Romcy-Pereira and Pavlides (2004), LTP maintenance in the medial prefrontal cortex was enhanced after 48 h of recording whereas LTP in the dentate gyrus returned to baseline levels after the same period (Romcy-Pereira and Pavlides, 2004). However, measurements of the population spike amplitude in the medial prefrontal cortex indicate increased neuronal excitability but not necessarily a change in synaptic efficacy. Taken together, current evidence suggests that sleep loss impairs the maintenance of LTP, at least in the CA1 and dentate gyrus regions of the hippocampus. The effects of sleep loss on cortical LTP have not been studied in detail.

Sleep loss impairs cognitive functioning

The impact of sleep loss on L-LTP is consistent with accumulating evidence regarding the benefits of sleep to cognitive functioning, including long-term memory formation (for a review, see Diekelmann and Born, 2010). Studies in rodents not only show that memory depends on sleep, but also that sleep must occur within a specific time window following learning. Indeed, mice subjected to 6 h sleep deprivation immediately after a complex object recognition task exhibit impaired memory retrieval, while memory is intact if sleep deprivation is performed 6 h after learning (Palchykova et al., 2006). A similar time-window is seen in contextual fear conditioning based on single-trial learning (Graves et al., 2003). Sleep deprivation from 0 to 5 h after conditioning impaired memory consolidation, whereas sleep deprivation from 5 to 10 h after training had no effect.

Earlier experiments in rats showed that REM sleep shortly after learning is necessary for the consolidation of memory. Selective REM sleep deprivation for 12 h immediately after or between 5–8 h after place (but not cue) learning in a Morris watermaze impaired long-term memory. REM sleep deprivation at other time points did not impair long-term memory formation. Notably, no impairment of place learning in the Morris watermaze occurred when REM sleep deprivation was applied 6 h after learning (Walsh et al., 2011). REM sleep periods increase in number and duration in active avoidance learning, and deprivation of post-trial REM during the period of enhanced REM sleep impairs long-term memory (Smith and Butler, 1982; Smith, 1996; Smith and Rose, 1996). These studies point to the existence of a time-window of REM sleep-dependent memory consolidation. To our knowledge, the possibility of a similar time-sensitive role for REM sleep in L-LTP maintenance remains to be explored.

Most studies in rodents have examined the role of total sleep or specific contributions of REM sleep in memory consolidation. In humans, specific functions for NREM and REM sleep have been proposed. While REM sleep mainly benefits the consolidation of procedural memories (skills), NREM sleep is implicated in consolidation of declarative and working memories (Gais and Born, 2004; Rasch and Born, 2013). Memory consolidation following tasks consisting of simple declarative material usually shows low susceptibility to REM sleep deprivation. In contrast, consolidation following tasks of higher complexity, or tasks which integrate procedural or emotional components, are more vulnerable to REM sleep deprivation (Rasch and Born, 2013). An interesting paradox originates from two studies where pharmacological (selective noradrenaline or serotonin re-uptake inhibitors) suppression of REM sleep enhanced, rather than impaired, memory consolidation (Vertes and Eastman, 2000; Rasch et al., 2009).

Overall, the evidence from rodents suggests that total sleep and REM sleep support cellular mechanisms that are used in the generation of stable LTP and long-term memory. As L-LTP and long-term memory depend on *de novo* gene transcription, it is

important to consider how gene expression is regulated during normal sleep and sleep deprivation.

GENE EXPRESSION DURING SLEEP AND AFTER SLEEP LOSS

Studies on how sleep and sleep loss affect the regulation of gene expression have yielded insights into the molecular mechanisms at play during sleep. In addition, a handful of studies have explored sleep stage-specific regulation of gene expression.

The advent of genome-wide expression profiling (i.e., blood, brain tissue) allowing the screening of thousands of transcripts has given researchers the opportunity to look at specific effects of sleep vs. sleep loss in the brain (Cirelli et al., 2004; Mackiewicz et al., 2007). Sleep specific changes in the mouse cortex involve as many as 2090 mRNAs which increase or decrease in their steady-state expression (Mackiewicz et al., 2007). Most of the genes increased during sleep are linked to macromolecular biosynthesis and transport, supporting a restorative function of sleep at the cellular level. In healthy humans, just 1 week of insufficient sleep (6 h per day) affects the expression of 711 different mRNAs in whole blood relative to subjects getting sufficient sleep (8.5 h per day; Möller-Levet et al., 2013). Genes altered by sleep restriction in this study were involved in sleep homeostasis, circadian rhythms, oxidative stress, and metabolism. Moreover, the insufficient sleep was associated with poor cognitive performance in a vigilance test. These studies support the idea that biosynthetic pathways are "recharged" during sleep for optimal function during wakefulness. However, it is also possible that metabolic changes are required to support bursts of protein synthesis or other energy-expensive processes during sleep.

Only a few IEGs have been causally linked to late LTP and long-term memory. These genes include the activity-dependent cytoskeletal-associated protein (Arc, a.k.a Arg3.1), the transcription factor zif268 (a.k.a. egr-1, krox24, Ngfi-A), and the neurotrophin brain-derived neurotrophic factor (BDNF; Guzowski et al., 2000; Jones et al., 2001; Plath et al., 2006; Messaoudi et al., 2007; Bekinschtein et al., 2008; Penke et al., 2014). Given their essential role in consolidation mechanisms, these genes could be expected to be induced at one point or another during the NREM-REM sleep cycle.

Arc and zif268

Surprisingly, a few hours of sleep has been associated with decreased expression of various IEGs, including Arc and zif268, throughout the cerebral cortex (Cirelli and Tononi, 1999; Cirelli et al., 2004). Thompson et al. (2010) mapped Arc and zif268 expression, among other candidates, across mouse brain regions following sleep deprivation. Arc mRNA expression in the hippocampus and neocortex was higher during spontaneous wakefulness and after 6 h of sleep deprivation compared to time-matched sleeping controls. A similar pattern has been shown for Arc and zif268 mRNA after 8 h of sleep deprivation (Cirelli and Tononi, 2000).

Following bursts of synaptic activation, a fraction of the newly transcribed Arc mRNA is transported to dendritic processes for local storage, translation, or decay. Arc protein is implicated in LTP, LTD as well as homeostatic scaling (Bramham et al., 2010; Korb and Finkbeiner, 2011; Shepherd and Bear, 2011). In LTP, Arc functions to stabilize nascent F-actin (Lyford et al., 1995; Messaoudi et al., 2007). In LTD and scaling, Arc recruits the endocytic machinery (binds endophilin and dynamin) to facilitate endocytosis of synaptic AMPARs (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). zif268 regulates late response genes but a causal role for zif268-dependent gene expression in LTP or memory formation has not been established (Davis et al., 2003a; Knapska and Kaczmarek, 2004; Baumgärtel, 2009; Penke et al., 2014). Genetic or pharmacological (antisense oligodeoxynucleotide) inhibition of Arc and zif268 expression severely impairs long-term memory (Guzowski et al., 2000; Jones et al., 2001; Plath et al., 2006; Messaoudi et al., 2007).

Sleep disturbances may affect gene expression through decreased activation of several signal transduction pathways. Short sleep deprivation (5–6 h) reduces ERK and cAMP-PKA signaling (Guan et al., 2004; Vecsey et al., 2009). Both pathways regulate gene expression at the transcriptional level via CREB, a cAMP-responsive transcription factor. The transcription of Arc and zif268 underlying L-LTP is both ERK and cAMP/PKA-dependent (Davis et al., 2000; Waltereit et al., 2001; Ying et al., 2002; Kawashima et al., 2009). Mechanistically, Vecsey et al. (2009) showed that 5 h of sleep deprivation in mice enhances the expression of an enzyme that degrades cAMP, namely phosphodiesterase 4, thus reducing PKA activation. Five hours of sleep deprivation altered PKA signaling to CREB while impairing PKA-dependent forms of hippocampal LTP and long-term memory. Importantly, all these effects were rescued by treatment with phosphodiesterase inhibitors (Vecsey et al., 2009). Reduced activity in these signaling cascades might be predicted to dampen IEG expression, yet enhanced expression of many IEGs has been observed after sleep deprivation. The basis for the enhanced expression of IEGs is not clear but there is evidence for regional and gene-specific regulation. Arc mRNA is upregulated in the cortex and hippocampus (Thompson et al., 2010; Grønli et al., 2012; Vecsey et al., 2012), though expression of the plasticity-associated IEGs Homer1a and zif268 in the hippocampus is not changed by 5 h of sleep deprivation (Vecsey et al., 2012). High-resolution mapping of gene expression in the study by Thompson et al. (2010) also identified subcompartments of the cortex in which the IEG expression was decreased in sleep deprived mice.

Post-learning REM sleep is associated with enhanced expression of zif268 in the rat neocortex and hippocampus (Ribeiro et al., 1999, 2007; Ribeiro and Nicolelis, 2004). Remarkably, enhanced zif268 mRNA expression also occurs during REM sleep after LTP induction in the dentate gyrus (Ribeiro et al., 2002). This important finding shows that plastic changes evoked at a single afferent pathway during the waking state are sufficient to alter REM-linked gene expression. Romcy-Pereira et al. (2009) further identified several genes with enhanced hippocampal expression specific to post-stimulation REM sleep. The genes included a putative dendritically localized mRNA (J01878) and calcium/calmodulin-dependent protein kinase I (CaMKI), a protein involved in dendritic spine remodeling and calcium signaling in ERK-dependent LTP induction (Wayman et al., 2008).

Ponto-geniculo-occipital (PGO) waves are prominent phasic events of REM sleep, and recent evidence links increases in PGO

wave activity to gene expression and memory formation (Mavanji and Datta, 2003; Ulloor and Datta, 2005; Datta et al., 2008). Following learning, an increase in PGO waves and enhanced expression of phospho-CREB, Arc and zif268 mRNA is found in the dorsal hippocampus and amygdala (Datta et al., 2008). Pharmacological suppression of PGO waves blocks the REM sleep-associated expression of these genes, while pharmacological activation of the PGO wave generator activates their expression. Taken together, these data implicate PGO waves as mediators of REM sleep-associated IEG induction of potential importance for the consolidation of synaptic plasticity during sleep.

NREM and REM sleep are associated with distinct neurotransmitter milieus which may differentially regulate gene expression (Brown et al., 2012). Cholinergic activity, in particular, is high during REM sleep compared to NREM sleep. Pharmacological stimulation, aimed at mimicking REM sleep-associated cholinergic activity, induced Arc expression in human neuroblastoma cells and somatodendritic expression of Arc protein in cultured rat hippocampal slices (Soulé et al., 2012). In neuroblastoma cells, Arc expression was controlled at the level of transcription, translation, proteosomal degradation, and mRNA decay. Although Arc is induced during REM sleep, nothing is known about the function of Arc in relation to LTP and LTD during sleep (see section "Working Model of Synaptic Plasticity Regulation During Sleep" for discussion).

Brain-derived neurotrophic factor

Expression of the secretory peptide, BDNF, is susceptible to sleep alterations. Unlike Arc and zif268, BDNF mRNA and protein are enhanced following sleep deprivation (of 8 and 48 h) in the rat hippocampus (Guzman-Marin et al., 2006).

BDNF is stored and released pre- and postsynaptically from glutamatergic synapses (Edelmann et al., 2014). BDNF activates TrkB receptors and induces protein synthesis-dependent LTP *in vitro* and *in vivo* (Kang and Schuman, 1996; Messaoudi et al., 2002, 2007; Panja and Bramham, 2014). In the dentate gyrus, BDNF triggers Arc expression on which L-LTP critically depends (Messaoudi et al., 2007). BDNF is rapidly transcribed following LTP induction (Castrén et al., 1993; Bramham et al., 1996; Wibrand et al., 2006), and BDNF protein synthesis is necessary for some forms of LTP (theta burst; Pang et al., 2004).

Sleep deprivation for 8 and 48 h decreases expression of several potential downstream gene targets of BDNF-TrkB signaling (Synapsin I, CREB, CaMKII, and BDNF itself) in the hippocampus, but not in the neocortex (Guzman-Marin et al., 2006). This suggests a differential susceptibility of these brain regions to sleep deprivation at the level of transcription and signaling. Consistent with the impact of sleep loss on both BDNF expression and maintenance of LTP, a recent study reports that 24 h of sleep deprivation abolishes the increase in BDNF expression associated with L-LTP in hippocampal region CA1 (Alhaider et al., 2011).

Waking exploratory behavior in rats is positively associated with cortical expression of BDNF as well as greater NREM slow-wave activity (SWA; Huber et al., 2007). Faraguna et al. (2008) show that local, unilateral cortical infusion of BDNF during wakefulness increases NREM SWA in the infused hemisphere without affecting REM sleep. Infusion of anti-BDNF antibody or K252a (which blocks TrkB kinase activity) during waking prevented the exploration-related increase in local SWA. The authors suggest that synaptic potentiation induced by local BDNF infusion results in local sleep regulation. In humans, a functional Val66Met polymorphism in the pro-BDNF gene causes impaired activity-dependent dendritic trafficking and secretion of mature BDNF protein. Supporting a role for BDNF in human sleep regulation, a recent study reported impaired intensity of SWA in NREM sleep in Val66Met carriers relative to Val/Val homozygotes under basal conditions and immediately following a 40 h period of waking (Bachmann et al., 2012). The Val/Met genotype is also associated with poorer performance in verbal working memory (Egan et al., 2003; Ninan, 2014).

To conclude, gene expression in the brain appears to be dynamically regulated across wakefulness and sleep. Microarray studies have identified families of genes implicated in metabolism, macromolecular biosynthesis and transport as important targets of state-transition. Plasticity-related IEGs such as Arc, zif268 and BDNF are also subject to differential transcriptional modulation during periods of sleep and sleep deprivation. Overall, sleep seems to downregulate IEG expression in the cerebral cortex, while stage (REM sleep)- and region-specific increases occur after learning and LTP induction. Such local and temporal modulations in gene expression may underlie variations in the direction, strength, and persistence of synaptic changes elicited during wakefulness by learning events.

PROTEIN SYNTHESIS, TRANSLATION CONTROL, AND SLEEP

A recent global quantification of gene expression in mammalian cells concludes that the cellular abundance of proteins is predominantly determined at the level of translation (Schwanhäusser et al., 2011). Translation proceeds in three phases: initiation, elongation, and termination. Translation initiation is the process whereby the mRNA is recruited to the ribosome. The translation factor, eukaryotic initiation factor 4E (eIF4E), is required for translation of most mRNAs. eIF4E binds to the 5′ terminal m^7GpppN cap structure on mRNA and serves to recruit the scaffolding protein, eIF4G, and other factors to form the translation initiation complex. The critical interaction between eIF4E and eIF4G is regulated by eIF4E-binding proteins (4E-BPs). In its unphosphorylated state, 4E-BP is bound to eIF4E and translation is inhibited. Phosphorylation of 4E-BP catalyzed by the mammalian target of rapamycin complex 1 (mTORC1) triggers the release of 4E-BP and enhances translation (Gingras et al., 2001; Proud, 2007). ERK signaling to mitogen-activated protein kinase-interacting kinases (MNKs) is associated with enhancement of translation though the mechanisms are not fully understood. MNKs bind directly to eIF4G and catalyze the phosphorylation of eIF4E at Ser209. Phosphorylated eIF4E has decreased affinity for mRNA binding, which, in theory, could facilitate protein synthesis by recruiting initiation complexes and therefore more ribosomes to the RNA (Buxade et al., 2008). During translation elongation the polypeptide chain is formed as the ribosome moves along the mRNA. Eukaryotic elongation factor 2 (eEF2) plays a key role in catalyzing the translocation of peptidyl-tRNAs from the A-site to the P-site on the ribosome. When phosphorylated, eEF2 does

not bind the ribosome and global translation is slowed down. By mechanisms that are not fully understood, translation of certain synaptic proteins (Arc, αCaMKII) is maintained or enhanced under conditions of eEF2 phosphorylation (Scheetz et al., 2000; Chotiner et al., 2003; Soulé et al., 2006; Park et al., 2008; Gal-Ben-Ari et al., 2012). eEF2 kinase, the only known kinase for eEF2, is regulated by calcium/calmodulin, mTORC1, and ERK signaling (Proud, 2007).

Several lines of evidence implicated sleep in the regulation of the protein synthesis. Two early studies involving *in vivo* incorporation of radioactive leucine in the brain revealed that global rates of protein synthesis were regulated during sleep (Ramm and Smith, 1990; Nakanishi et al., 1997). Both studies concluded that rates of protein synthesis correlate positively with the amount of NREM sleep. More recently, Vazquez et al. (2008) performed a proteomics screen of spontaneous sleep-wake state dependent changes in cortical protein expression and demonstrated rapid changes on the order of minutes. Sleep is associated with upregulation of numerous genes in the rodent cortex, including genes encoding translation initiation factors (eIF4b, eIF5, eIF3 subunits 3, 8 and 12), and eEF2 (Cirelli et al., 2004; Mackiewicz et al., 2007, 2008). Not surprisingly, proteomic studies indicate that changes in protein expression patterns depend on the duration of sleep deprivation. Short periods (6 h) of sleep deprivation altered expression of 11 proteins associated with synaptic function or cytoskeletal regulation in the basal forebrain cholinergic region (Basheer et al., 2005), while 7 days of sleep deprivation was associated with enhanced cortical expression of cytochrome C, the latter possibly indicative of metabolic stress (Cirelli et al., 2009).

In addition, a small subset of transcripts involved in tRNA activation is upregulated during sleep (Mackiewicz et al., 2007). Microarray analysis of mouse hippocampal tissue obtained after 5 h of sleep deprivation identified a decrease in the expression of mRNAs associated with protein translation (Vecsey et al., 2012). Independent validation confirmed decreased expression of total and phosphorylated mTOR following sleep deprivation. This difference was absent in mice permitted to sleep for 2.5 h after the sleep deprivation (rebound sleep). This upregulation of translation-related genes has been taken in support of active protein synthesis during sleep. Alternatively, translation factor synthesis in sleep may be restorative in nature, preparing the translational machinery for waking protein synthesis.

A recent study examined the relationship between sleep quality and quantity of home cage housed rats with the activity-state (phosphorylation) of translation factors eIF4E and eEF2 (Grønli et al., 2012). In the hippocampus, no association was found between sleep and translation factor activity. In the prefrontal cortex, more NREM sleep was associated with higher eIF4E and eEF2 phosphorylation. eEF2 phosphorylation correlated positively with sleep quality (total time spent in SWS) and negatively with poor sleep quantity (number of waking episodes). Levels of phosphorylated eIF4E correlated positively with the number of SWS and REM sleep episodes. Taken together, this suggests that sleep quality (based on the amount and number SWS episodes) correlates positively with phospho-eEF2 and phospho-eIF4E levels. These changes provide only an indirect measure of (enhanced) translational activity and more work is needed to profile the impact on translation and protein expression. However, dual eIF4E/eEF2 phosphorylation is mechanistically linked to protein synthesis-dependent forms of LTP in the dentate gyrus and plasticity (ODP) in the visual cortex (Kanhema et al., 2006; Panja et al., 2009; Seibt et al., 2012; Dumoulin et al., 2013).

Following 8 h of sleep deprivation, phosphorylation of eIF4E decreased in the dentate gyrus, but not in the CA region (Grønli et al., 2012). In contrast, eEF2 phosphorylation was elevated in both hippocampal regions and the prefrontal cortex. Thus, sleep deprivation has brain region-specific effects on translation initiation and elongation activity. Surprisingly, sleep deprivation increased Arc mRNA levels in the rat prefrontal cortex without affecting Arc protein expression. This dissociation between Arc mRNA and protein expression in sleep-deprived rats might be explained by enhanced ubiquitination and proteasomal degradation of Arc protein (Soulé et al., 2012). When Arc transcription is persistently stimulated, protein degradation imposes a powerful brake on protein expression.

Interestingly, sleep quality and quantity prior to sleep deprivation predicted the effects of sleep on translational factor activity in the prefrontal cortex, but not in the hippocampal regions. Phosphorylation of eEF2 was associated with previous SWS (positive) and waking episodes (negative), while levels of phosphorylated eIF4E were associated with prior episodes of SWS and REM sleep (positive). The implication may be that a good nights' sleep prior to sleep loss diminishes the impact of sleep deprivation on protein synthesis (Grønli et al., 2012).

Direct evidence for protein synthesis-dependent consolidation of synaptic plasticity has come from studies of ODP in the cat visual cortex (Aton et al., 2009; Seibt et al., 2012; Seibt and Frank, 2012). Sleep consolidates ODP by strengthening cortical responses to non-deprived eye stimulation (Aton et al., 2009). In a recent study (Seibt et al., 2012), cats were given monocular deprivation for 6 h in either wakefulness or sleep, combined with 6 h of intracortical infusion of the mTORC1 inhibitor, rapamycin. Vehicle-infused controls exhibited enhanced phosphorylation of 4E-BP1 and enhanced cortical expression of Arc and BDNF (and other proteins). Rapamycin blocked the sleep-related protein expression and consolidation of ODP, but did not affect plasticity induced during wakefulness. Dumoulin et al. (2013) further showed that consolidation of ODP requires ERK-MNK signaling leading to eIF4E phosphorylation (Dumoulin et al., 2013), as was found during LTP consolidation in the dentate gyrus (Panja et al., 2009). Taken together these results suggest that mTORC1 and ERK-MNK signaling are both required for sleep-dependent protein synthesis and consolidation of ODP.

In sum, gene and protein expression during sleep is likely important for changes in synaptic efficacy and consolidation of waking experience during sleep. The next section discusses recent insights into how functional and structural plasticity are regulated during sleep.

SYNAPTIC EFFICACY AND MORPHOLOGY DURING SLEEP

A decade ago, Tononi and Cirelli (2003) proposed a theory for a function of sleep in synaptic processes linked to cognitive

functioning. Binding together a significant part of current knowledge of sleep, the synaptic homeostasis hypothesis highlights the role of sleep in the downscaling of synaptic strength after prolonged wakefulness. In this view, synaptic strengthening during wakefulness occurs via LTP-like mechanisms, while NREM SWA induces mechanisms of LTD or depotentiation throughout the cerebral cortex (Tononi and Cirelli, 2003, 2006, 2012). According to this model, SWA results in a global homeostatic downscaling of synaptic weights in which the synapses enlarged by LTP during wakefulness are reduced in size during sleep and the weakest synapses are eliminated. Such scaling may enhance signal-to-noise ratios for information encoded during waking. By preventing saturation of input strength, homeostatic downscaling may serve to retain the information encoding capacity of networks. In addition, morphological scaling of spines would offset the metabolic expense of maintaining large synapses.

Electrophysiological recording of miniature excitatory postsynaptic currents (mEPSCs; currents which reflect spontaneous release of neurotransmitters from single vesicles) of layer II/III pyramidal neurons in the frontal cortex of mice and rats demonstrates wake-related increases and sleep-related decreases in synaptic efficacy (Liu et al., 2010). The frequency and amplitude of mEPSCs was enhanced after the dark period (wakefulness) and decreased after the sleep period. Matching these electrophysiological changes, the abundance of GluA1-containing AMPARs in biochemically fractionated synaptosomes was 40% higher after wakefulness than after sleep (Vyazovskiy et al., 2008; Hinard et al., 2012). Dephosphorylation of GluA1 on Ser845 is associated with decreases in channel open probability and decreased surface expression of AMPARs. Hinard et al. (2012) show that Ser845 phosphorylation is enhanced according to time spent awake, which appears compatible with the lack of synaptic depression at the level of AMPAR regulation during wakefulness. The fact that these changes are detected in synaptosomes from whole cortex and hippocampus is consistent with global scaling at the synaptic level.

Two recent studies provided evidence for distinct roles for NREM and REM sleep in modulation of synaptic plasticity. Chauvette et al. (2012) measured local field potentials in the rat somatosensory cortex of head-restrained cats during wake, comparing responses obtained before (wake 1) and immediately after (wake 2) a period of NREM. The responses were enhanced in wake 2, and longer periods of NREM were associated with larger evoked responses. A large transient increase in the response was observed in wake 2 but not after additional periods of NREM or REM sleep. The fact that delta power was increased in wake 2 compared to wake 1 is indicative of sleep inertia ("sleepiness"). Hence, it is possible that state-dependent modulation of synaptic efficacy contributes to the transient enhancement of the response (Winson and Abzug, 1978; Bramham and Srebro, 1989). However, a smaller stable increase in the evoked response was present in wakefulness after several sleep cycles, and this stable increase was mimicked by *in vitro* stimulation and intracellular hyperpolarization designed to mimic cortical slow wave "downstates" of NREM sleep (Chauvette et al., 2012). In sum, the work suggests that rapid upscaling (potentiation of the evoked responses) can occur in a cortical network during NREM sleep.

Grosmark et al. (2012) reported a prominent role of REM sleep in sleep-related neuronal plasticity. They show that overall firing rates of hippocampal pyramidal cells and interneurons increase moderately during NREM sleep periods, but decrease more during REM sleep, giving an overall net decrease in global firing from the neuronal population across a sleep cycle. Of major significance is the observed difference in pyramidal neuron firing during and between the intermittently occurring phasic events of NREM sleep known as sharp wave-ripples (SWRs). SWRs are irregular, synchronized bursts of neuronal activity in the hippocampus which are synchronized with thalamo-cortical spindle activity (Buzsáki et al., 2013). Grosmark et al. (2012) observed overall firing decreases in the periods between SWRs, but the synchrony and mean firing rate during ripple events increased across sleep in correlation with the power of the REM sleep theta rhythm. These findings are compatible with global downscaling of neuronal firing between SWRs and upscaling during SWRs. It is currently unclear whether these changes in neuronal firing and synchrony are mediated by LTP/LTD-type events on hippocampal pyramidal and interneurons. For further discussion see Born and Feld (2012), Tononi and Cirelli (2012), Cirelli (2013), Frank (2013), and Rasch and Born (2013).

At the anatomical level, two-photon microscopy has been used to visualize changes in dendritic spines of cortical neurons during sleep. Maret et al. (2011) showed that wakefulness is associated with a net increase in dendritic spines while sleep is associated with net spine loss. Yang and Gan (2012) ascribed the loss of spines during sleep to higher rates of turnover. However, it is not known whether spines size and density varies in a sleep-stage specific manner as shown for synaptic field potentials and neuronal firing activity.

WORKING MODEL OF SYNAPTIC PLASTICITY REGULATION DURING SLEEP

The evidence reviewed suggests that sleep-stage specific changes in synaptic efficacy and plasticity, firing activity, and network synchrony develop over the course of sleep. However, no consensus exists on how synaptic efficacy is regulated across the sleep cycle. In **Figure 1**, we offer a scenario for how synaptic plasticity is regulated during sleep. The working hypothesis is an attempt to integrate the cell biology of synaptic plasticity with the electrophysiological data.

We propose that specific cell biological events underlying homeostatic scaling and synaptic potentiation are parsed to specific stages of sleep and stage-specific population events. Downscaling during NREM sleep is supported by electrophysiological, biochemical and morphological data (**Figure 1D**; Grosmark et al., 2012; Cirelli, 2013). During REM sleep, immediate early genes such as Arc and zif268 are triggered by PGO-waves (**Figure 1E**; Ribeiro et al., 2002; Ulloor and Datta, 2005). Pharmacological cholinergic activity mimicking phasic REM sleep epoch also drives Arc expression in glutamatergic neurons (Soulé et al., 2012). Hence one function of REM sleep in this model is to provide immediate early gene induction in a broad population of cortical and hippocampal project neurons. A large body of work suggests that neuronal ensemble activity representing recent learning during the wake state is replayed in a time-compressed

format during the SWRs of NREM sleep (Lee and Wilson, 2002; Skaggs et al., 2007; O'Neill et al., 2010). Grosmark et al. (2012) showed that firing synchrony during SWRs develops gradually over successive NREM-REM sleep cycles. It follows that sparse, but synchronous synaptic firing, is repetitively replayed during NREM SWRs (**Figure 1F**). Thus, in NREM sleep an interplay may exist between synaptic potentiation and homeostatic scaling, with synaptic potentiation occurring during SWRs and scaling during the inter-ripple periods. Restorative macromolecular synthesis of the translational machinery (ribosomal proteins, translation factors, tRNA) occurs during sleep and may function to support bursts of synaptic protein synthesis.

As a multifunctional dendritically translated protein, Arc could play a role in coordinating diverse forms of plasticity during sleep. In REM sleep, Arc mRNA would be synthesized and transported to dendritic processes (**Figure 1E**). During NREM sleep, the synaptic activity of SWR events is proposed to drive local translation of Arc and other dendritically localized mRNAs. Repetitive bursts of translation during the night would ensure synapse-specific, protein synthesis-dependent potentiation (**Figure 1F**). Extrapolating from LTP studies, local Arc synthesis would consolidate synaptic potentiation through regulation of actin cytoskeletal dynamics and enlargement of dendritic spines (Fukazawa et al., 2003; Messaoudi et al., 2007). The extremely rapid rates of Arc mRNA and protein degradation are well-suited for mediating bursts of protein expression during SWRs. Arc mRNA is subject to rapid translation-dependent decay (perhaps limiting synthesis to translation by a single ribosome), while Arc protein is rapidly ubiquitinated and targeted for degradation in the proteasome (Rao et al., 2006; Giorgi et al., 2007; Soulé et al., 2012). In the same neurons, Arc protein could function to mediate homeostatic scaling and LTD. Dendrite-wide downscaling might be achieved through nuclear import of Arc leading to downregulation of GluA1 transcription (Korb et al., 2013), or through selective targeting of Arc to inactive or weakly activated synapses resulting in Arc-dependent endocytosis of AMPARs (Shepherd et al., 2006; Beique et al., 2010; Okuno et al., 2012). Clearly, it will be important to elucidate the time-dependent functions of Arc, and the possible role of post-translational modifications of the protein in dictating its localization and function (Bramham et al., 2010; Craig et al., 2012).

As summarized in the above sections, sleep loss can actively affect synaptic plasticity, synaptic efficacy and cognitive functioning. Such outcomes are sensitive to the various protocols used to induce sleep loss. Enforcing wakefulness when the brain is programmed to sleep may induce effects unrelated to sleep loss per se. Sleep restriction or sleep loss are often associated with an increase (but temporary) in the activity of the neuroendocrine stress systems by altering the state or function of the hypothalamo-pituitary-adrenal (HPA) axis. Most of the studies investigating the impacts of sleep loss on synaptic plasticity are performed in brain regions sensitive to stress. Among them is the hippocampus, which is involved in the negative feedback response to stress and helps to determine whether stress is ongoing. Since sleep deprivation can be stressful, it is important that studies aim to control for such non-specific effects. Several studies discussed in this review controlled for hormonal stress response following sleep deprivation (Palchykova et al., 2006; Hagewoud et al., 2010; Süer et al., 2011; Grønli et al., 2012), signifying that sleep loss rather than stress perturbs the changes in synaptic plasticity. From this emerges the question of how stress impacts sleep and synaptic plasticity, to which we now turn our attention.

IMPACT OF STRESS ON SLEEP (AND SYNAPTIC PLASTICITY)

Many people report that they feel stress due to perceived demands that exceed their resources. Our modern "24-h society" is one of several environmental stressors which disturb sleep. On work days we sleep about 38 min less than we did only a decade ago (Roenneberg, 2013). Stress is inevitable and has many (positive and negative) effects on the central nervous system. Stress is a perceived situation or experience which requires immediate compensatory responses for the maintenance of homeostasis. Importantly, if controllable, the body will adapt to stress, induce a fast energy input and improve cognitive achievements.

Stressful stimuli release stress hormones (glucocorticoids; cortisol in humans and corticosterone in rodents) that may have beneficial or detrimental physiological effects. Glucocorticoid receptors are widely expressed throughout the body and have a particularly dense distribution in the brain (De Kloet et al., 2005). Basal levels of corticosterone support LTP expression in hippocampus, whereas higher levels, stress or exposure to a new environment favor LTD (Pavlides et al., 1996).

Stress itself often disturbs sleep. Moreover, experiencing sleep loss following stress exposure may further potentiate changes in brain functioning at the level of synaptic plasticity. Vice versa, stress exposure after sleep loss alters the HPA response. In rats, the HPA response to restraint stress is reduced after 48 h sleep deprivation and 8 days of restricted sleep (but not after 1 day; Meerlo et al., 2002). In humans, partial (04–08 a.m.) and total (11 p.m. to 08 a.m.) sleep loss increases cortisol levels and delays the recovery of the cortisol release from the HPA axis (Leproult et al., 1997). Hence, sleep loss may affect the resilience of the stress response and potentiate the cognitive consequences of glucocorticoid excess.

The various paradigms employed in stress research rely either on acute or chronic, predictable or unpredictable stress. At present, little is known regarding the synaptic effects of stress-sleep relationship. However, it is expected that disturbed sleep and/or synaptic plasticity resulting from stress or manipulations of stress hormones depends on the intensity and duration of the treatment. Few studies have addressed this so far and no conclusion can yet be made. In the next sections we point to evidence that stress, either acute or chronic, predictable (controllable) or unpredictable (uncontrollable), can influence sleep and synaptic plasticity, but differently.

ACUTE STRESS, SLEEP AND SYNAPTIC PLASTICITY

Acute stress like social defeat, tail suspension, restraint, forced swim, or foot shock are developed as tools to mimic an immediate threat resulting in despair-like behavior. The behavioral effects (e.g., sleep changes) are often transient, typically gone within 1–3 days after termination of the stressor (Meerlo et al., 1997; Kinn et al., 2008).

Exposure to social defeat induces changes in NREM sleep but leaves REM sleep unaffected. An immediate increase in deep NREM sleep which dissipates during the following 12 h has been reported (Meerlo et al., 1997). Such an increase re-occurs 4 days after defeat and dissipates again within 14 days after social defeat (Kinn et al., 2008; Kinn Rød et al., 2014, in press). Inescapable foot-shock has been shown to increase wakefulness and then decrease REM sleep (Sanford et al., 2010; O'Malley et al., 2013). Additionally, Philbert et al. (2011) report long-lasting increases in sleep fragmentation (21 days after the stress exposure; Philbert et al., 2011). Similarly, wakefulness is also reported to increase after 1–2 h of restraint or forced swimming, while REM sleep increases during the sleep rebound (Cespuglio et al., 1995; Dewasmes et al., 2004). Hence, both NREM and REM sleep are differently affected by the nature of the stressor.

To the best of our knowledge, no study has directly focused on the acute stress–sleep interaction at the synaptic level. Studies of stress alone show that restraint suppresses maintenance of hippocampal LTP, enhances LTD *in vitro* and *in vivo*, and impairs cognitive functions like spatial memory (Foy et al., 1987; Bodnoff et al., 1995; Kim et al., 1996; Xu et al., 1997; Conrad et al., 2004; Krugers et al., 2006; Chen et al., 2010). Foot shock also facilitates LTD induction and slightly impairs learning of a spatial task directly after stress exposure, but enhances memory retrieval 5 days later (Xiong et al., 2003). Despite the transient sleep changes reported to occur after social defeat, this stressor is shown to produce long-lasting effects on hippocampal LTP and LTD. Artola et al. (2006) showed that the threshold for LTP induction is still raised and that for LTD lowered 7–9 months after defeat and individual housing (Artola et al., 2006).

Following a short stressful experience, *de novo* gene expression and protein synthesis, which are crucial to long-term synaptic changes, are rapidly but transiently altered. Activity of the ERK pathway and its downstream targets (including zif268) are increased after restraint and forced swimming (Gutièrrez-Mecinas et al., 2011), and ERK activation mediates the effects of restraint tail shock stress on hippocampal LTP (Yang et al., 2004). Other IEGs necessary for the stabilization of activity-dependent synaptic plasticity are also affected by acute stress. Arc and BDNF are among those and their expression varies in a region-specific manner. Cortical upregulation of both Arc mRNA and protein expression is indeed detected after restraint (Mikkelsen and Larsen, 2006). Restraint also induces a rapid, transient modification of BDNF expression across several brain regions. Importantly, the impact of stress depends on the individual's age. Defeat during adolescence and adulthood differentially regulates expression of several plasticity-related IEGs. A recent study shows that mRNA levels for Arc and BDNF (among others) are elevated following social defeat in adolescence, but not in adulthood (Coppens et al., 2011).

Compiled evidence suggests that acute stressful events have the capacity to induce sleep disturbances and alter long-term synaptic plasticity. Unfortunately, there is no data available on the effects of stress-sleep interactions on LTP, LTD, *de novo* gene expression or protein synthesis after acute stress.

CHRONIC STRESS, SLEEP AND SYNAPTIC PLASTICITY

When stress becomes chronic the physiological changes are more profound and long-lasting. Importantly, the changes vary accordingly with the intensity, frequency, and particularly the unpredictability of the stressor. The unpredictability is of importance to overcome stress habituation that occurs if the stressors are given repeatedly in a controllable manner.

The various protocols of repeated stress have been shown to affect sleep differently. Animals remain awake throughout a 6 h recording period after exposure to 2 days of inescapable foot shock; in contrast, 3–5 days exposure decreases wakefulness and REM sleep (Papale et al., 2005; O'Malley et al., 2013). Four days of forced swimming or restraint both decrease NREM sleep, and restraint additionally decreases REM sleep compared to baseline (Papale et al., 2005).

Repeated exposure to stressors may constitute an environmental risk factor for the development of anxiety and depression. When restraint is given repeatedly, 2 h for 10 days in rats, REM sleep is altered for at least 21 days after termination of the stressor (Hegde et al., 2011). Importantly, the impact of restraint stress on REM sleep was bimodally distributed. One group of rats manifested an increase in REM sleep and anxiety-like behavior, while the other group showed reduced REM sleep and no anxiety-like behavior (Hegde et al., 2011). One animal model that was developed to mimic minor daily hassles is chronic mild stress (Willner, 2005). Various mild stressors unpredictably given for 4 weeks decrease deep NREM sleep and increase time in REM sleep and wakefulness (Cheeta et al., 1997; Grønli et al., 2004, 2012). These sleep changes parallel those found in human depression. Although sleep alterations are one of the hallmark symptoms of depression and anxiety, there is limited research in rodents on the role of sleep in stress related depression and anxiety. Further research on sleep, depression- and anxiety related behaviors is an interesting direction for future investigation.

Hippocampal LTP maintenance is suppressed, and LTD enhanced *in vitro* and *in vivo* after acute and chronic restraint. This is also found when restraint is given in combination with tail shock as well as chronic corticosterone or chronic stress exposure (Foy et al., 1987; Bodnoff et al., 1995; Kim et al., 1996; Xu et al., 1997; Krugers et al., 2006; Chen et al., 2010). Brief exposure to mild stress also affects synaptic plasticity, as induction of LTP is blocked and LTD is facilitated (Xu et al., 1997). Moreover, this facilitation of LTD is abolished by acclimatization to, or removal from the mild stressors (Xu et al., 1997).

Chronic stress, in general, is associated with changes at the transcriptional and translational levels. At the transcriptional level, chronic stress has been shown to both increase (defeat and novel cage; Pardon et al., 2005) and impair CREB activity (glucocorticoid treatment; Föcking et al., 2003, chronic mild stress; Grønli et al., 2006). These contrasting results may relate to differences in glucocorticoid concentration release due to different intensity or chronicity of the stressor. At the translational level, chronic stress enhances phosphorylation of the translational regulators eIF4E and eEF2 in prefrontal cortex but not in the hippocampus or dentate gyrus (Grønli et al., 2012). This upregulation of translational activity may be taken as evidence in support for active protein synthesis after stress in the cortical areas.

Recently, sleep-stress interaction was examined at the translational level using the chronic mild stress model. Being chronically stressed abolishes associations between an individuals' sleep quality/quantity and translational activity. The sleep parameters are no longer predictive for cortical activity of initiation factor eIF4E and elongation factor eEF2 (Grønli et al., 2012). Similarly, chronic stress abolishes associations between sleep parameters measured *prior to* 8 h sleep deprivation and cortical translational activity as assessed after sleep deprivation. Given that persons experiencing chronic stress and depressed patients complain of non-restorative sleep, it is tempting to speculate that such lack of association between sleep quality and optimal rates of protein synthesis may be one of the underlying causes. Moreover, the effect of 8 h of sleep loss is modulated after chronic stress. In stressed rats, decreased activity of cortical eEF2 was found, whereas increased eEF2 activity occurs in non-stressed animals. No change of these translational regulators was observed in hippocampus. This may suggest that sleep deprivation counteracts the effect of chronic stress on eEF2 activity, in a region-specific manner. Interestingly, acute sleep deprivation has been reported to have antidepressant effects in humans (Wu and Bunney, 1990). The findings from animal studies raise the possibility that sleep deprivation may serve to restore or optimize rates of cortical protein synthesis in depressed patients.

Recent work shows that circadian changes in glucocorticoids are necessary for the formation and stabilization of dendritic spines in cortex after motor learning, and chronic and excessive exposure to glucocorticoids destabilizes learning-associated spines and impairs memory retention (Liston et al., 2013).

EARLY LIFE STRESS, SLEEP AND SYNAPTIC PLASTICITY

The brain is in constant change across the lifespan, starting from the early stages of life in utero. Early life (pre- and postnatal, as well as childhood and adolescence) hosts important developmental phases which allows the brain to mature. Being exposed to early life stress such as prenatal stress, maternal separation, low maternal care, or stress during adolescence has consistently been found to alter stress sensitivity in adulthood (Lupien et al., 2009).

Mammals show large amounts of active sleep (that parallels adult REM sleep) during early postnatal brain development. The predominance of REM sleep during early life is often taken in support of a role for REM sleep in processes of brain maturation and plasticity (Frank, 2011). The studies on sleep-related changes are scarce and the findings are divergent. Exposure of stress in utero may result in a prolonged first REM sleep episode and less NREM sleep in adulthood, compared to non-stressed controls (Rao et al., 1999). Long maternal separation (typically 3 h per day in the first 2 postnatal weeks) is reported to diminish the quality of deep NREM sleep, to alter total sleep time (decrease or increase), and to increase wakefulness compared to non-handled, handled, and brief maternally separated offspring. Moreover, the negative feedback regulation of the HPA axis in long maternal-separated offspring is suggested to be impaired and corticosterone level is elevated in long compared to brief maternal-separated offspring (Mrdalj et al., 2013).

Altered stress sensitivity in adulthood is also reflected in sleep changes. Exposure to later life stressor(s) affects sleep differently according to early life experience. Adult exposure to acute stress (2 h of cold) is followed by decreased REM sleep and elevated corticosterone levels, both in long maternal-separated offspring and handled controls (Tiba et al., 2004). Adult experience of chronic unpredictable mild stressors induces more time in sleep, more REM sleep episodes and more NREM sleep episodes ending in REM sleep in long, compared to brief, maternally separated offspring (Mrdalj et al., 2013). REM sleep deprivation in adult long maternal-separated offspring seems not to potentiate a present memory deficit (Garcia et al., 2013).

Independently of wakefulness, NREM or REM sleep, early life stress reduces brain activity measured by EEG, an effect potentiated by exposure to chronic stress as adults (Mrdalj et al., 2013). Offspring that receive low maternal care show poor LTP when they are adult, as opposed to those that were given high maternal care (Champagne et al., 2008). Single (short and prolonged), or repeated maternal separation can affect LTP expression in hippocampus and prefrontal cortex (Cao et al., 2013), without any change in the number of neurons and astrocytes (Baudin et al., 2012).

Activation of synaptic plasticity-related genes is assumed to represent an early step in the adaptation of neuronal networks to a stressful environment. Maternal separation after the first 2 postnatal weeks, at day 14–16, induces rapid increase in hippocampal Arc and zif268 mRNAs, accompanied by morphological changes such as an increase in spine number on CA3 dendrites (Xie et al., 2013). In rats exposed to isolation rearing, cortical upregulation of Arc mRNA and increase in both Arc and BDNF proteins is observed (Wall et al., 2012). Note that single (short and prolonged), or repeated maternal separation alter hippocampal BDNF expression (Roceri et al., 2002, 2004; Koo et al., 2003; Fumagalli et al., 2004; Nair et al., 2007). Notably, the prior history of maternal separation impacts the effect of adult stress on BDNF transcripts via modulation the upstream transcriptional activator CREB. An impact on neuronal progenitor proliferation is also reported, suggesting that alterations in CREB/BDNF may contribute to individual differences in hippocampal networks (Nair et al., 2007). In the cortex, the length of early life manipulations appears to be more important for these changes than their timing. Repeated early life stress induces a clear reduction of cortical BDNF levels in adult animals (Koo et al., 2003; Fumagalli et al., 2004; Roceri et al., 2004), whereas a single maternal deprivation does change BDNF expression (Roceri et al., 2002).

Stressful events early in life induce long-term sleep disturbances and alter long-term synaptic plasticity. Unfortunately, as for acute stress, there is no available data regarding the effects of stress-sleep interactions on LTP, LTD, *de novo* gene expression or protein synthesis after early life stress.

FACTORS IMPORTANT FOR STRESS-SLEEP INTERACTIONS

The findings on sleep changes after stress discussed above, raise an important issue that different stress modalities result in distinct sleep responses. Moreover, stress responses are mediated through the concerted activity of many brain areas and induce structural changes in neuronal networks. Changes can be short or long-lasting (Fuchs et al., 2006).

Brain areas involved in the stress response include areas important for sleep and wakefulness; the hypothalamus (including deep NREM sleep active neurons in the ventral lateral preoptic area), amygdala (activity is depotentiated during REM sleep), hippocampus (generating theta activity in REM sleep), prefrontal cortex (generating the highest voltage and the slowest NREM sleep waves compared to other cortical regions) and numerous brainstem regions promoting wakefulness like the locus coeruleus and raphe. Stress-induced changes in the activity of one or several of these brain regions may explain the different sleep changes.

The recovery from sleep loss is sensitive to stress. Likewise, recovery from stress is sensitive to sleep disturbances. If the individual has been exposed to stress prior to the sleep loss, the sleep recovery may be altered. Little data is available on how sleep recovery is affected by stress experience prior to sleep loss. One study has shown that exposure of rats to social defeat prior to 6 h of sleep deprivation potentiated changes in the recovery sleep by an increase in deep NREM sleep (Meerlo et al., 2001).

The available data on sleep disturbances and drive for sleep after acute stress suggests that stress accelerates the buildup of sleep need. NREM and REM sleep are differently affected by the nature of the stressor. Restraint increases REM sleep while social defeat increases deep NREM sleep. Cognitive functioning is considered to be potentiated and LTP-like changes facilitated after transient stress (Luine et al., 1996; Shors, 2001). However, more studies are needed to define the selective role of NREM and REM sleep rebound after stress. Moreover, knowledge on how prior stress may impair the sleep recovery after sleep loss is limited.

The recovery of stress may result in a long-lasting disruption of normal circadian sleep pattern by decreased or increased sleep throughout the 24 h period. Again, changes in specific sleep stage depend on the type of stressor. In the rats' *active* phase restraint decreases sleep efficiency, NREM and REM sleep, whereas foot shock, swimming, cold as well as chronic controllable stress increase REM sleep 4 days after the stress exposure (Kant et al., 1995; Papale et al., 2005).

Behavioral factors appear to be important for the understanding of the variations in sleep changes brought by stress. During a stress situation, the coping strategy may play a significant role. Animals fighting back during a social conflict before being defeated show fragmented NREM sleep, an effect becoming more robust in the long-term (day 21 post defeat) compared to animals showing quick submission and passivity (Kinn Rød et al., 2014, in press). Importantly, increases in REM sleep have been observed if the organism controls the stressor (e.g., escapable foot shock; Kant et al., 1995; Sanford et al., 2010) and LTP is impaired following inescapable, but not escapable, shock in a shuttle box avoidance task (Shors et al., 1989).

In summary, when an individual is subjected to environmental stressors in any phase of life, sleep is affected. Sleep disturbances after stress are modulated by several factors among which are the brain areas activated by stress, the ability to recover from stress, the behavioral coping strategy and ability to control the stressor.

CLINICAL PERSPECTIVES AND CLOSING COMMENTS

Sleep loss, sleep restriction, and the experience of being stressed are common place in our modern society. There is a broad consensus that insufficient sleep leads to a general slowing of response speed and increased variability in performance (Van Dongen et al., 2003). Whether sleep loss affects all cognitive processes and capacities, or specifically impairs some aspects of alertness, memory, perception and executive functions is a subject of debate (Killgore, 2010). Mood is especially sensitive to sleep loss. Chronic sleep disturbances are risk factors for developing anxiety and depression (Neckelmann et al., 2007), and vice versa, sleep disturbances are so frequently observed in patients experiencing psychological disorders that they form part of the diagnostic manual criteria for the disorders. Clinical studies of anxiety and depression indicate prevalence of both insomnia and hypersomnia (Ford and Kamerow, 1989; Ohayon, 2002; Riemann, 2007).

Modulation of recovery processes and neuroplasticity after brain trauma is sensitive to sleep loss. Insufficient sleep may compromise neuronal function and contribute to neurodegenerative processes. Disturbed sleep 3 days after focal cerebral ischemia is shown to reduce axonal sprouting, expression of synaptophysin, and the ischemia-stimulated neural and vascular cell proliferation in rats (Zunzunegui et al., 2011). The data suggests a role of sleep in the modulation of recovery processes and neuroplasticity after traumatic brain injury.

As the cell biological regulation of synaptic plasticity during sleep comes into view, new fundamental insights are likely to be gained regarding how information is processed and stored during the sleep cycle. Convergent evidence from electrophysiological, molecular, and behavioral studies all point to the importance of cyclic, synergistic interactions between NREM and REM stages in fulfilling the cognitive functions of sleep. Stress, sleep quality, and cognitive performance are inexorably intertwined. As reviewed here, differential effects of stressors on sleep quality, synaptic plasticity, and molecular mechanisms associated with synaptic plasticity have been established. A major challenge is to determine how different forms of stress (acute and chronic, controllable and uncontrollable) specifically alter the sleep cycle and the quality of the interactions between NREM and REM sleep. And reciprocally, how altered sleep habits may predispose to stress and maladaptive cognitive responses. More studies are needed to identify the specific neural circuits mediating stress-sleep interactions.

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Vagus nerve stimulation enhances extinction of conditioned fear and modulates plasticity in the pathway from the ventromedial prefrontal cortex to the amygdala

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Fearful experiences can produce long-lasting and debilitating memories. Extinction of the fear response requires consolidation of new memories that compete with fearful associations. Subjects with posttraumatic stress disorder (PTSD) show impaired extinction of conditioned fear, which is associated with decreased ventromedial prefrontal cortex (vmPFC) control over amygdala activity. Vagus nerve stimulation (VNS) enhances memory consolidation in both rats and humans, and pairing VNS with exposure to conditioned cues enhances the consolidation of extinction learning in rats. Here we investigated whether pairing VNS with extinction learning facilitates plasticity between the infralimbic (IL) medial prefrontal cortex and the basolateral complex of the amygdala (BLA). Rats were trained on an auditory fear conditioning task, which was followed by a retention test and 1 day of extinction training. Vagus nerve stimulation or sham-stimulation was administered concurrently with exposure to the fear-conditioned stimulus and retention of fear conditioning was tested again 24 h later. Vagus nerve stimulation-treated rats demonstrated a significant reduction in freezing after a single extinction training session similar to animals that received 5× the number of extinction pairings. To study plasticity in the IL-BLA pathway, we recorded evoked field potentials (EFPs) in the BLA in anesthetized animals 24 h after retention testing. Brief burst stimulation in the IL produced LTD in the BLA field response in fear-conditioned and sham-treated animals. In contrast, the same stimulation resulted in potentiation of the IL-BLA pathway in the VNS-treated group. The present findings suggest that VNS promotes plasticity in the IL-BLA pathway to facilitate extinction of conditioned fear responses (CFRs).

Keywords: anxiety, PTSD, local field potentials, *in vivo*, LTP, LTD

INTRODUCTION

Extinction of conditioned fear is the process of attenuating fearful behavioral responses to neutral stimuli when they no longer predict aversive outcomes. Therefore, extinction requires new learning about the conditioned stimuli (CS). About 30% of individuals who experience traumatic life events develop posttraumatic stress disorder (PTSD; Nemeroff et al., 2006), which is characterized by a general impairment in the ability to extinguish fear responses (Jovanovic et al., 2010; Norrholm et al., 2011). Patients suffering from PTSD exhibit reduced ventromedial prefrontal cortex (vmPFC) activation and heightened amygdala activation (Hayes et al., 2012; Stevens et al., 2013). Similarly, experiments in rat models of fear learning suggest that the vmPFC is required for the modulation and expression of extinction memory (Sierra-Mercado et al., 2011) and that plasticity in the vmPFC-amygdala pathway underlies the suppression of fear via attenuation of central amygdala activity (Marek et al., 2013). Neuroplasticity has been observed in the basolateral complex of the amygdala (BLA) following both the formation of conditioned fear responses (CFRs; Schafe and LeDoux, 2000) and the suppression of those responses during extinction (Parkes and Westbrook, 2010; Vouimba and Maroun, 2011). Activation of the infralimbic (IL) mPFC during extinction maintains extinction plasticity in the amygdala via its projections to the BLA and intercalated cells that inhibit central nucleus activity (Pape and Paré, 2010; Amano et al., 2011; Knapska et al., 2012). Thus the encoding of fear memory and extinction results in functional changes in neurons in the BLA (Amano et al., 2011). Vagus nerve stimulation (VNS) enhances memory in rats and in humans (Clark et al., 1998, 1999). Pairing VNS with discrete stimuli or behaviors has been used to induce targeted cortical plasticity for the treatment of tinnitus (Engineer et al., 2011) and motor deficits (Porter et al., 2012), raising the possibility that VNS might also be used to direct the neural plasticity underlying extinction memory. We previously showed that pairing VNS with non-reinforced CS presentations facilitates extinction of fear responses (Peña et al., 2013). The encoding of learned events results in synaptic changes that modulate subsequent induction of plasticity, or

metaplasticity. Here we examined metaplasticity in the IL-BLA pathway in animals that demonstrated VNS-enhanced extinction of conditioned fear.

MATERIALS AND METHODS

ANIMALS AND SURGICAL PROCEDURES

All procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of The University of Texas at Dallas. Male Sprague-Dawley rats (Charles River, Wilmington, MA) ∼90 days old, weighing 250–300 g on arrival were housed on a 12 h light/dark cycle (lights on at 7:00 am) with access to food and water *ad libitum*. Rats were anesthetized with isoflurane (2% at an oxygen flow rate of 600–800 ml/min) and mounted in a stereotaxic frame (World Precision Instruments, Sarasota, FL). A headstage was constructed using bone screws and dental cement to fix the platinum-iridium wires used for VNS (Sigmund-Cohn Inc., Mount Vernon, NY) and a four-channel strip connector (Omnetics, Minneapolis, MN) in place. Animals were then removed from the stereotaxic and a small (1–2 cm) incision was made on the left ventral side of the neck over the jugular vein. The opening was blunt dissected until the sternomastoid, sternohyoid, and omohyoid muscles were apparent. The muscles were pulled apart using muscle retractors and the deep cervical fascia was transected to reveal the carotid sheath, containing the carotid artery and vagus nerve. The sheath was opened and connective tissue holding the vagus nerve to the carotid artery was separated for 1 cm using modified glass micropipettes. A custom-made platinum-iridium wire electrode in micro-renathane "cuff" (0.04" ID, 0.08" OD, 4 mm long) was placed around the vagus nerve (**Figures 1B–D**). The platinum-iridium wire leads were tunneled subcutaneously to the headstage where they connected with the inputs from an isolated pulse stimulator (MS4, Tucker Davis TechnologiesAlachua, FL). Optimal vagal fiber activation was assessed before and after completion of the study by applying VNS (0.2 mA, 60 Hz, 10 s) and observing brief cessation of breathing in anesthetized rats. Once electrode function was established, the leads were permanently fixed to their input site on the headstage with dental cement. Sham-stimulated rats were subjected to the same surgical procedure; however, cuffs were designed to not deliver current. During the 1 week recovery period, animals were handled 5 min/day for 5 days.

AUDITORY FEAR CONDITIONING

Auditory fear conditioning and extinction trials were performed in a plexiglas operant box (20 × 20 × 20 cm, with stainless steel grid floor, Vulintus, Plano, TX) housed in a sound-attenuated chamber (Day 1; **Figure 1A**). Rats were presented eight tones (9 kHz, 85 dB SPL) as CS overlapping with a 1 s footshock (0.5 mA; DSCK-C Dual Output, scrambled shocker, Kinder Scientific Co., Poway, CA), serving as the unconditioned stimulus (UCS). To prevent the development of a specific temporal association with the footshock, a single 1 s footshock was administered at a randomized time during each 30 s tone presentation. To produce robust conditioned fear, rats were again given eight tones paired with footshock on a second conditioning day 24 h later. The inter-tone-interval (ITI) was varied between 3 and 5 min, averaging 4 min for every tone presentation, in order to prevent development of a specific temporal association with the footshock.

CONDITIONED FEAR TESTING

To measure VNS effects on extinction of conditioned fear, freezing was measured during tone presentations before and after VNS treatment. Conditioned fear was first measured 1 day after conditioning. After a habituation period of 10 s four tones were presented with an ITI of 3, 4, or 5 min (4 min average), but no footshock was administered during this test session (Day 3; **Figure 2A**). The session was video recorded and the rats' behavior was assessed by two independent observers who were blind to treatment conditions. The rats' freezing response was used as a measure of the CFR and expressed as the percent time spent freezing of total duration of exposure to the conditioned cue. Freezing was defined as a period of complete immobility, characterized by a lowered head, spread paws, and rapid respiration. In order to test whether VNS also has effects on behavior outside of the conditioned response to the tone we also analyzed freezing behavior during the intervals between tone presentations. Analysis of freezing behavior during the ITIs was done in the same manner as that for the CFR to the CS described above.

EXTINCTION TRAINING AND VNS/SHAM TREATMENT

Extinction trials were administered in the same context as training and testing trials. On Day 4, rats were presented four tones in the absence of footshock. During this treatment trial, VNS (30 s duration, 0.4 mA, 500 μs pulse width at 30 Hz, starting 150 ms before the onset of the tone) or sham-stimulation overlapped with the conditioned 30 s tone. These VNS parameters were selected because they were optimized for enhancing memory consolidation in rats (Clark et al., 1998). Another group received extended extinction (EE) training but no VNS. Rats in this group received 5× the number of tone presentations (i.e., 20 tones) during the treatment trial (Day 4, **Figure 2A**). Finally, to control for unspecific effects of VNS on synaptic plasticity, one group of animals that was not fear conditioned received the same amount of VNS in their home cages as the experimental group during extinction training.

POSTTREATMENT CONDITIONED FEAR TEST

On Day 5, freezing was assessed in all fear-conditioned animals as described above for the initial conditioned fear test; VNS and sham-stimulation were not administered and the level of freezing to the tone, in the absence of footshock, was again measured. Post-treatment freezing was analyzed as percent of each individual rat's CFR.

IN-VIVO ELECTROPHYSIOLOGICAL RECORDINGS

On day 6, evoked field potentials (EFPs) were recorded in the BLA (D/V: 7.2, A/P: 2.7, M/L: 4.9 from bregma) of isoflurane-anesthetized rats using glass microelectrodes (2M KCl; 1–2 MOhms resistance). A bipolar matrix stimulation electrode (FHC, Bowdoin, ME) was placed in the IL region of the vmPFC (D/V: 4.6, A/P: 3.0, M/L: 0.7 from bregma). The width of the

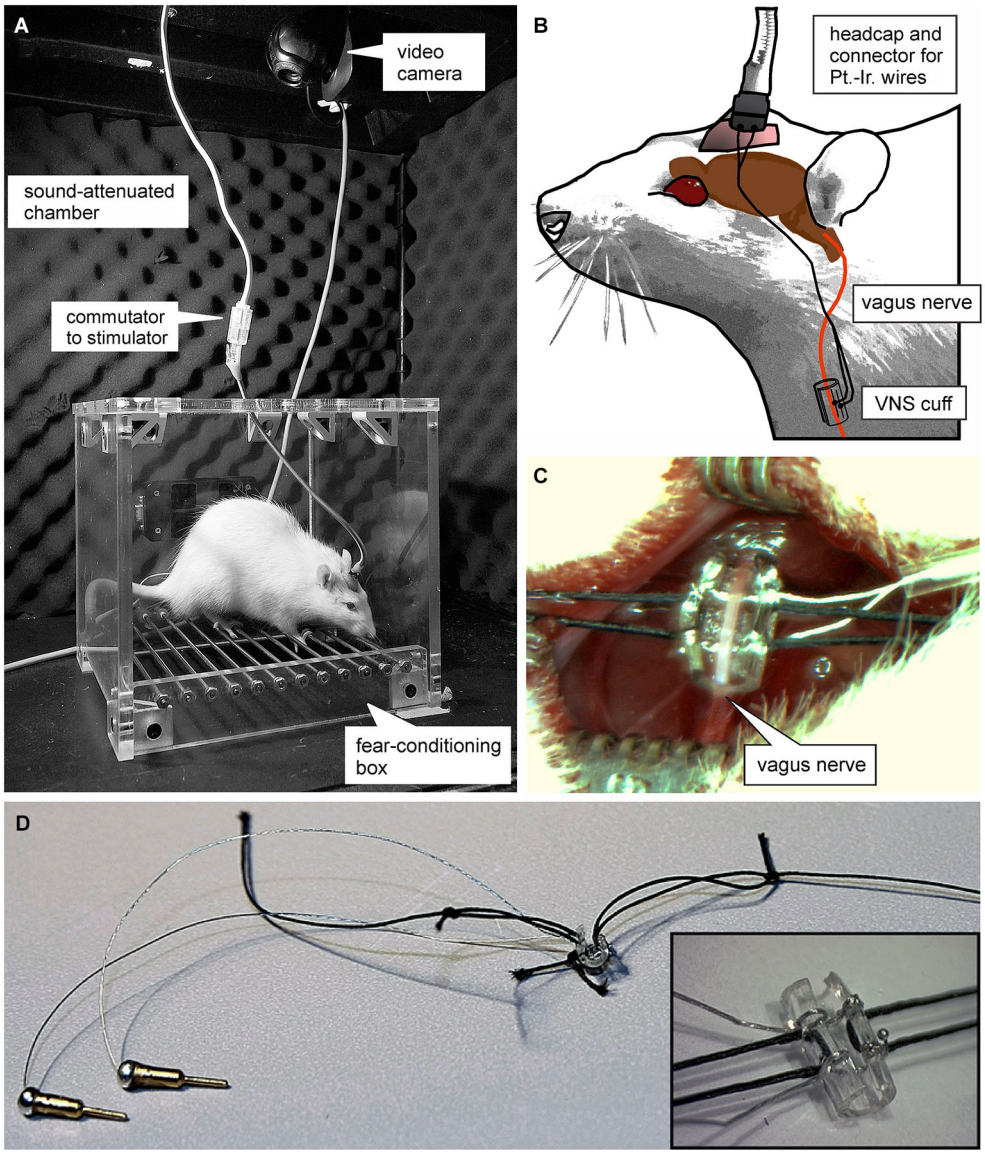

FIGURE 1 | Experimental set-up. (A) Photograph of the plexi-glass box used for auditory fear conditioning. **(B)** Schematic of the set-up used for vagus nerve stimulation (VNS). Animals were connected to a stimulation isolation unit via a headcap from which two platinum-iridum wires lead subcutaneously to a custom-made "cuff-electrode" that is wrapped around the vagus nerve. (**C** and **D**) Photomicrographs of the cuff electrode used for VNS. **(C)** shows the surgical incision and the exposed vagus nerve before the cuff electrode is sutured around it.

stimulation pulse was set to 0.3 ms and the stimulation intensity corresponded to 40% of the minimum current intensity that evoked a maximum field response (based on an input–output curve determined before collection of baseline data). Evoked responses were amplified using a Model 1600 Neuroprobe Amplifier (A-M Systems) and a BMA 200 Portable Bioamplifier (CWE, cwe-inc.com). Signals were digitized using a CED 1401 interface (Cambridge Electronic Design, Cambridge, England) and analyzed using Spike-2 (CED) and Axograph-X (Axograph Scientific, New South Wales, Australia). Data were collected every 15 s and averaged every 1 min for analysis. Baseline data were collected for a minimum of 10 min before synaptic plasticity was induced. The high-frequency stimulation (HFS) protocol used three bursts of 100 pulses at 50 Hz (2 s), with 20 s inter-burst intervals at the minimum current intensity that evoked the maximum field response. The low-frequency stimulation (LFS) protocol consisted of 900 pulses at 1 Hz. The amplitude of the EFP was measured as the difference between the mean of a 5 ms window before the stimulation artifact and the mean of a 5 ms window around 20–25 ms after the stimulation artifact, corresponding to the negative peak of the field potential. Data were normalized to baseline and the average of a 10 min baseline was set as 100% and the 10 min period 40–50 min after plasticity induction was used to analyze long-term plastic changes.

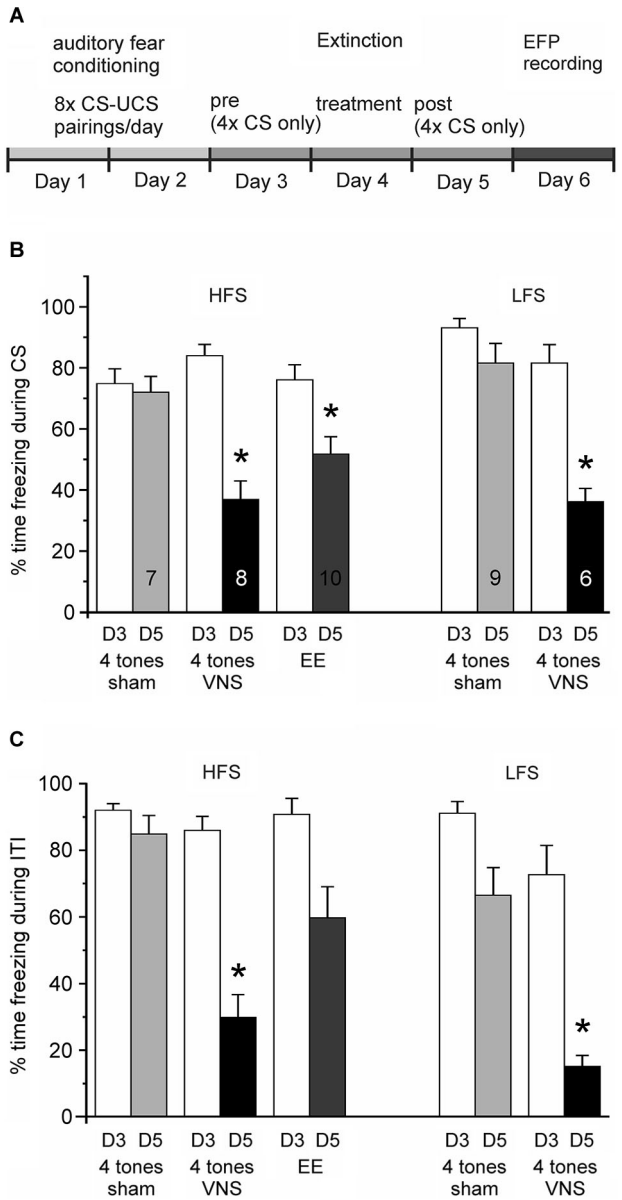

FIGURE 2 | Vagus nerve stimulation enhances extinction of auditory fear conditioning. (A) Experimental timeline. Auditory fear conditioning consisted of 2 days of training with eight pairings between a tone (CS) and a footshock (UCS) per day. On Day 3 rats were exposed four times to the conditioned tone in the absence of footshock and freezing levels were tested (Conditioned Fear Test). On Day 4 (Treatment) rats received group-specific treatment: four tone presentations were paired with either VNS or sham-stimulation. Rats in the extended-extinction group received 20 tone presentations. Freezing levels were tested again on Day 5 in response to four presentations of the CS alone (Post Treatment test). **(B)** Percentage of time spent freezing during presentation of the conditioned stimulus (CS) on Day 3 (D3, white bars) and Day 5 (D5), respectively, for the different treatment groups (4 tones + sham stimulation, four tones + VNS, and extended extinction, EE, with 20 tones + sham stimulation). Presentation of the Day 3 and Day 5 freezing responses is separated into groups of rats in which subsequently the plasticity of the IL-BLA pathway was tested with either high-frequency (HFS) or low-frequency stimulation (LFS) of the IL. After 1 day of extinction training paired with VNS (black bar) rats spent

(*Continued*)

FIGURE 2 | Continued
significantly less time freezing than sham controls (* $p < 0.05$). Similar levels of fear extinction were observed when rats were given extended extinction (gray bar). **(C)** Percentage of time spent freezing during the inter-tone intervals (ITI) on Day 3 (D3, white bars) and Day 5 (D5), respectively, for the same groups shown in **(B)**. Freezing levels during ITI might serve as a measure of whether extinction training generalizes to the context. Animals receiving extended extinction training did not differ significantly in their behavior from sham stimulated animals during ITIs; however, VNS animals also showed reduced freezing behavior outside the presentation of the conditioned tone.

HISTOLOGY

After the electrophysiological recordings, the stimulation and recording sites were marked by passing anodal currents (10 mA for 3 s and 10 mA for 2 min) through the electrodes. Rats were overdosed with urethane (3 g/kg) and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in PBS. Coronal sections were cut on a freezing microtome and Nissl-stained for the identification of stimulation and recording sites.

DATA ANALYSIS

Single trial behavioral data were analyzed with an ANOVA and pair-wise treatment group comparisons used Tukey-Kramer *post hoc* tests. Multiple trial experiments were analyzed with a partially repeated ANOVA (treatment group × trial), and group differences were determined using Tukey-Kramer *post hoc* tests. Changes in post-stimulation EFP amplitudes were compared using partially repeated-measures ANOVA with a treatment group × time interaction.

RESULTS

VAGUS NERVE STIMULATION ENHANCES EXTINCTION MEMORY

Animals in the three groups given extinction treatment (**Figure 2B**; VNS, $n = 14$; sham, $n = 16$; and EE, $n = 10$) showed a similar degree of CFR on the first test day (Day 3). An ANOVA revealed no significant group differences in CFR on the day before treatment ($F_{(2,37)} = 1.332$, $p = 0.276$). Analysis of the CFR measured 1 day after treatment (Day 5) revealed a significant main effect across treatment groups ($F_{(2,37)} = 20.682$, $p < 0.001$). Consistent with previous findings, the CFR of rats given only four non-reinforced exposures to the conditioned tone without VNS treatment on Day 4 changed only marginally, indicating no reduction in conditioned fear after the treatment trial. In contrast, a significant reduction in the percentage of CFR was seen in the group of rats in which a single extinction session (4 tone pairings) was paired with VNS (Tukey-Kramer, $p < 0.001$ vs. sham), as well as in the group of animals that received EE training (EE, 20 tone pairings) (Tukey-Kramer, $p = 0.002$ vs. sham). The magnitude of the reduction in CFR did not differ between the VNS and EE groups (Tukey-Kramer $p = 0.105$; **Figure 2B**). Similarly, an ANOVA showed no significant differences in freezing during the ITI on Day 3 ($F_{(2,37)} = 2.858$, $p = 0.070$), but after treatment a significant main effect was found across treatment groups ($F_{(2,37)} = 17.694$, $p < 0.001$) on Day 5. Rats treated with VNS showed reduced freezing behavior when compared to both sham and EE groups (Tukey-Kramer, $p < 0.05$ vs. sham and EE); in contrast, rats in the EE group

did not differ in freezing behavior from sham stimulated animals during Day 5 ITIs (Tukey-Kramer, $p = 0.308$; **Figure 2C**). Taken together, these findings indicate that VNS facilitated extinction of conditioned fear, bringing it to the level achieved by EE training, consistent with our previous results (Peña et al., 2013), and in addition, VNS also facilitated extinction to the context as evidenced by reduced freezing behavior during the inter-tone intervals.

VAGUS NERVE STIMULATION MODULATES PLASTICITY IN THE IL-AMYGDALA PATHWAY

Stimulation of the IL elicited negative field potentials in the BLA, which peaked after 20–25 ms (**Figure 3**). The current-voltage relationship between stimulation intensity in the IL and EFP amplitude in the BLA did not significantly differ between fear-conditioned sham-stimulated rats and rats that received VNS during extinction training ($F_{(2,30)} = 0.435$, $p = 0.651$; **Figure 3C**). To further examine learning- and VNS-induced synaptic plasticity in the IL-BLA pathway we used short burst stimulation (HFS) of the IL. A repeated measures ANOVA showed a main effect of treatment ($F_{(5,30)} = 5.983$, $p = 0.0003$). Consistent with previous reports (Maroun, 2006), this protocol did not induce synaptic plasticity in naïve rats ($n = 8$, +3.08 +/− 3.17% change, $F_{(1,12)} = 0.106$, $p = 0.75$). In rats given 2 days of fear conditioning without extinction training (FC, $n = 7$) and in rats that received only sham-stimulation during the treatment phase of extinction ($n = 10$), resulting in no reduction of the freezing response, HFS of the IL induced LTD of the EFP (FC −15.35 +/− 2.04% change, $F_{(1,12)} = 7.855$, $p = 0.016$; sham −25.28 +/− 2.39% change, $F_{(1,18)} = 17.6$, $p = 0.001$). In contrast, HFS induced LTP (+30.44 +/− 5.41% change, $F_{(1,14)} = 6.6063$, $p = 0.022$) in the IL-BLA pathway of rats given a single extinction session paired with VNS ($n = 8$) (**Figure 3D**). To determine whether the change in the sign of plasticity in the IL-BLA pathway was due to extinction of conditioned fear, the pairing of extinction training with VNS, or VNS alone, we tested two control groups. Increasing the amount of tone exposures during extinction (EE group) reversed the LTD in the IL-BLA pathway seen in fear conditioned and sham-stimulated rats (−0.64 +/− 2.62% change, $F_{(1,18)} = 0.008$, $p = 0.931$). On the other hand, applying VNS outside of the fear-conditioning context (i.e., applying VNS to untrained, never fear-conditioned rats in their home cages; $n = 6$) did not alter the ability to induce plasticity in the IL-BLA pathway (−4.30 +/−1.94% change, $F_{(1,10)} = 0.633$, $p = 0.455$; **Figure 3D**).

Finally, we tested whether context-specific modulation of synaptic plasticity is frequency-dependent. To this end we used a low frequency stimulation protocol that has previously been shown to induce robust LTD in the IL-BLA pathway in naïve rats (Maroun, 2006). A partially repeated-measures ANOVA revealed a significant difference in the EFP following LFS between rats which received VNS during extinction ($n = 6$) and sham-treated rats ($n = 9$) ($F_{(1,10)} = 5.156$, $p = 0.041$). In sham-stimulated rats LFS induced LTD (−21.7 +/− 2.6% change, $F_{(1,16)} = 11.662$, $p = 0.004$), but this was suppressed in rats that received VNS during extinction (+1.9 +/− 2.4% change, $F_{(1,10)} = 0.122$, $p = 0.734$), indicating that under these conditions VNS similarly reversed fear-conditioning-associated plasticity in the IL-BLA pathway (**Figure 4**).

DISCUSSION

Consistent with our previous results (Peña et al., 2013), we found that administration of VNS during a single session of exposure to conditioned cues facilitates extinction of conditioned fear. Here we expand on these behavioral findings by examining the effects of fear conditioning and extinction on synaptic plasticity in the IL-BLA pathway. Both the IL and the BLA are implicated in fear conditioning and extinction (Marek et al., 2013). Here, we found evidence that fear conditioning and extinction produce metaplasticity in the IL-BLA pathway, modulating conditions for the induction of synaptic plasticity. Fear conditioning predisposed synapses toward depression and extinction reversed this effect. Importantly, VNS delivered during extinction training further potentiated the evoked responses in the BLA, resulting in LTP in the IL-BLA pathway. Thus, VNS enhances extinction and has lasting effects on synaptic plasticity in a pathway crucial for extinction learning. Neural activity in the vmPFC is increased during recall of extinction memory (Milad and Quirk, 2002), and HFS of the IL following retrieval of a conditioned fear memory enhances subsequent fear extinction learning (Maroun et al., 2012). On the other hand, previous reports have shown that successful fear extinction reduces the efficacy of excitatory synaptic inputs from the vmPFC to the BLA (Vouimba and Maroun, 2011; Cho et al., 2013). The reasons for this discrepancy are not clear, but may reflect compensatory mechanisms and cell type specific projections of the vmPFC to the BLA and the intercalated cells, respectively (Cho et al., 2013). Here, using recordings in anesthetized rats more than 24 h after the last behavioral testing, we observed no significant differences in the input-output relationship of EFPs before plasticity-induction between animals that received sham-stimulation or VNS. Nevertheless, behavioral training altered the conditions for the induction of synaptic plasticity in this pathway. Consistent with a previous report (Maroun, 2006) we found that the pathway from the IL to the BLA is resistant to the induction of LTP in naïve animals. However, following fear conditioning, burst stimulation of the IL induced LTD in the BLA of fear conditioned and sham-stimulated rats. Extended extinction training or VNS during a single extinction session reversed this plasticity. In VNS-treated rats this resulted in the promotion of LTP in response to HFS. In a follow-up experiment we tested whether pairing VNS with extinction generally promoted LTP within the circuit or, alternatively, can also enhance LTD induced by low frequency stimulation of the IL. Under these conditions LTD induction was inhibited, suggesting that pairing VNS with extinction facilitates the ability of IL stimulation to potentiate synapses in the BLA. Importantly, this shift occurred only in combination with behavioral training. VNS provides network-specific modulation of experience-driven synaptic actions to promote lasting circuit-specific plasticity (Engineer et al., 2011; Porter et al., 2012). Accordingly, and consistent with our previous behavioral data (Peña et al., 2013), VNS only altered the plasticity in the pathway between the IL and the BLA when it was delivered in an extinction context. In contrast, VNS delivered to untrained animals in the home cage had no effect. It is interesting to note that

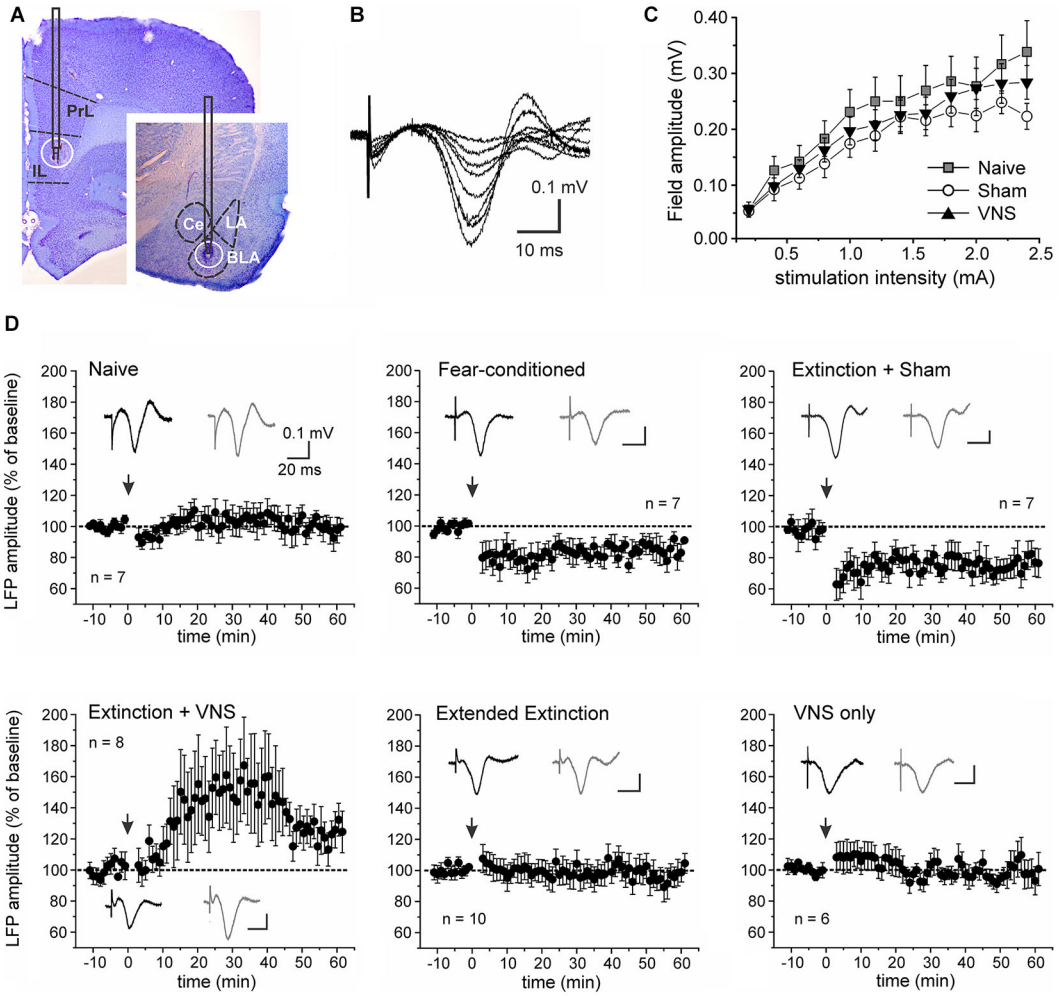

FIGURE 3 | Vagus nerve stimulation alters metaplasticity in the IL-BLA pathway. **(A)** Representative stimulation and recording sites in the IL and BLA. **(B)** Single-pulse stimulation targeted to the infralimbic (IL) PFC elicited negative field potentials in the BLA that peaked after 20–25 ms. Representative traces (average of 10 consecutive sweeps) of an input-output curve from a sham-stimulated rat. **(C)** Input-output curves from naïve rats (n = 7), fear-conditioned sham-stimulated rats (n = 7) and rats receiving VNS during extinction (n = 8). **(D)** Synaptic plasticity in the IL-BLA pathway in response to short burst stimulation in six groups of rats. Top row: In naïve rats burst stimulation does not induce synaptic plasticity. In fear-conditioned only and rats that received sham-stimulation during a single extinction session (4 tone presentations), burst stimulation induces LTD. Bottom row: In rats that received extended extinction training (20 tone presentations) and showed reduced freezing behavior, LTD induction is abolished. In rats treated with VNS during a single extinction session (4 tone presentations), synaptic strength is further potentiated, leading to LTP. This effect on synaptic plasticity appears to be context-specific as VNS stimulation by itself (VNS only) in untrained animals receiving VNS in their home cages did not affect plasticity in the IL-BLA pathway. PL, prelimbic cortex; BLA, basolateral nucleus of the amygdala, LA, lateral nucleus of the amygdala; CE, central nucleus of the amygdala.

VNS paired with extinction training, unlike EE training by itself, also facilitated the extinction of freezing behavior outside of the presentation of the CS. Because animals underwent extinction training in the same context as the auditory fear conditioning, this change of behavior during the ITI might be an indicator that VNS facilitated generalization of extinction learning to the context. It is tempting to speculate whether these behavioral differences are also reflected in the different magnitudes of potentiation of the EFP seen after (extended) extinction training by itself and extinction training paired with VNS, respectively.

The mechanisms through which VNS modulates activity in the central nervous system are poorly understood, but proposed mechanisms include alteration of norepinephrine (NE) release by projections from the nucleus tractus solitaris to the locus coeruleus (LC), elevated levels of inhibitory GABA related to vagal stimulation, and inhibition of aberrant cortical activity by reticular system activation (Ghanem and Early, 2006; Manta et al., 2009). Thus VNS may modulate cortical plasticity and memory via the synergistic action of multiple neuromodulators, which also include acetylcholine, serotonin, and brain-derived neurotrophic factor (Dorr and Debonnel, 2006; Nichols et al., 2011; Manta et al., 2013). Acute VNS increases NE and serotonin release in both the medial PFC and the amygdala (Hassert et al., 2004; Roosevelt et al., 2006; Manta et al., 2009) and enhances

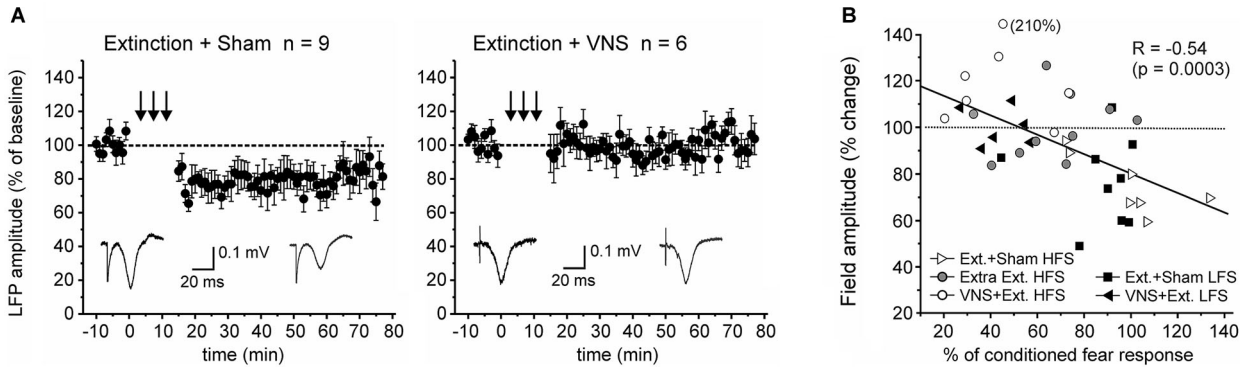

FIGURE 4 | Pairing VNS with extinction prevents the induction of LTD. (A) In sham-stimulated rats (n = 9) low-frequency stimulation resulted in LTD in the IL-BLA pathway. In rats in which extinction training was paired with VNS (n = 6), induction of LTD was inhibited. **(B)** Plot of the correlation between the amount of conditioned fear response on day 6 and the change in EFP amplitude following high-frequency (HFS, c.f. **Figure 2**) or low-frequency stimulation (LFS).

synaptic transmission in the hippocampus (Zuo et al., 2007; Shen et al., 2012; Ura et al., 2013). Norepinephrine has previously been shown to be involved in the modulation of fear expression. Locus coeruleus neurons fire in response to unexpected changes in stimulus-reinforcement contingencies (Sara and Segal, 1991; Sara, 2009). Lesions of the NE projections from the LC to the forebrain impair the extinction of active avoidance without altering acquisition or retention of the original learning (Fibiger and Mason, 1978; Mason and Fibiger, 1978). Whereas consolidation of conditioned fear depends on β-adrenoceptor activation within the BLA (McGaugh, 2002), previous evidence suggests a role for both α- and β-adrenergic receptors in the medial PFC in memory consolidation of extinction training (LaLumiere et al., 2010; Mueller and Cahill, 2010; Smith and Aston-Jones, 2011; Buffalari et al., 2012). Thus pairing VNS with extinction may set the stage for synaptic plasticity and consolidation of extinction and through β-adrenergic receptor-PKA mediated phosphorylation of AMPA receptors, marking them for membrane insertion (Mueller and Cahill, 2010; Shen et al., 2012). Similarly, the relative activation of neuromodulator receptors coupled to adenylyl cyclase and phospholipase C may result in phosphorylation of postsynaptic glutamate receptors at sites that specify induction of LTP or LTD, respectively. Thus, cholinergic and adrenergic neuromodulation associated with the behavioral state of the animal can control the gating and the polarity of cortical plasticity (Seol et al., 2007).

Several anxiety disorders, including PTSD, are associated with poor vagal tone and an altered balance of activity between the vmPFC and amygdala (Friedman, 2007; Milad and Quirk, 2012). Activation of the vmPFC during therapy is correlated with positive patient outcomes (Bryant et al., 2008). A hallmark of anxiety disorders is impaired extinction of traumatic memories (Jovanovic et al., 2010; Norrholm et al., 2011). Our findings that VNS enhances the extinction of a conditioned fear and changes synaptic strength in the IL-BLA pathway suggest that VNS can be used to overcome an insufficient vagal response to conditioned cues in order to enable the consolidation of extinction memory. Thus VNS, which is clinically approved for the treatment of depression and the prevention of seizures, could be used as an adjunct treatment to exposure therapy because it produces pairing-specific plasticity and enhances the effect of exposure on extinction of conditioned fear responding.

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Deep brain stimulation of the ventral striatum increases BDNF in the fear extinction circuit

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Deep brain stimulation (DBS) of the ventral capsule/ventral striatum (VC/VS) reduces the symptoms of treatment-resistant obsessive compulsive disorder (OCD), and improves response to extinction-based therapies. We recently reported that DBS-like stimulation of a rat homologue of VC/VS, the dorsal-VS, reduced conditioned fear and enhanced extinction memory (Rodriguez-Romaguera et al., 2012). In contrast, DBS of the ventral-VS had the opposite effects. To examine possible mechanisms of these effects, we assessed the effects of VS DBS on the expression of the neural activity marker Fos and brain-derived neurotrophic factor (BDNF), a key mediator of extinction plasticity in prefrontal-amygdala circuits. Consistent with decreased fear expression, DBS of dorsal-VS increased Fos expression in prelimbic and infralimbic prefrontal cortices and in the lateral division of the central nucleus of amygdala, an area that inhibits amygdala output. Consistent with improved extinction memory, we found that DBS of dorsal-VS, but not ventral-VS, increased neuronal BDNF expression in prelimbic and infralimbic prefrontal cortices. These rodent findings are consistent with the idea that clinical DBS of VC/VS may augment fear extinction through an increase in BDNF expression.

Keywords: anxiety disorders, obsessive compulsive disorder, prefrontal cortex, amygdala, Fos, high-frequency stimulation, fear expression

INTRODUCTION

Deep brain stimulation (DBS) of the ventral capsule/ventral striatum (VC/VS) reduces the symptoms of refractory obsessive compulsive disorder OCD, (Denys et al., 2010; Greenberg et al., 2010), but little is known about the mechanisms. Many OCD compulsions consist of avoidance of stimuli interpreted as threatening (Pietrefesa and Coles, 2009). Avoidance behaviors persist in the absence of danger, suggesting a deficit in extinction of fear (Rasmussen and Eisen, 1992; Milad et al., 2013). We recently observed in rats that DBS-like high frequency stimulation of the VS, a rodent homologue of the VC/VS, either enhanced or weakened extinction of fear, depending on the specific site within the VS (Rodriguez-Romaguera et al., 2012). DBS of the dorsal portion of the VS (dorsal-VS) reduced fear expression and enhanced extinction memory, whereas DBS of the ventral portion of the VS (ventral-VS) impaired extinction. The opposite effects of DBS at these VS sites offer a unique opportunity to understand the mechanisms of DBS in extinction. For example, DBS of dorsal-VS, but not ventral-VS, increased expression of the plasticity marker pERK in prefrontal and amygdala regions associated with extinction (Rodriguez-Romaguera et al., 2012).

While induction of plasticity by DBS is consistent with enhancement of extinction memory, it tells us little about the mechanisms involved. It is well established that BDNF is a key mediator of synaptic plasticity in fear circuits (see Monfils et al., 2007; Andero and Ressler, 2012 for reviews). BDNF in the basolateral amygdala (BLA) and the infralimbic (IL) prefrontal cortex have been associated with extinction learning (Chhatwal et al., 2006; Bredy et al., 2007; Peters et al., 2010; Soliman et al., 2010). Thus, BDNF could play a role in extinction-modulation by DBS. Furthermore, OCD is associated with reduced BNDF function (Maina et al., 2010; Fontenelle et al., 2012), and patients expressing a BDNF genetic polymorphism show poor response to extinction-based therapies (Fullana et al., 2012).

We therefore used an immunocytochemical approach to compare the effects of DBS of dorsal-VS vs. ventral-VS on the expression of BDNF in the medial prefrontal cortex (mPFC) and amygdala. In addition to neurons, BDNF is expressed in microglia, astrocytes, and endothelial cells (Rudge et al., 1992; Bejot et al., 2011). Therefore, to assess neuronal BDNF, we co-labeled BDNF antibodies with the neuronal marker NeuN. Furthermore, we measured the expression of Fos protein in the mPFC and amygdala as a marker of recent neuronal activity (Morgan et al., 1987; Dragunow and Faull, 1989).

MATERIALS AND METHODS

SUBJECTS

Male Sprague-Dawley rats ($n = 37$, Harlan Laboratories) weighing ~320 g and 12–16 weeks old were used. Animals were housed individually in transparent polyethylene cages with standard environmental conditions (73–75°F and a 12 hrs light/dark cycle, light on at 7:00 A.M.) and free access to food and water. All procedures were approved by the Institutional Care and Use Committee from University of Puerto Rico School of Medicine, in compliance with the National Institutes of Health.

SURGERY

Rats were anesthetized with isofluorane inhalant gas (5%) in an induction chamber and positioned in a stereotaxic frame. Isofluorane (2–3%) was delivered through a facemask used for anesthesia maintenance throughout the surgery. Animals were stereotaxically implanted with concentric bipolar stimulating electrodes (NEX-100; Rhodes Medical Instruments) as previously described (Rodriguez-Romaguera et al., 2012). Electrodes were aimed at the dorsal portion of the ventral striatum (−6.5 mm dorsoventral from the skull surface, ±2.0 mm mediolateral from midline, and +1.2 mm anteroposterior from bregma) or at the ventral portion of the ventral striatum (−8.0 mm dorsoventral, ±2.0 mm mediolateral, and ±1.2 mm anteroposterior) (Paxinos and Watson, 1997). After surgery, rats were allowed to recover for one week before experiments initiated.

DEEP BRAIN STIMULATION

Rats were initially connected to the stimulation cable in their home cage and habituated for 3 hrs on 2 consecutive days. On the following day, rats were randomly divided to receive bilateral monophasic DBS (100 μA, 0.1 ms pulse duration, 130 Hz, bipolar) continuously during 3 hrs (DBS group) or no stimulation (Sham control group). These parameters of stimulation were the same as those used to facilitate extinction in our previous study (Rodriguez-Romaguera et al., 2012). A stimulator (S88X, Grass Instruments, USA) connected to a constant-current unit (SIC-C Isolation Unit, Grass Instruments, USA) was used.

IMMUNOCYTOCHEMISTRY

Rats were deeply anesthetized with sodium pentobarbital (450 mg/Kg i.p.) immediately after receiving 3 hrs of DBS or sham stimulation in their home cages. They were perfused transcardially with 100 ml of 0.9% saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. Brains were removed from the skull and fixed overnight in the same fixative solution. The next day, brains were transferred to a solution of 30% sucrose in 0.1 M phosphate buffer at 4°C during 48 hrs for cryoprotection. The brains were frozen and series of coronal sections (40 μm) were cut on a cryostat (CM 1850; Leica) and collected at different levels of mPFC and amygdala. Sections at the level of VS were also collected, mounted in coated-gelatin slides, stained for Nissl bodies, cover-slipped and used to determine electrode placement. Immunohistochemistry for VS sections was not assessed because previous studies have shown that DBS of VS does not induce local changes (McCracken and Grace, 2009; van Dijk et al., 2011).

For Fos immunocytochemistry experiments, alternate sections were initially blocked in a solution of 2% normal goat serum (NGS, Vector Laboratories®, USA) plus 0.3% triton (Triton X-100, Sigma-Aldrich®, USA) in 0.12 M potassium buffer saline for 1 hr, as previously described in our lab (Padilla-Coreano et al., 2012). The sections were then incubated overnight at room temperature with anti-Fos serum raised in rabbit (Ab-5, Oncogene Science®, USA) at a dilution of 1:20,000. The primary antiserum was localized using a variation of the avidinbiotin complex system. Sections were then incubated for 2 hrs at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories®) and placed in a mixed avidin biotin horseradish peroxidase complex solution (ABC Elite Kit, Vector Laboratories®) for 90 min. Black immunoreactive nuclei labeled for Fos were visualized after 10 min of exposure to a chromogen solution containing 0.02% 3,3' diaminobenzidine tetrahydrochloride with 0.3% nickel ammonium sulphate (DAB-Ni) in 0.05 M Tris buffer, pH 7.6, followed by a 10 min incubation period in a chromogen solution with glucose oxidase (10%) and D-Glucose (10%). The reaction was stopped using potassium phosphate buffered saline (PBS) (pH 7.4). Sections were mounted in coated-gelatin slides, dehydrated and cover slipped. Counter sections were collected, stained for Nissl bodies, cover slipped and used to determine the anatomical boundaries of each structure analyzed.

For BDNF immunocytochemistry, alternate sections were initially blocked in a solution of 2% normal goat serum (NGS, Vector Laboratories®) plus 0.3% triton (Triton X-100, Sigma-Aldrich®) in 0.12 M potassium buffer saline for 1 hr, as previously described (Ou et al., 2010). Sections were then incubated overnight at room temperature with sheep anti-BDNF antibody (1:200, Millipore®, USA) plus anti-NeuN (1:200, conjugated with rabbit polyclonal Alexa Fluor 488, Millipore®). The next day, slices were incubated with anti-sheep fluorescent secondary antibody (1:200, Alexa Fluor 594, Invitrogen®) for 2 hrs, mounted in coated-gelatin slides, dehydrated and then cover-slipped with a mounting medium to avoid fluorescence fading (Vectashield, Vector Laboratories®).

IMMUNOREACTIVITY QUANTIFICATION

Counts of the number of Fos-immunoreactive neurons were carried out at 20X magnification with an Olympus microscope (Model BX51) equipped with a digital camera. Images were generated for prelimbic cortex (PL), IL cortex, basal nucleus of the amygdala (BA), lateral portion of the central nucleus of the amygdala (CeL), which also included the intercalated cells (ITC), and the medial portion of the central nucleus of the amygdala (CeM). To be considered positive for Fos-like immunoreactivity, the nucleus of the neurons had to be of appropriate size (area ranging from 100 to 500 μm^2) and shape (at least 50% of circularity), show brown-black staining of oxidized DAB-Ni, and be distinct from the background. Fos positive cells were automatically counted and averaged for each hemisphere at 2–3 distinct rostrocaudal levels of each structure (Metamorph software version 6.1). For prefrontal cortex sections, the antero-posterior levels were +2.7 mm, +3.2 mm and +3.7 mm from bregma. For amygdala sections, the antero-posterior levels were +2.3 mm and +2.8 mm from bregma. The density of Fos positive neurons was calculated by dividing the number of Fos positive neurons by the total area of each region.

BDNF images were obtained using the same microscope equipped with a fluorescent lamp (X-Cite®, Series 120 Q) and a digital camera, for the same structures quantified for Fos immunoreactivity. Image pairs were acquired at 20X magnification using the appropriate filter sets for green Alexa Fluor 488 or red Alexa Fluor 594 fluorescence, respectively for NeuN or BDNF labeling. Background luminescence for all images was digitally removed. The threshold was automatically adjusted and the

percentage of overlapping area between NeuN and BDNF images (co-labeling) was determined.

STATISTICAL ANALYSIS

Statistical significance was determined with Student's t-test (unpaired, two-tailed). The average of BDNF-NeuN overlapping area or Fos positive cells for each brain hemisphere was calculated and used for group comparisons. The level of statistical significance adopted was $p < 0.05$. All statistical analyses were performed using the Statistica software package (Version 6.0, Statsoft®, Tulsa, USA).

RESULTS

We previously reported that 3 hrs of continuous DBS (130 Hz) of dorsal-VS enhanced extinction memory, whereas the same duration of stimulation of ventral-VS impaired extinction memory (Rodriguez-Romaguera et al., 2012). **Figure 1** shows electrode placements and behavioral data of individual rats from our previous study. As reported, rats receiving DBS of dorsal-VS on Day two showed reduced freezing at the start of extinction (DBS on), and reduced freezing during the extinction memory test on Day three (DBS off). In contrast, DBS of ventral-VS increased freezing on Day two and impaired extinction memory on Day three.

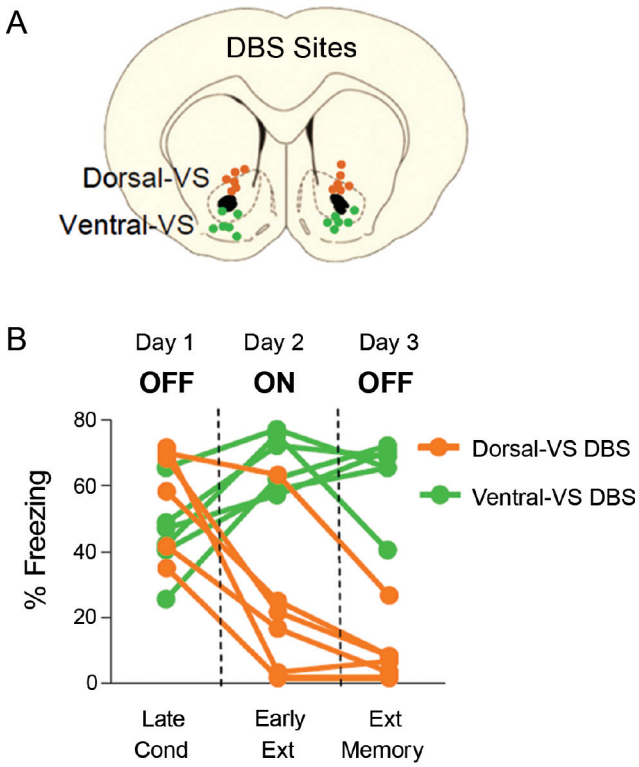

FIGURE 1 | DBS of the ventral striatum can either enhance or impair fear extinction, depending on the site of stimulation (modified from Rodriguez-Romaguera et al., 2012). (A) Placement of DBS electrode tips within the dorsal-VS (orange circles) and ventral-VS (green circles). **(B)** Individual data showing that DBS of dorsal-VS ($n = 6$) decreased fear expression on Day 2 (DBS ON) compared to Day 1 (DBS OFF), and enhanced extinction memory, as shown by the maintenance of low levels of freezing on Day 3 with DBS OFF. In contrast, DBS of ventral-VS ($n = 6$) increased fear expression on Day 2 (DBS ON) as compared to Day 1 (DBS OFF) and impaired extinction memory, as shown by the maintenance of high levels of freezing on Day 3 with DBS OFF. Data shown in blocks of two trials.

DBS OF THE DORSAL-VS, BUT NOT VENTRAL-VS, INCREASES FOS EXPRESSION IN PL AND IL

In a different set of rats, we compared the effects of 3 hrs of continuous DBS in the dorsal- or ventral-VS on the expression of Fos protein in the PL and IL subregions of the mPFC, as well as in different amygdala subnuclei: the basal nucleus (BA), CeL, which also included ITC, CeM. As illustrated in **Figure 2**, DBS of the dorsal-VS significantly increased the number of Fos positive neurons in PL [Sham: 10; DBS of dorsal-VS: 20; $t_{(16)} = -3.13$; $p = 0.007$], IL [Sham: 8; DBS of dorsal-VS: 17; $t_{(16)} = -3.02$; $p = 0.008$], and CeL/ITC [Sham: 3; DBS of dorsal-VS: 7; $t_{(14)} = -2.48$; $p = 0.03$]. No significant differences were observed in the number of Fos positive neurons in BA [Sham: 2; DBS of dorsal-VS: 4; $t_{(14)} = -1.12$; $p = 0.28$] or CeM [Sham: 2; DBS of dorsal-VS: 2; $t_{(14)} = 0.26$; $p = 0.80$].

In contrast to dorsal-VS, DBS of ventral-VS did not alter Fos expression in PL [Sham: 9; DBS of ventral-VS: 12; $t_{(30)} = -0.98$; $p = 0.34$], IL [Sham: 9; DBS of ventral-VS: 11; $t_{(30)} = -0.90$; $p = 0.38$], or CeM [Sham: 1; DBS of ventral-VS: 1; $t_{(27)} = 0.89$; $p = 0.38$]. However, a significant increase in Fos positive neurons was observed in both BA [Sham: 2; DBS of ventral-VS: 4; $t_{(27)} = -2.20$; $p = 0.04$] and CeL/ITC [Sham: 1; DBS of ventral-VS: 6; $t_{(27)} = -2.67$; $p = 0.01$, see **Figure 2**]. Thus, using Fos expression as an indicator of neuronal activity, DBS of dorsal-VS increased activity in PL and IL, whereas DBS of ventral-VS increased activity in BA. DBS of either VS site increased activity in CeL/ITC.

DBS OF THE DORSAL-VS, BUT NOT VENTRAL-VS, INCREASES BDNF EXPRESSION IN PL AND IL

Similar to Fos expression, levels of neuronal BDNF in mPFC and amygdala were altered by DBS. As illustrated in **Figure 3**, DBS of the dorsal-VS significantly increased the percentage of overlap (co-labeling) between BDNF and the neuronal marker NeuN in both PL [Sham: 1.3%; DBS of dorsal-V: 3.3%; $t_{(18)} = -3.57$; $p = 0.002$] and IL [Sham: 1.4%; DBS of dorsal-VS: 3.3%; $t_{(18)} = -4.52$; $p < 0.001$]. Notably, no group differences in BDNF-NeuN overlap were observed in BA [Sham: 0.6%; DBS of dorsal-VS: 0.6%; $t_{(18)} = -0.30$; $p = 0.77$], CeM [Sham: 2.5%; DBS of dorsal-VS: 2.0%; $t_{(18)} = 1.02$; $p = 0.32$], or CeL/ITC [Sham: 2.1%; DBS of dorsal-VS: 2.3%; $t_{(18)} = -0.30$; $p = 0.77$].

In contrast to dorsal-VS, DBS of the ventral-VS did not alter the neuronal levels of BDNF in PL [Sham: 1.8%; DBS of ventral-VS: 1.6%; $t_{(18)} = 0.57$; $p = 0.58$] and IL [Sham: 1.9%; DBS of ventral-VS: 1.8%; $t_{(18)} = 0.40$; $p = 0.70$]. In addition, no changes in BDNF levels were observed in CeM [Sham: 1.9%; DBS of ventral-VS: 2.0%; $t_{(18)} = -0.19$; $p = 0.85$] and CeL/ITC

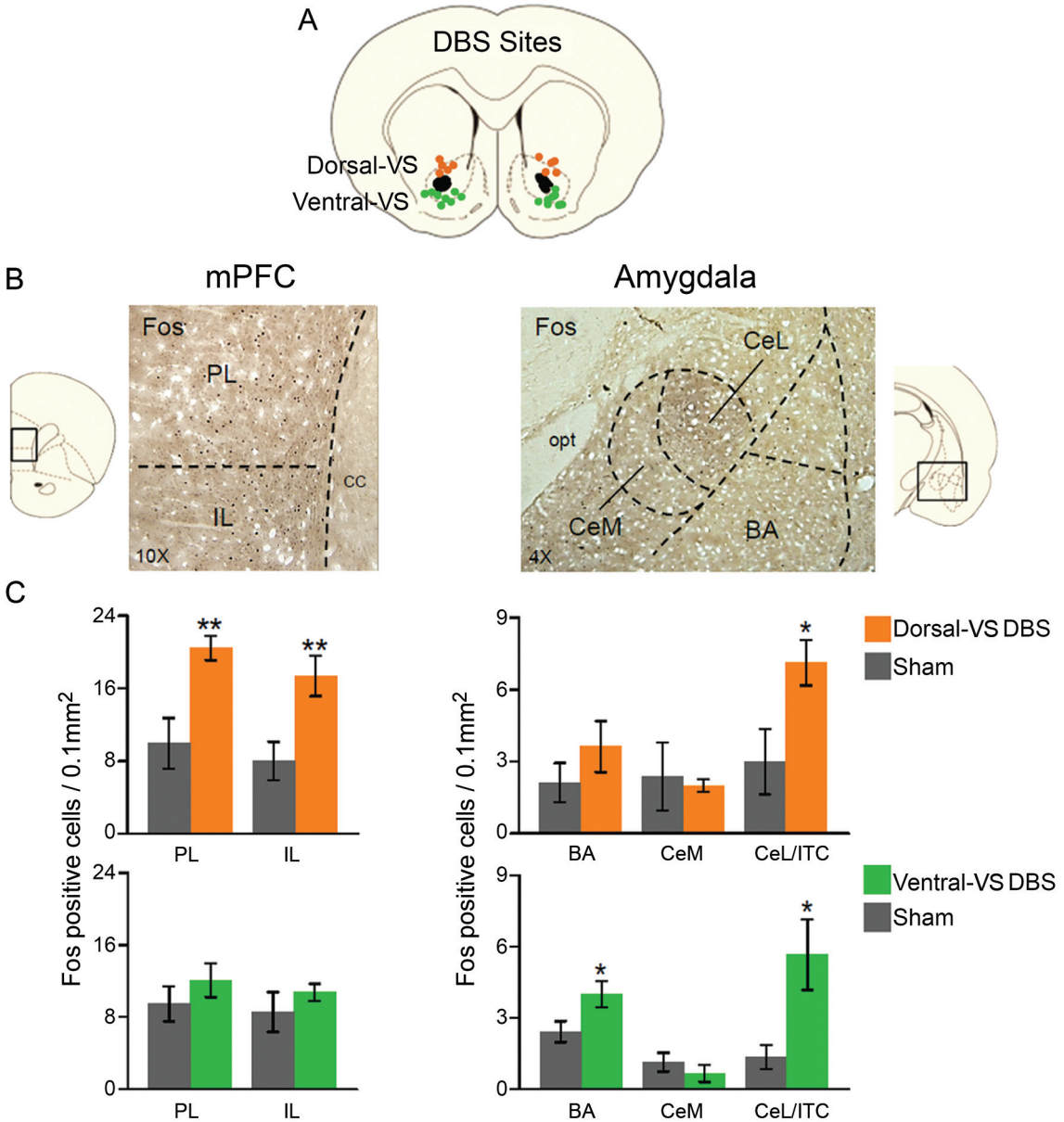

FIGURE 2 | DBS of dorsal-VS increases neuronal activity within extinction circuits. (A) Placement of DBS electrode tips within the dorsal-VS (orange circles) and ventral-VS (green circles). **(B)** Representative micrographs showing Fos labeled neurons in prelimbic (PL) and infralimbic (IL) regions of medial prefrontal cortex (mPFC, 10x magnification, left), and the lateral portion of the central nucleus of the amygdala, including the intercalated cells (CeL/ITC), in rats administered DBS in the dorsal-VS (Amygdala, 4x magnification, right). **(C)** DBS of dorsal-VS increased Fos expression in PL, IL and CeL/ITC, but not in BA or CeM (Sham, $n = 5$; DBS of dorsal-VS, $n = 4$). In contrast, DBS of ventral-VS increased Fos in the basal nucleus of the amygdala (BA) and CeL/ITC, but not in PL, IL or CeM (Sham, $n = 8$; DBS of ventral-VS, $n = 8$). Legend: CeM = medial portion of the central nucleus of the amygdala, cc = corpus callosum, opt = optic tract. Data shown as mean and SEM. $^{*}p < 0.05$ $^{**}p < 0.01$.

[Sham: 2.7%; DBS of ventral-VS: 2.2%; $t_{(18)} = 0.71$; $p = 0.48$]. However, DBS of ventral-VS significantly increased the neuronal levels of BDNF in BA [Sham: 0.7%; DBS of ventral-VS: 2.1%; $t_{(18)} = -3.56$; $p = 0.002$, see **Figure 3**], in agreement with Fos expression. In fact, all the areas that showed increased Fos also showed increased BDNF, with the exception of CeL/ITC which showed increased Fos but no increase in BDNF.

DISCUSSION

Following up on our study of DBS in dorsal-VS and ventral-VS (Rodriguez-Romaguera et al., 2012), we used an immunocytochemical approach to uncover possible mechanisms of DBS effects on fear expression and extinction memory. We found that DBS of dorsal-VS increased expression of Fos and neuronal BDNF in both PL and IL subregions of the mPFC. In contrast, DBS of ventral-VS increased the

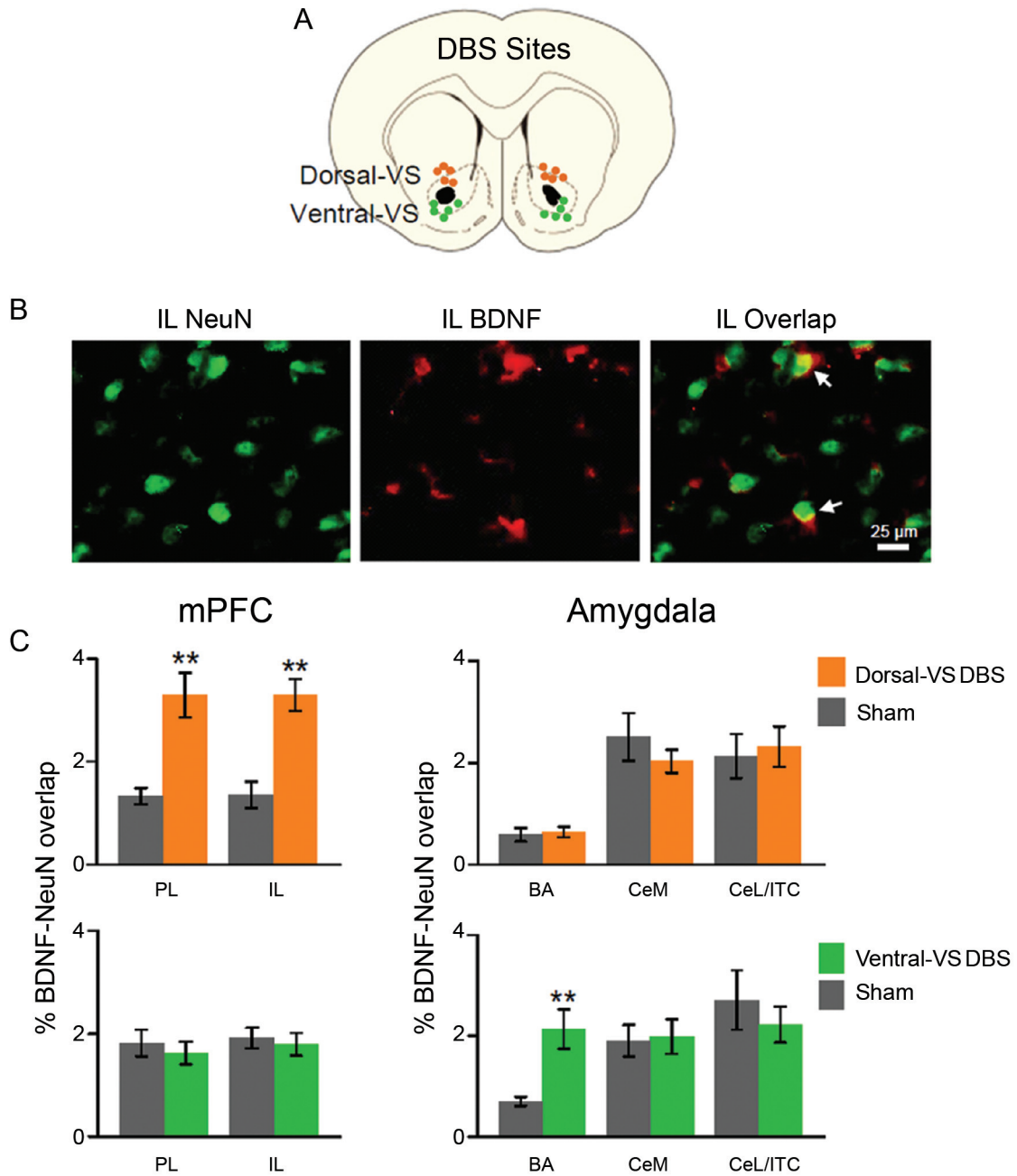

FIGURE 3 | DBS of dorsal-VS increases neuronal BDNF in PL and IL. (A) Placement of DBS electrode tips within the dorsal-VS (orange circles) and ventral-VS (green circles). **(B)** Representative micrographs showing IL labeling of neuronal marker NeuN (left), BDNF (middle), and BDNF-NeuN overlap (right, white arrows). **(C)** DBS of dorsal-VS increased BDNF-NeuN overlap in PL and IL subregions of the mPFC, but not in the amygdala (Sham, $n = 4$; DBS of dorsal-VS, $n = 6$). In contrast, DBS of ventral-VS increased BDNF-NeuN overlap in BA, but not in PL, IL, CeM or CeL/ITC (Sham, $n = 5$; DBS of ventral-VS, $n = 5$). Legend: CeM = medial portion of the central nucleus of the amygdala, CeL = lateral portion of the central nucleus of the amygdala, ITC = intercalated cells. Data shown as mean and SEM. $^{**}p < 0.01$.

expression of Fos and BDNF only in BA. Increased Fos expression in CeL/ITC was observed after DBS of both dorsal-VS or ventral-VS. Our data suggest that enhanced extinction memory observed with DBS of dorsal-VS may be due to increased BDNF levels in IL neurons, leading to increased activation of inhibitory neurons in CeL/ITC (**Figure 4**).

Recent findings suggest that PL and IL cortices have opposite effects on fear responses (Vidal-Gonzalez et al., 2006; Laurent and Westbrook, 2009; Sierra-Mercado et al., 2011). PL sends excitatory projections to BA (Vertes, 2004; Likhtik et al., 2005), a region necessary for fear expression (Anglada-Figueroa and Quirk, 2005), whereas IL projects

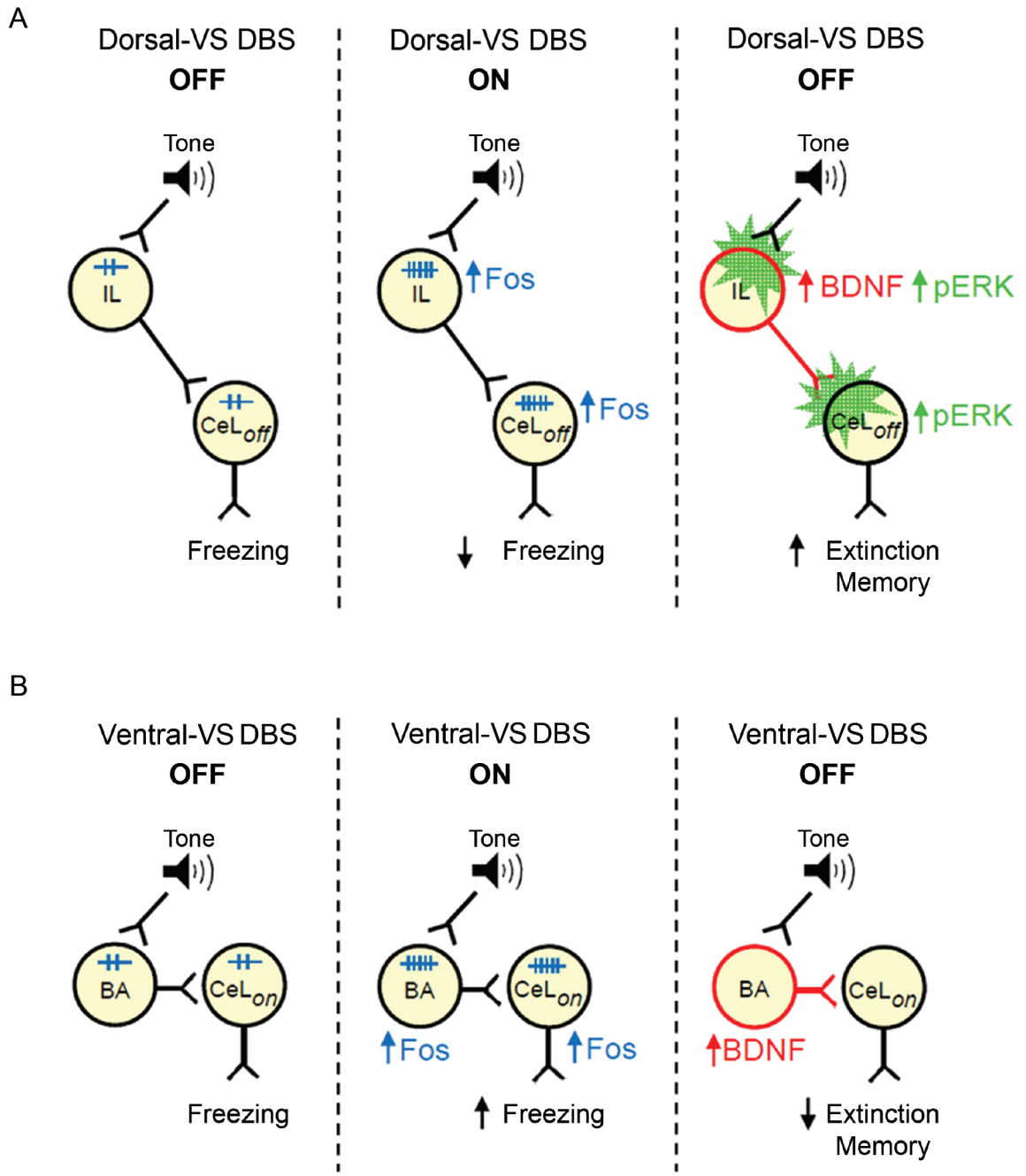

FIGURE 4 | Suggested models of how DBS of dorsal-VS and ventral-VS affect fear. (A) Middle: DBS of dorsal-VS (ON) increases neuronal activity (Fos) in the IL-CeL$_{off}$ circuit, decreasing freezing to a conditioned tone. Right: DBS of dorsal-VS also increases BDNF in IL and induces plasticity (pERK) in IL and CeL$_{off}$ (Rodriguez-Romaguera et al., 2012), thereby enhancing extinction memory in the absence of DBS. **(B)** Middle: DBS of ventral-VS increases neuronal activity (Fos) in the BA-CeL$_{on}$ circuit, increasing freezing to a conditioned tone. Right: DBS of ventral-VS also increases BDNF in BA, thereby impairing extinction memory in the absence of DBS.

to GABAergic (gamma-aminobutyric acid) cells in CeL, which inhibits CeM outputs and consequently fear expression (Royer and Pare, 2002; Quirk et al., 2003; Amano et al., 2010). It was somewhat surprising, therefore, that dorsal-VS DBS increased Fos expression in PL as well as IL. Optogenetic activation of IL pyramidal neurons has been shown to reduce PL activity through feed-forward inhibition (Ji and Neugebauer, 2012). Therefore, increased Fos in PL may reflect augmented GABAergic activity in PL. In addition, extinction training increases Fos expression in both PL and IL, as well as CeL/ITC (Santini et al., 2004; Knapska and Maren, 2009; Kim et al., 2010; Plendl and Wotjak, 2010), similar to what we observed for

DBS of dorsal-VS. In contrast, DBS of ventral-VS increased Fos expression in BA, consistent with increased fear expression (Amano et al., 2011; Sangha et al., 2013). BA projections to VS terminate in the ventral-VS, rather than the dorsal-VS (Kelley et al., 1982; Mcdonald, 1991), suggesting that increased BA activity may be due to antidromic activation of BA fear neurons (Herry et al., 2008), by DBS of ventral-VS. Surprisingly, DBS of ventral-VS also increased Fos expression in CeL/ITC, suggesting that DBS at this site may be activating a different population of CeL neurons mediating fear expression (Ciocchi et al., 2010). Alternatively, both PL and IL project through the VS to reach the amygdala (St Onge et al., 2012). Thus, stimulation of distinct prefrontal-amygdalar fibers passing through the VS could explain the opposite effects of adjacent DBS sites, however more studies are needed to address this possibility.

Previous studies have shown that extinction training increases BDNF gene expression in the mPFC (Bredy et al., 2007), and infusion of BDNF into IL facilitates extinction learning (Peters et al., 2010). Furthermore, a common polymorphism in the BDNF gene has been associated with deficits in extinction memory in both mice and humans (Soliman et al., 2010). In particular, the same polymorphism was associated with reduced NMDA-glutamatergic transmission specifically in IL (Pattwell et al., 2012). Therefore, the increase in IL BDNF following DBS of dorsal-VS, but not ventral-VS, may mediate the enhancement of fear extinction by DBS. DBS of dorsal-VS also increased BDNF levels in PL, however prior studies have demonstrated that extinction is unaffected by intra-PL infusion of BDNF (Rosas-Vidal et al., 2012), or deletion of the BDNF gene in PL (Choi et al., 2010).

In contrast to DBS of dorsal-VS, DBS of ventral-VS modified BDNF in BA, but not in prefrontal cortex. Previous studies have shown that BDNF levels in BA are significantly increased after fear conditioning (Rattiner et al., 2004; Ou and Gean, 2006). In addition, blockade of BDNF signaling in the BA disrupted acquisition of conditioned fear (Rattiner et al., 2004), and expression of BDNF in this area is necessary for maintenance of fear memories (Ou et al., 2010). Therefore, increased BDNF levels in BA could contribute to augmented fear memory with DBS of ventral-VS, leading to impaired fear extinction memory in the next day.

Extinction-based therapy is currently among the most effective treatments for OCD (Rasmussen and Eisen, 1997; Franklin and Foa, 2011; Olatunji et al., 2013), and DBS of the VC/VS increases the effectiveness of such therapies (Denys et al., 2010; de Koning et al., 2011). Serum levels of BDNF are reduced in OCD (Maina et al., 2010; Fontenelle et al., 2012), and a BDNF polymorphism is correlated with impaired response to extinction-based therapy (Fullana et al., 2012), suggesting a role of BDNF in OCD pathophysiology. OCD patients show impaired fear extinction and reduced activity in the vmPFC, a homologue of rodent IL (Milad et al., 2013). Thus, DBS-induced increases in prefrontal BDNF, as well as prefrontal monoamines (van Dijk et al., 2012), suggests that DBS of the VC/VS in OCD patients may repair faulty prefrontal circuits (Figee et al., 2013), thereby improving extinction-based therapy.

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Appetitive behavioral traits and stimulus intensity influence maintenance of conditioned fear

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Individual differences in appetitive learning have long been reported, and generally divide into two classes of responses: cue- vs. reward-directed. The influence of cue- vs. reward-directed phenotypes on aversive cue processing, is less well understood. In the current study, we first categorized rats based on their predominant cue-directed orienting responses during appetitive Pavlovian conditioning. Then, we investigated the effect of phenotype on the latency to exit a familiar dark environment and enter an unfamiliar illuminated open field. Next, we examined whether the two phenotypes responded differently to a reconsolidation updating manipulation (retrieval+extinction) after fear conditioning. We report that the rats with a cue-directed ("orienting") phenotype differentially respond to the open field, and also to fear conditioning, depending on US-intensity. In addition, our findings suggest that, regardless of appetitive phenotype or shock intensity, extinction within the reconsolidation window prevents spontaneous recovery of fear.

Keywords: orienting, open field, fear conditioning, reconsolidation, extinction

INTRODUCTION

When pairing a conditioned stimulus (CS) with a biologically significant event such as food (unconditioned stimulus, US), rats develop conditioned responses (CR). In the case of light-food pairings, some rats develop both CS- and US-directed responses, that is, they orient/rear toward the light cue and approach the site of food delivery, while other rats develop only the food cup approach behavior. Because both groups exhibit an approach to the food cup and only a subset develops an orienting response to the light, we characterize these groups based on their conditioned orienting response to the CS and classify them as Non-orienters and Orienters, respectively.

Numerous reports, including our own, have indicated that these two phenotypes differ in measures of risky decision making, delay discounting, novelty preference, dopaminergic response to cues, and response to drug exposure (Flagel et al., 2011; Lovic et al., 2011; Olshavsky et al., 2012; Yager and Robinson, 2012). Orienters and Non-orienters also behave differently in their susceptibility to appetitive memory updating (Olshavsky et al., 2013). Monfils et al. (2009) previously showed that presenting an isolated retrieval trial (CS) prior to an extinction session led to a persistent reduction in fear expression, which did not leave the fear memory susceptible to spontaneous recovery (SR), reinstatement, or renewal. Unlike standard extinction, the retrieval+extinction procedure has been proposed to involve an updating of a memory during the reconsolidation window (Monfils et al., 2009; Schiller et al., 2010). The isolated retrieval trial is thought to induce memory destabilization for a limited time period during which the memory is labile (Monfils et al., 2009; Nader et al., 2000). Using a procedure based on this paradigm (Monfils et al., 2009; Schiller et al., 2010), Olshavsky et al. (2013) observed that rats receiving a retrieval trial prior to extinction showed attenuated conditioned responding during tests for SR (Olshavsky et al., 2013). Interestingly, this effect was dependent on whether the rats were Orienters or Non-orienters—only Orienters showed attenuation of conditioned responding after the retrieval-extinction procedure. This result is particularly important in light of the fact that many (Clem and Huganir, 2010; Schiller et al., 2010; Rao-Ruiz et al., 2011) but not all (Chan et al., 2010) labs have observed the persistent fear memory updating described in Monfils et al. (2009), prompting a need to investigate the boundary conditions that surround this form of memory updating. To this effect, for the present study we first classified rats as either Orienters or Non-orienters based upon their expression of either CS-directed or US-directed responses during light-food pairings, we then compared their behaviors within an open field task, then tested whether expression of conditioned fear differs in rats that show robust cue-oriented responding and those that do not, and finally, examined whether fear memory could be persistently attenuated in those groups using the retrieval+extinction paradigm (Monfils et al., 2009).

MATERIALS AND METHODS

SUBJECTS

Sixty-six Long-Evans male rats (250–275 g upon arrival, Charles River Laboratories) were used. Rats were maintained on a 12-h regular light-dark cycle with lights on at 7am. For the open field and appetitive conditioning portions of the experiment, rats were maintained at 90% free-feeding weight; water was available *ad libitum*. During fear conditioning procedures, food and water were both provided *ad libitum*. All experiments were conducted according to *the National Institutes of Health's Guide for*

the Care and Use of Laboratory Animals, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Initially, rats were trained to retrieve food pellets from a food cup located within an appetitive conditioning chamber. Eight individual conditioning chambers (30.5 W × 25.4 D × 30.5 H in cm, Coulbourn Instruments, Allentown, PA) with aluminum sidewalls and ceiling, clear acrylic front and back walls and stainless steel rod floors (rods 0.5 cm in diameter, spaced 1.0 cm apart) comprised the appetitive conditioning context. A wall-mounted magazine delivered grain pellets (Test Diet, 45 mg) to a recessed food cup mounted 2.5 cm above the floor. Each chamber was enclosed in a light- and sound-attenuated box (58.4 × 61 × 45.7 cm); a ventilation fan provided masking noise. A video camera was mounted within each box and images were recorded during behavioral training. During the initial food cup training a total of 30 pellets were delivered to the food cup at a variable inter-trial interval (ITI) averaging 60 s over a 30-min session. After one session, all rats reliably retrieved the grain pellets.

OPEN FIELD

After food cup training, both rats' latency to enter an illuminated open field and their preference for the illuminated open field vs. a familiar dark compartment were assessed. Two open field chambers consisting of white acrylic floors surrounded on all sides by clear acrylic walls were used (43.2 W × 43.2 D × 30.5 H in cm). On day 1, rats were restricted to an opaque black insert (43.3 W × 21.6 D × 30.5 H in cm) for 10 min. The following day rats were initially placed within the black insert, but were free to exit into the illuminated portion of the open field and had 10 min of free access to both sides. Activity in both sides of the field was detected by infrared beam motion detectors (**Figure 1**).

APPETITIVE CONDITIONING

Forty-eight hours after completing the open field test, rats began appetitive conditioning. The first day of appetitive conditioning consisted of two parts. In order to habituate the unconditioned orienting response to light, the stimulus light (2-Watt white light mounted 20 cm above the magazine) was illuminated eight times, for 10 s each time, without any food pellets being delivered to the magazine. Then, during the second half of the session, 10 s light-CS illuminations were followed by grain pellet delivery into the food cup. For the next three days of conditioning, sessions consisted of 16 light–food pairings with a variable ITI averaging 120 ± 50 s.

Nosepoke to the food cup was detected by an infrared beam at the opening, while orienting behavior was scored by a blind observer from DVD recordings of sessions. Orienting measures were directly adapted from the ones used by Holland and colleagues (Gallagher et al., 1990; Lee et al., 2005, 2010, 2011). Even though the light-CS was a localized cue, it still provided diffuse illumination of the entire chamber. Thus, an orienting response was defined as any rearing response in which both forepaws were lifted from the floor of the training box, but did not include grooming behavior. For each light-food trial, behavior was sampled at every 1.25 s resulting in 12 observations: 4 times during the 5 seconds immediately preceding the onset of the CS (preCS), 4 times during the first 5 s of the CS (CS1), and 4 times during the last 5 s of the CS (CS2). Because orienting response and food cup approach occur predominantly during CS1 and CS2, respectively (Holland, 1977), we report orienting response from CS1 and food cup approach behavior from CS2. Their behaviors during preCS are subtracted to account for any baseline differences (**Figure 1**).

FEAR CONDITIONING

Following appetitive training, rats were transferred to a new colony and after a 3–5 days of acclimation, all rats were fear conditioned in a second context. All remaining procedures (fear conditioning, long-term memory test, and the test for SR) were conducted in this second context. Rats were fear conditioned in chambers equipped with two metal walls, two clear plexiglass walls, and stainless-steel rod floors connected to a shock generator (Coulbourn Instruments, Allentown, PA). Each conditioning chamber was enclosed in an acoustic isolation box (Coulbourn Instruments) and lit with a red house light. Behavior was recorded with digital cameras mounted on the top of each unit. Stimulus delivery was controlled using Freeze Frame software (Coulbourn Instruments). The CS used for fear conditioning was a 20-s tone (5 kHz, 80 dB). The US was either a 0.7 or 1.0 mA footshock 500 ms in duration. Orienters and Non-orienters, as determined by the orienting response during the last eight trials of appetitive training, were divided into two shock intensity groups for fear conditioning (0.7 and 1.0 mA). On the fear-conditioning day, after a 2-min habituation period, all rats received three 20-s presentations of the tone CS (variable ITI = 120 s), each co-terminating with either a 0.7 or 1.0 mA foot-shock. An experimenter blind to group assignment

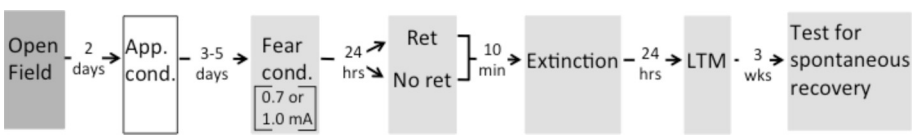

FIGURE 1 | Timeline of experimental design. Rats were first tested for their willingness to enter an illuminated open field. Rats then received appetitive conditioning (App. cond.) with 56 light-food pairings in Context A. On their last day of appetitive conditioning rats were classified as Orienters and Nonorienters. After 3–5 days, both groups were fear conditioned (Fear cond.) with 3 tone-shock pairings of either 0.7 or 1.0 mA in Context B (indicated by gray shading). 24 h after fear conditioning, rats were exposed to a single cue retrieval trial (Ret) or a typical extinction session (No ret). For rats in the Ret group that received a cue exposure and those in the No ret group that received a context exposure, the exposure occurred 10 min prior to beginning the extinction session. 24 h after extinction, rats were tested for long-term memory (LTM), and 3 weeks later tested for spontaneous recovery. Context change is indicated by shading.

scored freezing behavior manually from video recorded during each session. Freezing was defined as the absence of any movements, excluding those required for respiration. The total number of seconds spent freezing throughout the CS presentation was expressed as a percentage of CS duration.

Twenty-four hours after fear conditioning, all subjects underwent either extinction (ext only) or retrieval+extinction (ret+ext). For the extinction session, rats were placed in the fear-conditioned context and exposed to 19 non-reinforced presentations of the tone CS (variable ITI = 120 s). A subset of these rats ($n = 21$ out of 37) in the extinction only group were placed in the context 10 min prior to the extinction session but received no CS presentations. Context-exposed and non-context-exposed rats from the No Retrieval groups were not significantly different and these groups were collapsed for the remainder of analyses. Rats in the ret+ext group were first exposed to a single CS presentation in the fear-conditioned context, returned to the home-cage for 10 min, and then returned to the same context for the remaining 18 extinction trials. This resulted in eight groups for analysis - Orienter 0.7 mA ret+ext $n = 8$; Orienter 0.7 mA ext only $n = 9$; Non-orienter 0.7 mA ret+ext $n = 9$; Non-orienter 0.7 mA ext only $= 7$; Orienter 1.0 mA ret+ext $n = 8$; Orienter 1.0 mA ext only $n = 9$; Non-orienter 1.0 mA ret+ext $n = 9$; Non-orienter 1.0 mA ext only $n = 8$ (**Figure 1**).

RESULTS

APPETITIVE CONDITIONING

Based on their average number of orienting bouts during the last eight trials of training, rats were divided into two groups. Rats scoring at or above the median (0.38 bouts/trial) were classified as Orienters ($n = 34$), while those rats that scored below the median were classified as Non-orienters ($n = 32$). The mean conditioned orienting levels, 0.85 ± 0.07 and -0.01 ± 0.04, were significantly different between Orienters and Non-orienteres, respectively, $t_{(64)} = 9.84$, $p < 0.0001$ (**Figure 2A**). Groups of rats, however, did not differ in displaying conditioned food cup approach (**Figure 2B**). Furthermore, the groups did not differ in unconditioned orienting response during the first 8 trials, in which light was presented without any food: Mean orienting bouts during those trials were 0.36 for Orienters and 0.35 for Non-orienters ($p = 0.91$) (data not shown).

As stated in the materials and methods section (2.3. Appetitive conditioning), these reported numbers reflect elevated scores, in which the behaviors in the absence of CS were subtracted from the ones during CS presentation. Analyses of preCS responses (i.e., orienting and food-cup behavior during the 5-s immediately before the CS onset) revealed no differences between Orienters and Non-orienters ($p > 0.05$). PreCS orienting bouts were 0.24 (Orienters) and 0.35 (Non-orienters) and preCS food-cup numbers were 0.46 (Orienters) and 0.49 (Non-orienters). Furthermore, orienting scores during the first half (CS1) and food-cup scores from the second half (CS2) of CS presentation are presented in **Figure 2**, due to the predominant display of these behaviors in respective time points. Further analyses of these two behaviors in both CS1 and CS2 with repeated ANOVA of two CS time points still revealed the same trend in which there was an overall significant difference in orienting response between Orienters and Non-orienters, $F_{(1, 64)} = 54.4$, $p < 0.001$, but not in food-cup behavior, $F_{(1, 64)} = 3.47$, $p > 0.05$. As expected, the overall orienting levels were significantly higher during CS1 compared to CS2, $F_{(1, 64)} = 8.60$, $p < 0.01$, and the food-cup response was significantly higher during CS2 compared to CS1, $F_{(1, 64)} = 42.4$, $p < 0.001$.

OPEN FIELD

Analysis of data collected during the dark-light open field task indicated that Orienters exited the dark insert (and entered the illuminated field) more quickly than Non-orienters, $t_{(64)} = 1.98$, $p = 0.05$ (**Figure 2C**). There was also a trend for Orienters to spend more time in the illuminated field than Non-orienters, $t_{(64)} = 1.85$, $p = 0.07$. These results cannot be attributed to a difference in general activity levels, as the ambulatory distance traveled of the two groups were comparable, $t_{(64)} = 0.91$, $p = 0.37$ (**Figure 2C**).

FEAR CONDITIONING

Freezing during the fear conditioning session was analyzed using mixed factor ANOVAs with fear conditioning cue (3 cues total) as the repeated measure and orienting classification (Orienter or Non-orienters) and shock intensity (0.7 or 1.0 mA) as the between subjects factors. There was a significant within-subjects effect

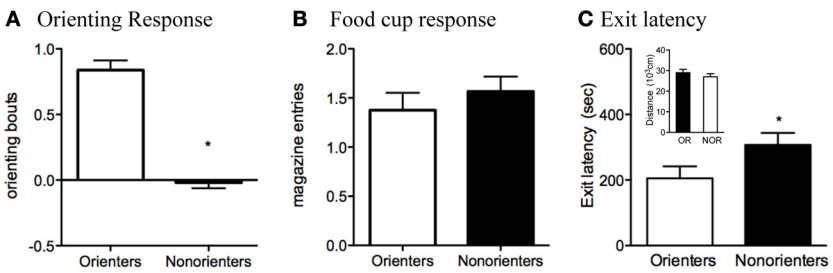

FIGURE 2 | (A,B) Conditioned orienting and food cup approach for the Orienters and Nonorienters. Mean ± s.e.m number of orienting bouts (*$p < 0.0001$) **(A)** or food cup entries ($p = 0.42$) **(B)** averaged for last 8 trials of training. Orienters showed significantly more orienting than Nonorienters, but the food cup response was equivalent between groups. **(C)** Latency to exit the dark insert and enter the illuminated open field. Orienters exited significantly more quickly than the Nonorienters ($p = 0.05$). Activity, as measured by the total distance traveled within both fields **(C)**, did not differ between Orienters and Nonorienters ($p = 0.37$).

of fear conditioning cue, $F_{(2,\ 116)} = 391.58$, $p < 0.001$, indicating that rats froze significantly more toward the end of the fear conditioning session than at the beginning. Additionally, overall rats froze significantly more throughout conditioning to the 1.0 mA than the 0.7 mA. In addition the Orienters and Non-orienters were differentially affected by shock intensity. There was a significant fear conditioning cue x shock intensity interaction, $F_{(2,\ 116)} = 3.74$, $p = 0.027$ as well as an overall main effect of both orienting classification, $F_{(1,\ 58)} = 4.17$, $p = 0.046$, and shock intensity, $F_{(1,\ 58)} = 5.36$, $p = 0.024$. Follow up ANOVAs for each shock intensity revealed that for the 0.7mA fear conditioning group (**Figure 3A**), there were no differences in freezing levels during acquisition between the Orienters ($n = 15$) and Non-orienters ($n = 14$), $F_{(1,\ 27)} = 0.49$, $p = 0.49$. However, rats classified as Orienters who were fear conditioned to the 1.0 mA shock ($n = 17$) froze significantly less than rats classified as Non-orienters ($n = 16$) evidenced by an overall main effect of orienting on freezing levels during the fear conditioning session, $F_{(1,\ 31)} = 4.57$, $p = 0.041$ (**Figure 3B**). However, a comparison of the mean freezing of Orienters and Non-orienters in the 1.0 mA group revealed that the groups were not significantly different during the last trial of conditioning.

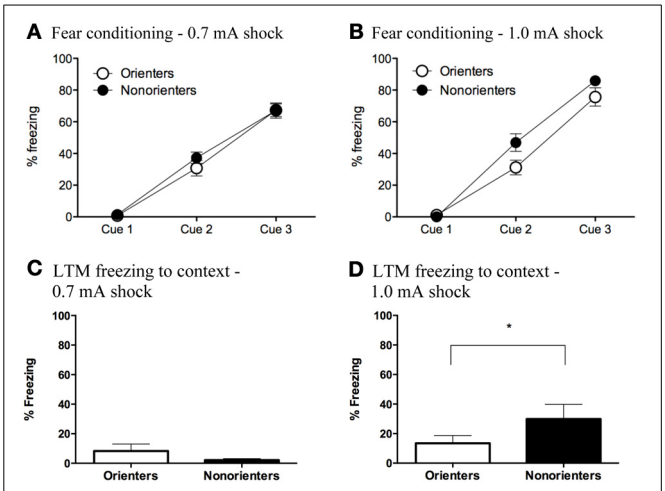

FIGURE 3 | (A,B) Freezing during fear conditioning with a 0.7 mA and 1.0 mA footshock. **(A)** Orienters and Non-orienters showed no differences in freezing during conditioning when the US was a 0.7 mA footshock ($p = 0.49$). **(B)** Non-orienters froze significantly more than Orienters during fear conditioning when the US was 1.0mA footshock ($p = 0.04$). Each conditioning session involved three CS-US pairings. (**C,D**) Contextual freezing 24 h after fear conditioning to either a 0.7 or 1.0 mA footshock. **(C)** There were no significant differences between Orienters and Non-orienters in freezing to the fear conditioning context when the US was 0.7 mA footshock ($p = 0.09$) and overall context freezing was extremely low. **(D)** Non-orienters froze significantly more than Orienters to the fear conditioning context when the US was a 1.0 mA footshock (*$p = 0.03$).

CONTEXTUAL FEAR

Contextual fear was measured by scoring freezing during a 20 s sample within the first 2 min that the rat was placed in the fear conditioning context the day after fear conditioning. For rats that received a CS retrieval, freezing to the context was measured in the 20 s immediately preceding the CS onset. In the ext only group, rats that received a context exposure only, freezing to the context was measured for 20 s at the same time point as the retrieval group. In the subset of animals that did not receive a context exposure, context freezing was measured in the 20 s preceding the first CS of extinction. All of these measurements took place at the same time point during the rat's first exposure to the fear conditioning context. A 2 × 2 ANOVA with orienting classification and shock intensity as the factors revealed a significant main effect of shock intensity, $F_{(1,\ 62)} = 15.96$; $p < 0.001$, no main effect of orienting classification, $F_{(1,\ 62)} = 1.90$; $p = 0.173$, and an orienting classification X shock intensity interaction, $F_{(1,\ 62)} = 7.73$; $p = 0.007$. Follow up t-tests revealed that there were no significant differences between Orienters and Non-orienters after conditioning to a 0.7mA shock, $t_{(31)} = 1.74$; $p = 0.092$, and overall contextual freezing levels were very low (<10%) as seen in **Figure 3C**. However, after conditioning to a 1.0 mA footshock, Non-orienters showed significantly more freezing to the context than Orienters, $t_{(31)} = 2.27$; $p = 0.03$ (**Figure 3D**).

EXTINCTION/RETRIEVAL+EXTINCTION

Given the differences between Orienters and Non-orienters in freezing during the 1.0 mA fear conditioning session, we compared the mean of the first four trials of extinction and tested whether our groups differed in their fear conditioning retention. Neither orienting classification, shock level, nor retrieval group resulted in any significant differences in freezing during the first 4 trials of extinction ($ps > 0.05$) suggesting that the differences observed during fear acquisition are a result of differential responses to the immediate presence of the foot-shock as opposed to differences in the ability to acquire and retain CS-US association. Freezing during the extinction session was initially analyzed with a 2 × 2 × 2 mixed factor ANOVA with extinction cue as the repeated measure and retrieval group (ext only, ret+ext), orienting classification (orienters or non-orienters), and shock intensity (0.7 or 1.0 mA) as the between subjects factors. Rats did show a significant reduction in freezing over the course of extinction as evidenced by a significant within-subjects effect of extinction cue, $F_{(18,\ 1026)} = 62.53$, $p < 0.001$, with no overall main effect of either orienting classification, $F_{(1,\ 57)} = 0.05$, $p = 0.831$, or retrieval group, $F_{(1,\ 57)} = 2.40$, $p = 0.127$ (**Figure 4**).

LONG TERM MEMORY OF FEAR

Twenty-four hours after extinction, rats were tested for long-term memory (LTM) by presenting 4 tone-only trials (variable ITI = 120 s) in the same context as fear conditioning and extinction. Freezing behavior during these trials was scored and averaged During the LTM test, none of the experimental groups showed a significant increase in freezing, as compared to their own freezing at the end of extinction (all p's > 0.1). For rats conditioned with a 0.7 mA shock, no between-group differences existed in LTM freezing. For rats conditioned with a 1.0 mA shock, the freezing levels of Orienters and Non-orienters receiving typical extinction treatment (ext only) were comparable; however, Non-orienters in the ret+ext group showed significantly higher freezing than Orienters in the ret+ext group, $t_{(15)} = 2.89$, $p = 0.011$ (**Figure 4**).

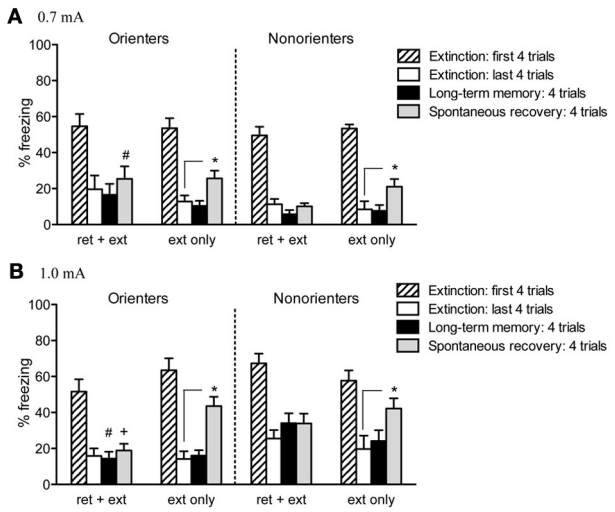

FIGURE 4 | Cue-induced freezing at the beginning of extinction, end of extinction, during LTM test, and spontaneous recovery. (A) For rats conditioned with a 0.7 mA shock, a retrieval trial prevented spontaneous recovery (i.e., there was no significant increase in freezing from the end of extinction to spontaneous recovery test; Orienters $p = 0.206$, Non-orienters $p = 0.732$). While neither group showed significant spontaneous recovery, Non-orienters froze significantly less than Orienters during the test for spontaneous recovery ($^{\#}p = 0.041$). Rats receiving typical extinction treatment did show a significant increase in freezing (Orienters $p = 0.014$, Non-orienters $p = 0.032$). **(B)** Rats conditioned with a 1.0 mA shock showed the same pattern of results: a retrieval trial prior to extinction attenuated spontaneous recovery (Orienters $p = 0.524$, Nonorienters $p = 0.235$). Rats exposed to typical extinction showed a significant increase in freezing (Orienters $p < 0.001$, Non-orienters $p = 0.032$). While neither Orienters nor Non-orienters that received ret+ext showed significant increases in freezing from the end extinction to LTM or spontaneous recovery tests, Orienters showed significantly less freezing than Non-orienters at both time points (LTM $^{\#}p = 0.011$, spontaneous recovery $^{+}p = 0.045$).

SPONTANEOUS RECOVERY OF FEAR

Twenty-one days after extinction, rats were returned to the chambers and tested for SR of freezing by playing 4 tone-only trials (variable ITI = 120 s). An overall ANOVA with orienting classification, shock intensity, and retrieval group as the factors revealed no overall effect of orienting classification, $F_{(1, 57)} = 0.19$; $p = 0.661$, but did reveal a significant overall effect of both retrieval group, $F_{(1, 57)} = 10.02$; $p = 0.002$, and shock intensity, $F_{(1, 57)} = 16.05$; $p < 0.001$, as well as a significant orienting classification X shock intensity interaction, $F_{(1, 57)} = 5.75$; $p = 0.02$, and a trend toward an orienting classification X shock intensity X retrieval group interaction, $F_{(1, 57)} = 3.73$; $p = 0.058$ (**Figure 4**). Rats receiving typical extinction treatment (ext only) showed recovery of freezing, regardless of orienting classification or shock intensity, i.e., freezing was significantly increased from extinction to the SR test [Orienters—0.7 mA: $t_{(8)} = 3.133$, $p = 0.014$; Non-orienters—0.7 mA: $t_{(5)} = 2.96$, $p = 0.032$; Orienters—1.0 mA: $t_{(8)} = 7.73$, $p < 0.001$; Non-orienters—1.0 mA: $t_{(6)} = 2.785$, $p = 0.032$]. In contrast, rats exposed to a retrieval trial prior to extinction did not show significant recovery of freezing during the SR test regardless of orienting classification or shock intensity (all p's > 0.2). Although neither Orienters nor Non-orienters receiving a retrieval trial prior to extinction showed a significant increase in freezing from extinction to SR test, for either shock intensity, Non-orienters showed more freezing behavior during SR test than Orienters after conditioning to a 1.0mA shock, $t_{(15)} = 2.18$, $p = 0.045$, and less freezing behavior than Orienters after conditioning to a 0.7mA shock, $t_{(15)} = 2.23$, $p = 0.041$.

DISCUSSION

Fear conditioning provides a controlled means to investigate aversive associations that underlie many pathological fear conditions. Memory update methods such as ret+ext, where an extinction session is presented within the reconsolidation window show promise for reducing fear non-invasively; however, individual differences between subjects and methodological variations across laboratories leaves the efficacy of such paradigms in question. Here we consider how individual differences in response style during an appetitive conditioning task (i.e., propensity for conditioned orienting to a light stimulus predictive of food) relate to individuals' hesitance to enter an open field and how they affect freezing after fear conditioning. We report that Non-orienters show more reluctance to enter an illuminated open field, indicating an enhanced fear of unfamiliar open environments, as compared to Orienters. Additionally, we report that when conditioned with a tone and 1.0 mA footshock, Non-orienters show heightened freezing. Groups do not differ in their response conditioning with a 0.7 mA shock.

After fear conditioning to a foot-shock of either standard intensity (0.7 mA) or increased intensity (1.0 mA), ret+ext prevented SR of freezing for both Orienters and Non-orienters. However, for the 1.0 mA experiment, Non-orienters in the ret+ext group froze significantly more than Orienters in the ret+ext group. We show that while retrieval+extinction prevents the significant return of fear for both phenotypes, the intensity of the US used in training and subjects' appetitive phenotype affect the magnitude of fear behavior that persists. A relationship between these two behaviors (conditioned orienting in an appetitive task and fear expression in a fear conditioning task) seems perhaps unsurprising given the overlap in the neural circuitry responsible for each. Projections from the central nucleus of the amygdala have been shown to be necessary for both the acquisition of conditioned orienting to a cue predictive of reward and the freezing response exhibited after fear conditioning (Ledoux et al., 1988; Gallagher et al., 1990; Han et al., 1997; Goosens and Maren, 2001; Choi and Brown, 2003; Duvarci et al., 2011). It is possible Orienters and Non-orienters have fundamental differences in central amygdala function and that the results reported here are evidence of that variation, but more investigation needs to be done.

Furthermore, we report that after conditioning to a strong 1.0 mA footshock, Non-orienters show increased susceptibility to condition to context than Orienters as evidenced by increased freezing in the absence of the CS when returned to the chamber 24 h after conditioning. This result replicates previous research indicating that goal-trackers show more context-induced freezing when placed in the conditioning context 24 h after aversive

conditioning (Morrow et al., 2011). However, the same study also reported that sign-trackers show more cue-induced freezing when first re-exposed to an aversive CS, while we report that the two groups show no difference when initially re-exposed to the tone. Morrow et al. (2011) reports freezing results during re-exposure to the CS in a novel context, 24 h after conditioning, while we report freezing during CS exposure in the original conditioning context both 24 h after conditioning and 21 days after extinction or retrieval+extinction. Another difference lies in characterization of sign-tracking phenotypes. Morrow et al., used insertion of an inactive lever as a CS which elicited a different form of sign-tracking behavior (i.e., engagement with the lever). Unlike Orienters that also displayed US-directed food-cup behavior, these rats engaged almost exclusively with the lever while others engaged almost exclusively with the food cup resulting in an inverse correlation between these two behaviors. These two types of sign-tracking behaviors (i.e., lever-engagement and orienting) might represent slightly different phenotypes. It has been shown that the central nucleus of the amygdala, which is crucial for acquisition of conditioned orienting (Gallagher et al., 1990), is not necessary for sign-tracking behavior toward the lever CS (Chang et al., 2012).

Non-orienters' apprehension about entering an open field, enhanced freezing during fear conditioning, and enhanced expression of contextual fear suggest that their expression of fearful behaviors differs from that of Orienters across modalities and circumstances. Although retrieval+extinction prevents SR in all cases, conditioning to a 1.0 mA footshock resulted in Non-orienters freezing more than Orienters during tests both 24 h (LTM) and 21 days (SR) after retrieval+extinction, whereas conditioning to a 0.7 mA foot shock resulted in Orienters freezing more than Non-orienters during a test 21 days after retrieval+extinction. These differences in freezing after conditioning to a 0.7 mA foot shock were not present 24 h after retrieval+extinction. Combined, our results suggest that time, orienting phenotype, and shock intensity all interact to influence the ability of an extinction session within the reconsolidation window to update an existing fear memory trace. The influence of these factors on the efficacy of retrieval+extinction may provide some explanation for the variation in reported results for fear memory updating studies. Despite the fact that, when systematically measured there is no significant effect of orienting phenotype on the efficacy of the retrieval+extinction paradigm to prevent the return of fear, it is plausible that, in the absence of explicitly observing and quantifying orienting phenotypes, these factors might still contribute to group differences. Orienting-driven effects could occur, for instance, in a case where we have an unintended uneven (and unnoticed) distribution of Orienters/Non-orienters across experimental groups. Interestingly, the orienting phenotype seems to differentially affect fear vs. appetitive memory updating. It would be important, going forward, to examine other potential factors that might contribute variability in orienting phenotype (e.g., rat strain). Ultimately, we believe that understanding individual differences and their neurobiological correlates is key to optimizing memory update techniques.

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Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus

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When presented with a light cue followed by food, some rats simply approach the foodcup (Nonorienters), while others first orient to the light in addition to displaying the food-cup approach behavior (Orienters). Cue-directed orienting may reflect enhanced attentional and/or emotional processing of the cue, suggesting divergent natures of cue-information processing in Orienters and Nonorienters. The current studies investigate how differences in cue processing might manifest in appetitive memory retrieval and updating using a paradigm developed to persistently attenuate fear responses (Retrieval-extinction paradigm; Monfils et al., 2009). First, we examined whether the retrieval-extinction paradigm could attenuate appetitive responses in Orienters and Nonorienters. Next, we investigated if the appetitive memory could be updated using reversal learning (fear conditioning) during the reconsolidation window (as opposed to repeated unreinforced trials, i.e., extinction). Both extinction and new fear learning given within the reconsolidation window were effective at persistently updating the initial appetitive memory in the Orienters, but not the Nonorienters. Since conditioned orienting is mediated by the amygdala central nucleus (CeA), our final experiment examined the CeA's role in the retrieval-extinction process. Bilateral CeA lesions interfered with the retrieval-extinction paradigm—did not prevent spontaneous recovery of food-cup approach. Together, our studies demonstrate the critical role of conditioned orienting behavior and the CeA in updating appetitive memory during the reconsolidation window.

Keywords: appetitive learning, fear learning, conditioned orienting, extinction, central amygdala

INTRODUCTION

When a neutral conditioned stimulus (CS) is paired with an unconditioned stimulus (US), animals often acquire cue-directed responses, for example, approaching/orienting to a light predictive of food (Brown and Jenkins, 1968; Holland, 1977). Under certain conditions, only a subset of animals acquires cue-directed behaviors (aka sign-tracking) in addition to, or at the cost of, developing US-directed behaviors (aka goal-tracking) that ultimately lead to the obtainment of a rewarding US. Cue-directed behaviors likely reflect enhanced attentional, emotional, and/or motivational processing of the cue (Holland, 1977; Robbins and Everitt, 1996; Cardinal et al., 2002) and represent how the cues themselves can acquire incentive value (Robinson and Berridge, 2001). Several brain regions/networks, including the amygdala and dopaminergic pathways, have been implicated in cue-directed behaviors (Gallagher et al., 1990; Parkinson et al., 2000, 2002; Lee et al., 2005, 2011; Mahler and Berridge, 2009; Flagel et al., 2011). In particular, the amygdala central nucleus (CeA) and nigrostriatal circuitry are critical in mediating the conditioned orienting response (OR) directed to CSs paired with food, but are not involved in conditioned approach behavior to the food delivery site (Gallagher et al., 1990; Han et al., 1997; Lee et al., 2005; El-Amamy and Holland, 2006). These studies suggest a separate neural mechanism for cue-directed behaviors and that the nature of CS-information processing may be different in animals displaying robust conditioned cue-directed behaviors. What is not clear is how the presumably different nature of acquired CS-information influences memory extinction, retrieval and updating.

Extinction (repeated exposure to a CS that no longer predicts a US) gradually attenuates conditioned responses; however, this response attenuation is not permanent, and the conditioned responses can return in the form of renewal, reinstatement, or spontaneous recovery (Pavlov, 1927; Rescorla and Heth, 1975; Bouton and Bolles, 1979; Robbins, 1990; Bouton, 2002). Thus, extinction does not generally modify the original CS-US association, but rather creates a separate CS-noUS memory that suppresses the original memory trace (Bouton, 2004). Recently, Monfils and colleagues (Monfils et al., 2009; Schiller et al., 2010)

designed an extinction paradigm for fear conditioning in rats and humans that could potentially target the original CS-US association (see also Chan et al., 2010; Clem and Huganir, 2010; Rao-Ruiz et al., 2011; Agren et al., 2012). Standard extinction trials within 6 h of a single CS exposure blocked return of conditioned fear responses. The CS exposure presumably retrieved the original CS-US memory, which was then in a labile state needing to be re-consolidated (Nader et al., 2000; Nader, 2003; Tronson and Taylor, 2007). Thus, an extinction session after the cue-induced memory retrieval possibly updated the original CS-US association to a CS-noUS association. Others have also shown that this retrieval-extinction paradigm was effective in attenuating drug-seeking behaviors (Xue et al., 2012) in both humans and rats and in suppressing conditioned reinforcement in rats (Flavell et al., 2011).

In the current study, rats were categorized as Orienters and Nonorienters based on their display of conditioned responses during the acquisition phase. Orienters displayed robust conditioned orienting/rearing to the light CS in addition to acquiring conditioned food-cup approach while Nonorienters acquired only the conditioned food-cup approach. Because both groups showed comparable goal-tracking behavior (i.e., food-cup approach), we termed them Orienters and Nonorienters (rather than sign- and goal-trackers) in order to more accurately describe their phenotypes. The first experiment examined whether the retrieval-extinction paradigm might be equally effective in blocking the return of Pavlovian appetitive responses directed to the CS (conditioned orienting/rearing response to the light) and to the US (conditioned food-cup approach). We further examined how individuals' predilections for the cue-directed ORs might manifest in memory retrieval and extinction. In the second experiment, we investigated whether fear conditioning rather than extinction after memory retrieval could update the appetitive memory. Finally, in the third experiment, we examined the role of the CeA in appetitive memory retrieval and extinction processes given the CeA's critical role in mediating conditioned OR.

MATERIALS AND METHODS

SUBJECTS

Adult male Long-Evans rats (Harlan—Experiment 1, Charles-River—Experiment 3) weighing 250–275 g upon arrival were singly housed in a reverse 14 h light/10 h dark cycle, with the lights going off at 10 am. For Experiment 2, subjects were adult male Sprague-Dawley rats (Harlan), weighing 250–275 g upon arrival and were housed in a 12 h standard light cycle with lights on at 7 am. During acclimation, water and food were available ad libitum. One week after arrival to the colony (Experiments 1 and 2) or 7–10 days post-surgery (Experiment 3), rats were put on restricted feeding to reduce weight to 90% of their free-feeding body weight; this weight was maintained throughout the study. All experiments were conducted according to the *National Institutes of Health's Guide for the Care and Use of Laboratory Animals*, and the protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin.

EXPERIMENTAL DESIGNS

Experiment 1: Effects of retrieval-extinction paradigm on conditioned OR and food-cup approach

In this experiment, extinction learning after memory retrieval was used to update the original appetitive memory. After animals were conditioned to light-food pairings, they received an extinction session within the reconsolidation window (i.e., a single CS exposure before standard extinction trials). Then, spontaneous recovery rate was used to measure whether the original memory was updated.

Appetitive conditioning and testing took place in eight individual conditioning chambers that had aluminum sidewalls and ceiling, with clear acrylic front and back walls (30.5 cm W × 25.4 cm D × 30.5 cm H, Coulbourn Instruments). The floor was made of stainless steel rods (0.5 cm in diameter, spaced 1.0 cm apart). The food magazine was located on the right wall of the chamber, 2.5 cm above the floor. Nose-poke entry into the magazine was detected by an infrared beam at the opening. A 2 w white light was mounted 20 cm above the food-magazine and its illumination served as a CS signaling grain pellet delivery. The left wall was concaved and had five ports with lights, which were not activated. Each chamber was enclosed in a light- and sound-attenuated box (58.4 cm × 61 cm × 45.7 cm) where the ventilation fan provided masking noise. Digital cameras were mounted within each box and images were recorded during behavioral training and testing.

Animals were first trained to eat a single grain pellet delivered to the magazine. A total of 30 pellets were delivered at a variable interval (averaging 60 s) over a 30 min session. After two pre-training sessions, all rats reliably retrieved grain pellets from the magazine. The first training session consisted of two parts. In order to habituate the unconditioned OR to light, the stimulus light was illuminated eight times, for 10 s each time, without any food pellets being delivered to the magazine. Then, during the second half of the session, eight trials of a 10 s light presentation were followed by a food pellet delivery to the magazine. For the next 3 days of conditioning, sessions consisted of 16 light—food pairings with a variable intertrial interval (ITI) averaging 120 s. Extinction occurred 24 h after the final training session. Prior to extinction, rats were pseudo-randomly divided into Retrieval and No Retrieval groups in order for each group to have similar levels of conditioned food-cup responding during acquisition. On the day of extinction, rats in the Retrieval group received one isolated CS presentation and were placed back in the home cage. After one h in the home cage, they were returned to the conditioning boxes and received 17 CS-alone presentations. Rats in the No Retrieval group underwent a typical extinction session consisting of 18 CS-alone presentations, again with a variable ITI averaging 120 s.

Both groups received a test session 24 h after extinction (Test 1), which consisted of four CS presentations, given at variable intervals (average 120 s) without delivery of a grain pellet. Three weeks after this first test session, the rats were again tested with 4 presentations of the CS alone (Test 2). In summary, training (4 days), extinction, and Test 1 were completed in 6 consecutive days. After completing Test 1, rats remained at 90% free feeding weight and were again tested 21 days after Test 1.

Experiment 2: Appetitive memory updating with fear conditioning after memory retrieval

Instead of using extinction learning to update the original appetitive memory, fear conditioning was used in this experiment. Thus, animals first received appetitive training, then received fear conditioning either within the appetitive memory reconsolidation window, or after appetitive memory consolidation. Subsequently, reacquisition rate of light-food pairings was used to measure the strength of the original appetitive memory.

Animals first underwent appetitive conditioning as described in Experiment 1 (Context A), except that they received an additional 16-trial training day. Forty-eight hours after the last appetitive training day, rats were fear conditioned in different conditioning chambers located in a different room (Context B). Animals were divided into Retrieval and No Retrieval groups. The same 2-w white light used during appetitive conditioning served as a CS. Rats in the Retrieval group received one CS exposure 10 min prior to fear conditioning. Rats in the No Retrieval group were placed in the conditioning context 10 min prior to the fear conditioning session, but were not exposed to a CS. Both groups of animals were held in their home cages between the CS/context exposure and fear conditioning. Then, rats were conditioned with three 10 s light CSs co-terminating with a 500 ms 0.7 mA footshock. ITI was variable, averaging 180 s. The behavior was recorded from digital cameras mounted within each chamber.

Forty-eight hours after fear conditioning, rats were placed in Context C to potentially extinguish both conditioned fear and appetitive responses to the light. Context C was created by modifying Context A chambers by inserting a smooth black floor and adding peppermint scent. Rats received 18 light-only CS presentations and conditioned appetitive (orienting and food-cup approach) and fear (freezing) responses were recorded. Seventy-two hours after extinction learning, rats were placed back in context A and received 16 light-food pairings to examine reacquisition rate.

Experiment 3: Role of CeA in appetitive memory updating within the reconsolidation window

Prior to behavioral training, rats first received bilateral CeA lesions. They were anesthetized with isoflurane gas (Vet Equip) and placed in a stereotaxic frame (Kopf Instruments). Two sites per hemisphere were targeted; AP −2.0/−2.4, ML 4.2, DV −8.2. Rats in the lesion group received 0.2 μL infusion (per site) of 10 mg/mL ibotenic acid dissolved in a 0.1 M phosphate buffered saline solution (PBS) (infused at 0.1 μL /min). Rats in the control group received a sham surgery consisting of either 0.2 μL infusion of PBS per site or lowering of the cannula into CeA with no infusion. Rats were allowed 7–10 days to recover before beginning food deprivation and training.

Training, retrieval-extinction, and test procedures for Experiment 3 were identical to those described in Experiment 1. However, for Experiment 3, the No Retrieval group was subdivided into a context exposure group and a no context exposure group. Animals in the context exposure group were placed in the conditioning box 1 h prior to extinction, but received no CS presentation. Animals in the no context exposure group remained in the home cage prior to extinction. As in Experiment 1, training (4 days), extinction, and Test 1 were completed in 6 consecutive days. After completing Test 1, rats remained at 90% free feeding weight and were again tested 21 days after Test 1.

Following behavioral testing, rats received an overdose of pentobarbital (86 mg/kg) and phenytoin (11 mg/kg) mix (Euthasol® by Virbac Animal Health) and were perfused transcardially with 0.9% saline followed by 4% Paraformaldehyde in 0.1 M phosphate buffer (PFA). Brains were removed, post-fixed, and cryoprotected overnight in a 20% sucrose PFA. Twenty-four hours later, brains were frozen in powdered dry ice and stored at −80°C. Brains were sliced on a freezing microtome and 30 μm sections were collected. In order to verify lesion size and placement, every fourth section was mounted on slides and Nissl-stained.

BEHAVIORAL ANALYSES

Previous work has shown that when presented with a 10 s light CS that predicts pellet delivery, rats typically show an OR towards the light during the first 5 s (CS1) and a food-cup approach response during the last 5 s (CS2) (Holland, 1977). For all experiments, number of OR bouts were counted by a blind observer from DVD recordings of all training sessions. An OR was defined as a rearing response in which both forelimbs were lifted from the floor of the conditioning box, and did not include grooming behavior. To account for within-groups variation in baseline orienting, we report the response difference in CS1 and pre-CS (the 5 s prior to the CS). Food cup approach is reported as bouts of nose-pokes into the magazine (Experiments 1 and 3) or percentage of time spent with the nose inserted in the magazine (Experiment 2), as measured by the infrared beam. We report the difference in CS2 and pre-CS food cup responding. Freezing was scored by a blind observer and calculated as percentage of CS duration spent devoid of movement, excluding breathing and whisker twitching.

STATISTICAL ANALYSES

For acquisition analyses of three experiments, orienting classification × trial repeated ANOVAs were conducted for orienting and food-cup responses. For extinction analyses, orienting classification × retrieval condition × trial repeated ANOVA was conducted. When appropriate (Experiment 1), it was followed with simple ANOVA within Orienters and Nonorienters for OR. For spontaneous recovery tests, orienting classification × retrieval condition × extinction/test days repeated ANOVA was conducted. When appropriate, it was followed up with separate ANOVAs with just Orienters or Nonorienters (Experiment 1) or a priori comparison (Experiment 3). In Experiment 2, for both fear acquisition and extinction, and appetitive reacquisition, an orienting classification × retrieval condition × trial repeated ANOVA was conducted. When appropriate, the significant interaction effects were followed up with one-way ANOVAs and then with Bonferroni tests.

RESULTS

EXPERIMENT 1

Acquisition

During the conditioning sessions, in which the light cue was repeatedly paired with food, there was an overall acquisition of conditioned OR and food-cup approach behavior. However, a

subset of rats did not acquire conditioned OR. Thus, based on their average number of OR bouts during the last eight trials of training, rats were divided into two groups. Rats scoring at or above the median number of OR bouts were classified as Orienters ($n = 26$), while those rats that scored below the median score were classified as Nonorienters ($n = 22$). As shown in **Figure 1A**, Orienters acquired conditioned OR to the light CS while Nonorienters did not show an increase in OR as training progressed. An orienting classification × trial block repeated ANOVA of OR showed a significant main effect of orienting classification, $F(1, 42) = 46.0$, $p < 0.0001$, a significant main effect of trial block, $F(6, 252) = 9.24$, $p < 0.001$, and significant interaction effects between the orienting classification and trial block, $F(6, 252) = 9.24$, $p < 0.001$. Importantly, Orienters and Nonorienters did not differ in their display of unconditioned OR. Both groups equally showed unconditioned OR at the beginning of the habituation trials and this unconditioned OR decreased over the course of the eight habituation trials: the average OR scores of the first four trials were 0.19 (Orienters) and 0.22 (Nonorienters), and the last four trials were 0.10 (Orienters) and 0.13 (Nonorienters). This was supported by a lack of main effect of orienting classification as well as orienting classification × trial interaction ($ps > 0.05$). Due to a video equipment malfunction, four rats were missing OR data from the eight habituation trials and first eight trials of training and were excluded from analysis of OR data during habituation and training. The generally low levels of conditioned OR by Orienters (**Figure 1A**) partly reflect the nature of OR scoring and analyses procedures. Rats typically rear once towards the light within the first 5 s but not at every trial, resulting the average score to be lower than one. In addition, even though it is not frequent, any baseline rearing during the 5 s prior to the light onset has been subtracted, resulting in negative OR scores at some trials.

In contrast to conditioned OR acquisition, both Orienters and Nonorienters showed an increase in food-cup responding as training progressed and there was no difference in acquisition rate between these two groups (**Figure 1B**). An orienting classification × trial block repeated ANOVA of food-cup responding showed only main effect of trial block, $F(6, 276) = 43.3$, $p < 0.001$.

Extinction

For an extinction session, animals were further divided into groups that received a single CS exposure an hour prior to standard extinction trials (Retrieval group) or only standard extinction trials (No Retrieval group). Thus, there were four groups of animals: Orienters-Retrieval ($n = 13$), Orienters-No Retrieval ($n = 13$), Nonorienters-Retrieval ($n = 11$), and Nonorienters-No retrieval ($n = 11$). As expected, Orienters showed more OR than Nonorienters, but the retrieval trial did not affect extinction rates (**Figure 2A**). An orienting classification × retrieval conditions × extinction trials repeated ANOVA supported this observation; there was a main effect of extinction trials, $F(8, 352) = 2.36$, $p < 0.05$ and a main effect of orienting classification, $F(1, 44) = 15.3$, $p < 0.0001$, but no interaction effects among orienting classification, retrieval conditions and/or extinction trials. Even though the interaction effect of orienting classification and extinction trials was not significant ($p = 0.17$), the main extinction trial effect seemed to be driven by Nonorienters. Thus, we ran separate ANOVAs on Orienters and Nonorienters. The results show that the trial effect was only significant among Nonorienters, $F(8, 160) = 3.43$, $p = 0.001$, but not among Orienters, $F(8, 192) = 0.98$, $p > 0.4$. In terms of conditioned food cup responding (**Figure 2B**), all animals showed a reduction of food cup responding over the course of extinction trials, $F(8, 352) = 4.31$, $p < 0.05$, and there was no difference among the four groups, as shown by no main or interaction effects, $ps > 0.1$.

Test

Both 24 h (Test 1) and 21 days (Test 2) after extinction, rats were tested with 4 CS exposures. In order to determine whether there was spontaneous recovery of OR and food-cup responding, the responses during the last four trials of

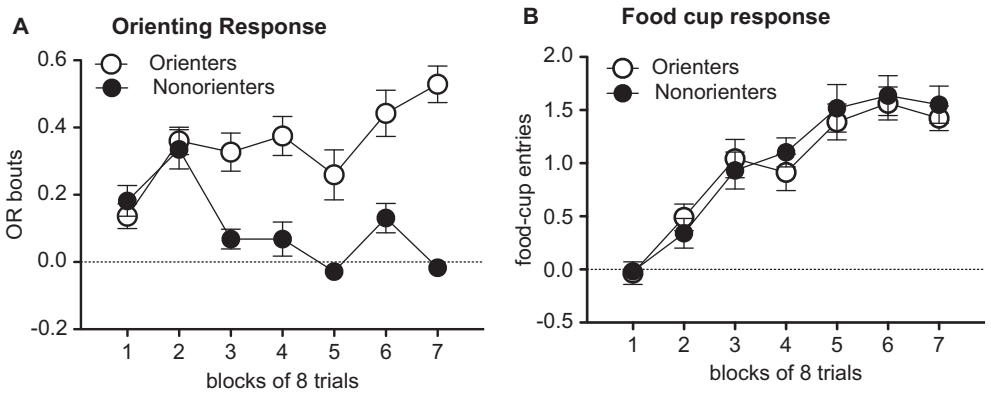

FIGURE 1 | Mean (±SEM) OR **(A)** and food-cup response **(B)** during training. OR bouts were measured during the first 5 s of each CS and food-cup entries were measured during the last 5 s CS period. The values shown are elevation scores, calculated by subtracting pre-CS baseline responding from responding during the CS. Orienters, but not Nonorienters, acquired conditioned OR to the light CS, $p < 0.0001$ **(A)**. In contrast, both Orienters and Nonorienters acquired conditioned food-cup responding **(B)**.

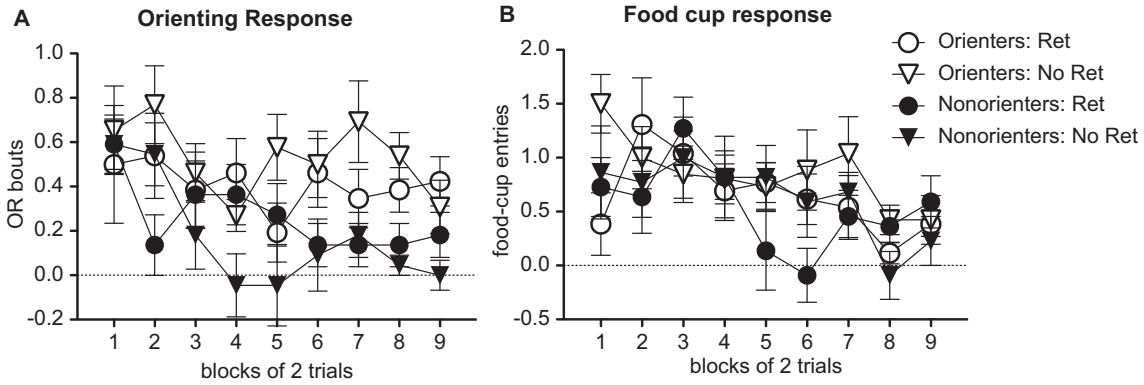

FIGURE 2 | Mean (±SEM) OR **(A)** and food cup response **(B)** during extinction. Orienters and Nonorienters refer to the animals that showed robust and no conditioned orienting, respectively, during conditioning phase. Ret refers to the extinction condition, in which a single CS was presented prior to regular extinction trials while No Ret refers to the regular extinction trials without a prior CS presentation. Orienters showed more OR than Nonorienters **(A)**. There was no difference in food-cup responding among four groups, and all showed comparable extinction rates **(B)**.

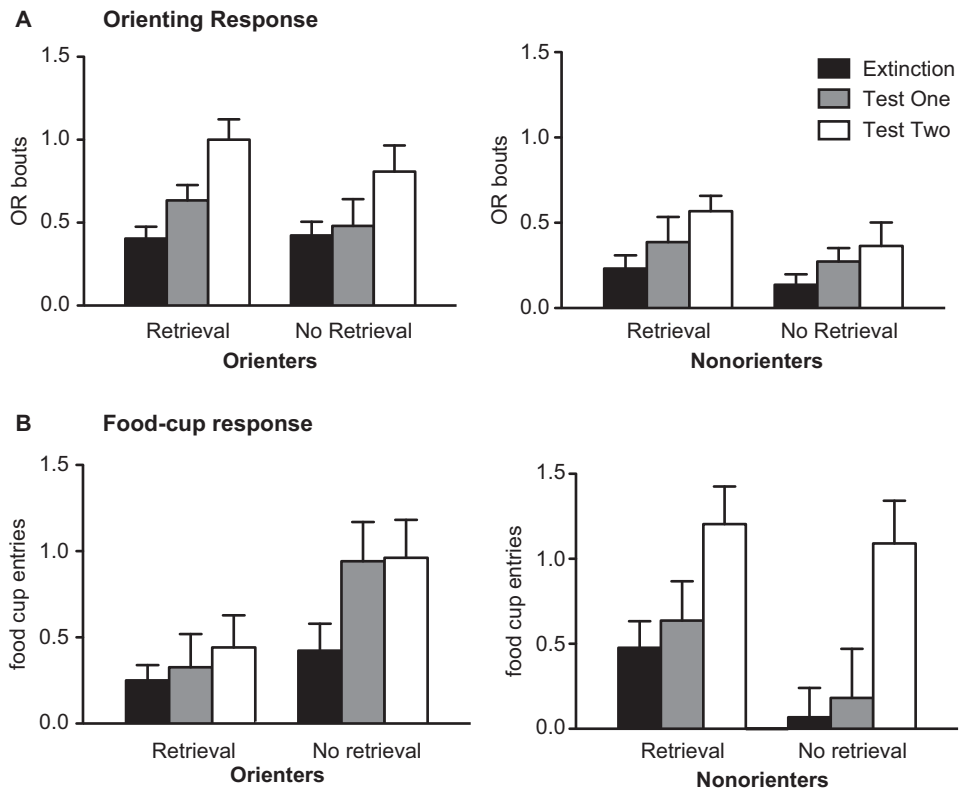

FIGURE 3 | Mean (±SEM) OR **(A)** and food cup response **(B)** for Orienters (left panels) and Nonorienters (right panels). The values are responses during the last four CS alone presentations in extinction session, four CS alone presentation 24 h (test 1) and 21 days (test 2) after extinction. A single CS presentation 1 h prior to extinction trials (retrieval condition) blocked return of spontaneous food-cup response only in Orienters.

extinction were compared to the responses during the test trials. Conditioned OR was observed in most of the animals regardless of extinction conditions (**Figure 3A**). As expected, Orienters generally showed higher levels of OR compared to Nonorienters. In support of this observation, an orienting classification × retrieval condition repeated ANOVA over extinction, Test 1, and Test 2 trials showed a main effect of orienting classification $F(1, 44) = 15.0$, $p < 0.0001$ and main effect of extinction-test days $F(2, 88) = 16.5$, $p < 0.001$, but no main effect of retrieval condition $F(1, 44) = 1.92$, $p > 0.1$.

Furthermore, there was no interaction of orienting classification × extinction-test days, $F(2, 88) = 1.25$, $p > 0.2$, no interaction of retrieval condition × extinction-test days, $F(2, 88) = 1.0$, $p > 0.3$, and no three way interaction of orienting classification, retrieval condition and extinction-test days, $F(2, 88) = 0.04$, $p > 0.9$.

Conditioned food-cup responding was different based on orienting classification and the retrieval condition (**Figure 3B**). Orienters in the No Retrieval group showed similarly increased food-cup responding at both Test 1 and Test 2. By contrast, Orienters in the Retrieval group did not show much food-cup responding at either test points. Food-cup responding of Nonorienters in both Retrieval and No Retrieval groups increased during Test 2. In support of these observations, orienting classification × retrieval conditions repeated ANOVA over extinction, Test 1 and Test 2 trials showed a main effect of extinction-test days, $F(2, 88) = 10.2$, $p < 0.0001$, an interaction effect of orienting classification × extinction-test days, $F(2, 88) = 3.16$, $p < 0.05$, and an interaction effect of orienting classification × retrieval conditions, $F(1, 44) = 9.37$, $p < 0.01$. The interaction effects were further examined with follow-up analyses (i.e., retrieval condition × extinction/test days repeated ANOVA) conducted on Orienters and Nonorienters separately. Among Orienters, there was a main effect of retrieval condition, $F(1, 24) = 6.74$, $p < 0.05$, but no longer a significant main effect of test days, $F(2, 48) = 2.53$, $p > 0.05$. Among Nonorienters, there was only a main effect of test days, $F(2, 40) = 8.82$, $p = 0.001$ and no main effect of retrieval condition $F(1, 20) = 3.14$, $p > 0.05$. The results suggest that the retrieval-extinction paradigm reduced food-cup responding among Orienters but not in Nonorienters.

EXPERIMENT 2

Appetitive conditioning

During the conditioning sessions, in which the light cue was repeatedly paired with food, a subset of rats did not acquire conditioned OR (**Figure 4A**). Thus, based on their average number of OR bouts during the last eight trials of training, rats were divided into two groups. Rats scoring above the median number of OR bouts were classified as Orienters ($n = 15$), while those rats that scored at or below the median score were classified as Nonorienters ($n = 31$). Because a large number of rats failed to acquire the conditioned OR and displayed zero or fewer bouts of orienting, there were more Nonorienters than Orienters. An orienting classification × trial block repeated ANOVA of OR showed a significant main effect of orienting classification, $F(1, 29) = 30.2$, $p < 0.0001$, and a significant interaction effect

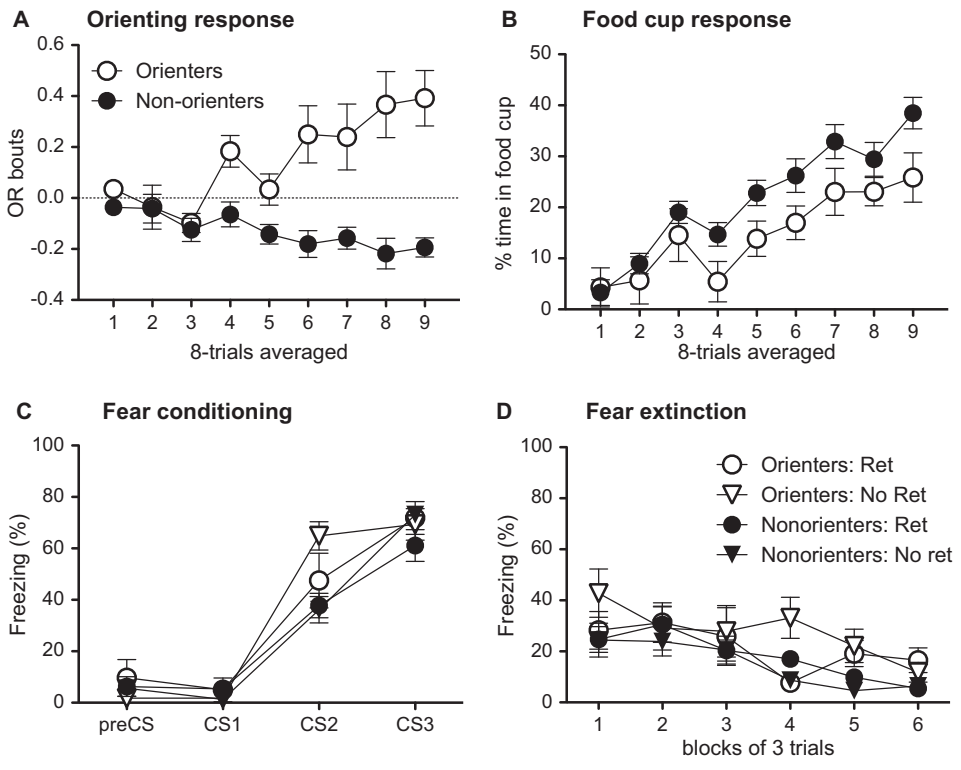

FIGURE 4 | Mean (±SEM) OR **(A)** and food-cup response **(B)** during appetitive training, and freezing response during fear conditioning **(C)** and subsequent extinction trials **(D)**. Orienter and Nonorienter designations refer to those rats that developed a robust OR during appetitive training (Orienters) and those that did not (Nonorienters). Ret refers to the condition in which rats received a single CS exposure 10 min prior to fear conditioning, while No ret designates those rats were only exposed to the conditioning context prior to fear conditioning. Both Orienters and Nonorienters acquired conditioned food cup response **(B)** while only Orienters showed conditioned OR **(A)**. Both Orienters and Nonorienters achieved comparable freezing levels by the end of fear conditioning trials **(C)** and displayed similar extinction rates **(D)** regardless of retrieval condition. However, the Orienters-No Retrieval group showed slightly increased freezing levels both during acquisition and extinction trials.

between the orienting classification and trial block, $F(8, 232) = 5.42$, $p < 0.0001$. In contrast to the acquisition of conditioned OR, both groups acquired conditioned food-cup (**Figure 4B**). However, animals in the Nonorienter group showed slightly higher acquisition rate than the ones in the Orienter group. This is not unusual in that slightly higher food-cup responses have been observed at times among rats displaying attenuated OR due to brain manipulations (Gallagher et al., 1990; Han et al., 1997). An orienting classification × trial block repeated ANOVA of food-cup responding supported this observation. There was a significant main effect of trial block, $F(8, 352) = 21.9$, $p < 0.0001$, as well as a main effect of orienting classification, $F(1, 44) = 5.65$, $p < 0.05$.

Fear conditioning

Fear conditioning was conducted in a different context and rats were further divided into two groups in which one received a single CS exposure prior to fear conditioning (Retrieval group) while the other was only exposed to the conditioning context without CS exposure prior to fear conditioning (No Retrieval group). Then, rats in all groups received three light-footshock pairings and showed an increase in freezing to the light across three trials (**Figure 4C**). An orienting classification × retrieval condition × trial repeated ANOVA of percent freezing revealed significant main effects of both orienting classification, $F(1, 42) = 6.10$, $p < 0.05$, and trial, $F(2, 84) = 155.7$, $p < 0.0001$, as well as an interaction between orienting classification and trial, $F(2, 84) = 3.67$, $p < 0.05$. One-away ANOVA for each trial revealed that the groups only differed at trial 2, $F(3, 42) = 4.65$, $p < 0.01$. Follow-up Bonferroni comparisons at trial 2 showed that Orienters in No Retrieval group, but not in Retrieval group, displayed significantly higher freezing compared to Nonorienters in both Retrieval ($p = 0.01$) and No Retrieval ($p = 0.01$) groups. However, all four groups of animals displayed comparable freezing by the end of fear conditioning as shown by non significant effect at the third trial, $F(3, 42) = 1.07$, $p > 0.1$.

Extinction

In a context that was different from the ones used for either appetitive and fear conditioning, an extinction session of 18 light-alone trials was given to assess both appetitive and fear responses as measured by conditioned OR, food-cup approach, and freezing. If fear conditioning after CS retrieval updated the original appetitive memory, then higher freezing levels and lower appetitive behaviors should be seen in the retrieval group, particularly among Orienters. We predicted that the rats in the no retrieval group would predominantly display fear responses initially, but might display appetitive responses as fear responses extinguished. Thus, we hypothesized that differences in fear and appetitive responses would be observed at the beginning and the end of extinction trials, respectively.

Contrary to our prediction, the retrieval condition neither yielded higher fear responses nor lower appetitive behaviors compared to no retrieval condition. Overall, all rats showed comparable freezing levels and extinction rate as shown by the main effect of trial block, $F(5, 195) = 10.9$, $p < 0.0001$ without any interaction effects (**Figure 4D**). Interestingly, there was a main effect of orienting classification, $F(1, 39) = 4.24$, $p < 0.05$, which is likely to be driven by higher freezing levels seen in the Orienters-No Ret group. One-way ANOVA for each trial revealed that the groups only differed at trial blocks 4 and 5, $F(3, 42) = 6.0$, $p < 0.01$ and $F(3, 42) = 3.97$, $p < 0.05$, respectively. A post-hoc Bonferroni revealed that the Orienter-No Ret group froze significantly more than Orienter-Ret and Nonorienter-No Ret groups at trial block 4 ($ps < 0.01$) and from the Nonorienter-No Ret group at trial block 5 ($p < 0.05$). In contrast to our prediction, appetitive responses did not re-emerge as freezing extinguished in any of the groups. Rats displayed very few appetitive behaviors throughout the session; the overall average of OR bout was −0.05 and percent food-cup response was 1.63.

Appetitive retraining

To test for savings of the original appetitive memory, rats were retrained in the original context with 16 light-food pairings. If fear conditioning after CS retrieval updated the original appetitive memory, then slower reacquisition of appetitive behaviors should be seen in the retrieval group, particularly among Orienters. Given that extinction after CS retrieval blocked spontaneous recovery only for Orienters in Experiment 1, we predicted that fear conditioning after CS retrieval would be more effective in updating appetitive memory with fear memory only for Orienters. In support of our hypothesis, the retrieval condition as well as orienting classification played an important role in reacquisition of conditioned food-cup approach (**Figure 5A**). An orienting classification × retrieval condition × trial repeated ANOVA revealed that there was an overall reacquisition of food-cup behavior among all four groups, $F(3, 126) = 6.11$, $p = 0.001$. However, the Orienters in the Retrieval condition showed a retarded reacquisition rate. This observation was supported by the interaction effect of orienting classification and retrieval condition, $F(1, 42) = 6.23$, $p < 0.05$. A follow-up one way repeated ANOVA among Orienters revealed a main effect of retrieval condition, $F(1, 13) = 4.71$, $p < 0.05$ but not among Nonorienters, $F(1, 29) = 1.0$, $p > 0.3$. When considering OR, the retrieval condition did not influence reacquisition rate. Overall, the Orienters displayed reacquisition of conditioned OR while the Nonorienters did not (**Figure 5B**). An orienting classification × retrieval condition × trial block repeated ANOVA confirmed this observation. There was a significant main effect of trial block, $F(3, 126) = 6.14$, $p < 0.001$, a main effect of orienting classification, $F(1, 42) = 21.7$, $p < 0.0001$, and an interaction effect of trial block and orienting classification, $F(3, 126) = 3.12$, $p < 0.05$. However, there was no interaction effect of orienting classification and retrieval condition, $F(1, 42) = 0.48$, $p = 0.5$.

As expected, minimal fear responses were displayed, but the freezing levels were slightly higher at the beginning of the trials as shown by the main effect of session, $F(3, 126) = 15.5$, $p < 0.001$ (**Figure 5C**) . This difference was mainly driven by the Orienters as shown by the interaction effect of orienting classification and session block, $F(3, 126) = 4.0$, $p < 0.05$. In particular, the Orienter-No Ret group showed slightly higher freezing levels at the beginning of reacquisition session. Post-hoc Bonferroni tests revealed that the Orienter-No Ret group was significant different from the two Nonorienter groups at the first trial block, $ps < 0.05$.

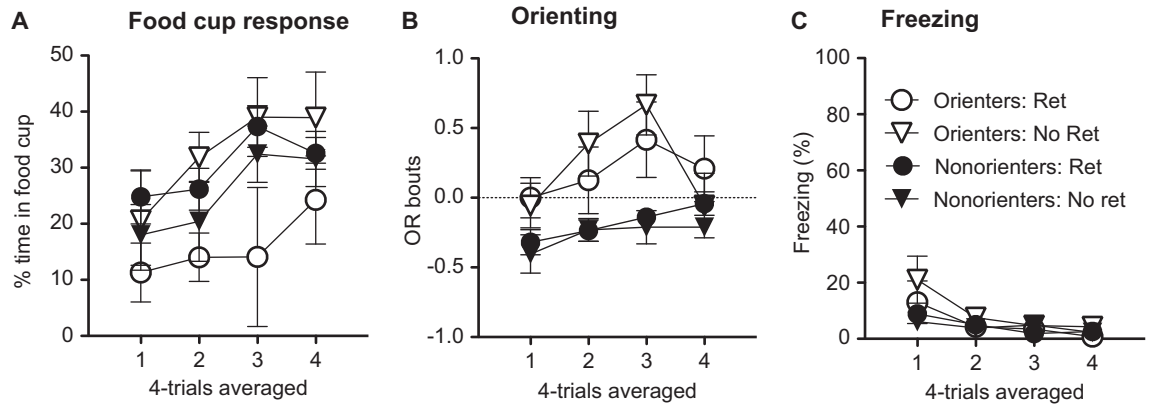

FIGURE 5 | Mean (±SEM) food cup response **(A)**, OR **(B)** and freezing **(C)** during the appetitive reacquisition phase. Orienter and Nonorienter designations refer to those rats that developed a robust OR during the original appetitive training (Orienters) and those that did not (Nonorienters). Ret refers to the condition in which rats received a single CS exposure 10 min prior to fear conditioning while No ret designates those rats that did not (context exposure only). Only Orienters in the retrieval condition showed retarded reacquisition of conditioned food cup response **(A)**, but intact reacquisition of conditioned OR **(B)** and no difference in the minimal levels of freezing **(C)**.

Importantly, the Orienters in the retrieval condition did not show any differences in the minimal display of conditioned freezing compared to the other three groups, suggesting that the retarded reacquisition of conditioned food-cup response was not simply due to higher freezing response.

EXPERIMENT 3

Histology

Twenty-four lesions were deemed acceptable. Lesions were rejected ($n = 10$) if there was less than 30% damage to the medial CeA of either hemisphere or if there was extensive damage to surrounding areas such as the basolateral nucleus (BLA) of the amygdala. Average bilateral lesion size was 65% damage of the entire CeA. **Figures 6A, B** show pictures of intact and lesioned CeA.

Acquisition

Rats with the CeA lesions were not expected to acquire conditioned OR. Thus, only rats in the sham surgery group were divided into Orienters and Nonorienters. This division provided three groups for analysis of training data: Lesion ($n = 24$), Orienter ($n = 18$), and Nonorienter ($n = 18$). As expected, Nonorienters as well as rats with good bilateral CeA lesions did not acquire conditioned OR. A group × trial block repeated ANOVA revealed a significant main effect of trial block, $F(6, 342) = 2.43$, $p < 0.05$, but also a significant group × trial block interaction, $F(12, 342) = 5.05$, $p < 0.001$. As seen in **Figure 6C**, by the end of training Orienters displayed significantly higher conditioned OR when compared to Lesion rats and Nonorienters. A one-way ANOVA on the mean OR scores of the last eight trials showed a main effect of groups, $F(2, 57 = 27.8$, $p < 0.001$, and a post-hoc Bonferroni test revealed that OR scores of Orienters were significantly higher from the ones of Nonorienters ($p < 0.001$) and Lesion rats ($p < 0.001$). As expected, there was no difference between Nonorienters and Lesion rats ($p > 0.3$).

Regardless of the lesion/orienting classifications, all animals acquired the conditioned food-cup response as training progressed and no differences in acquisition rates existed among these three groups. By the end of training, all reached the same levels of conditioned food-cup approach (**Figure 6C**). A group × trial block repeated ANOVA showed only a main effect of trial block, $F(6, 324) = 29.78$, $p < 0.001$. There was neither a main effect of lesion/orienting classifications, $F(2, 57) = 0.01$, $p = 0.99$ nor an interaction effect of trial block by lesion/orienting classification, $F(12, 342) = 1.31$, $p > 0.2$.

Extinction

At the end of training, Lesion rats, Orienters, and Nonorienters were further divided into the Retrieval and No Retrieval groups. Within the No Retrieval group, half of the rats were exposed to the context without the light CS while the others remained in their home cages. A lesion/orienting classification × retrieval condition (retrieval, context exposure, no context exposure) repeated ANOVA on food-cup response revealed only a main effect of extinction trials, $F(17, 782) = 3.03$, $p < 0.001$. Even though there was no main effect of retrieval condition, we did further analyses comparing just the context and no context exposure (i.e., orienting/lesion classification × context exposure repeated ANOVA with extinction trials) to make sure there was still no difference when these two factors were directly compared. There was neither a main effect of context exposure, $F(1, 27) = 0.67$, $p > 0.4$, nor an interaction effect of context exposure by orienting/lesion classification, $F(2, 27) = 2.39$, $p > 0.1$. Therefore, the context and no context exposure groups were collapsed as the No Retrieval group. There were thus six groups; Lesion-Retrieval ($n = 11$), Lesion-No Retrieval ($n = 13$), Orienters-Retrieval ($n = 9$), Orienters-No Retrieval ($n = 9$), Nonorienters-Retrieval ($n = 7$), Nonorienters-No Retrieval ($n = 11$).

As expected, Orienters displayed more OR responses at the beginning of the extinction session compared to Nonorienters or Lesion rats (**Figure 6D**). However, the overall OR decreased throughout extinction and groups were not significantly different

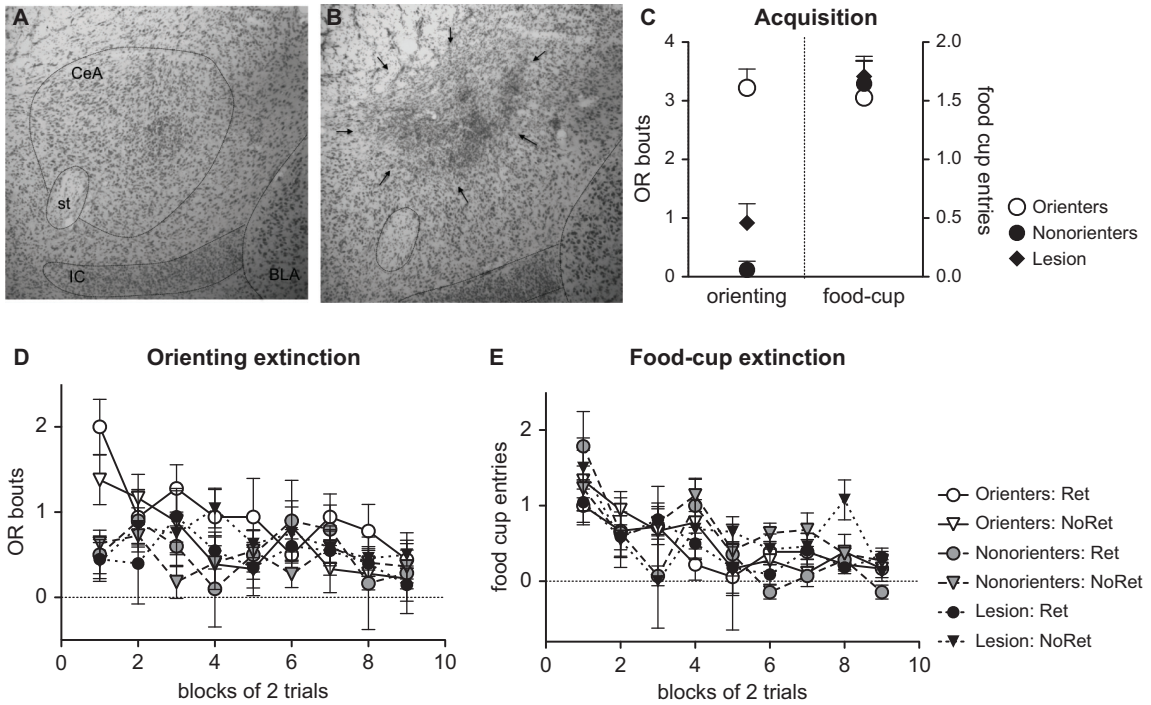

FIGURE 6 | Representative photomicrographs of the amygdala region from the animals with sham lesion **(A)** and ibotenic acid lesion **(B)**. Central amygdala (CeA), stria terminalis (ST), intercalated nucleus (IC), and BLA are highlighted. Average lesion size was 65% CeA damage, and rats with significant BLA damage were excluded. **(C)** Mean (±SEM) OR and food cup response during the last eight trials of training for Orienters, Nonorienters, and Lesion rats. Animals with CeA lesions showed minimal conditioned OR, but still showed intact conditioned food-cup response. **(D and E)** Mean (±SEM) OR and food cup response during extinction. Orienters showed more OR than Nonorienters and CeA Lesioned rats at the beginning but at the end. There was no difference in food-cup responding among six groups, and all showed comparable extinction rates.

at the end of the session. A lesion/orienting classification × retrieval condition × trial repeated ANOVA confirmed a significant main effect of trial, $F(17, 918) = 2.23$, $p < 0.05$, as well as a lesion/orienting classification × trial interaction, $F(34, 918) = 1.62$, $p < 0.05$. One-way ANOVA on the mean OR scores of the first two trials showed a main effect of groups, $F(2, 57) = 11.2$, $p < 0.001$, and a post-hoc Bonferroni test revealed that OR scores of Orienters were significantly higher from the ones of Nonorienters ($p = 0.001$) and Lesion rats ($p < 0.001$). When the last two trials of OR scores were analyzed, there was no main effect of lesion/orienting classification, $F(2, 57) = 0.29$, $p > 0.7$. In contrast to OR responding, the food-cup approach did not differ among Orienters, Nonorienters, and Lesion rats (**Figure 6E**). A lesion/orienting classification × retrieval condition × trial repeated measures ANOVA revealed only a significant main effect of trial, $F(17, 918) = 3.27$, $p < 0.001$.

Test

Four rats (2 in the Orienter-No retrieval group, 1 in the Nonorienter-Retrieval group, and 1 in the Lesion-Retrieval group) did not receive light-CS exposures during Test 1. They were placed in the context, but a computer malfunction resulted in no light exposures. Because their behaviors did not differ from their cohorts in Test 2, their Test 2 data were included. Thus, we ran orienting classification × retrieval conditions repeated ANOVA over extinction and Test 2 only. Including Test 1 as a repeated factor by eliminating those 4 rats did not change the results.

Table 1 | Mean (±SEM) orienting response during the last two trials of extinction and the first two trials of test done at 24-h (Test 1) and 21-days (Test 2) after extinction.

		Extinction	Test 1	Test 2
Orienters	Ret	0.44(0.23)	1.56(0.30)	1.17(0.30)
	No Ret	0.22(0.12)	1.14(0.43)	0.89(0.25)
Nonorienters	Ret	0.33(0.56)	1.00(0.39)	1.07(0.43)
	No Ret	0.36(0.19)	0.95(0.32)	1.45(0.18)
Lesion	Ret	0.18(0.21)	0.90(0.24)	0.85(0.22)
	No Ret	0.62(0.16)	1.04(0.18)	0.63(0.25)

Conditioned OR was observed in most of the animals regardless of retrieval condition or orienting/lesion classifications. There was only a main effect of extinction/test days, $F(1, 54) = 16.14$, $p < 0.001$ (see **Table 1** for the OR data). Similar results were found with the food-cup responses. There was only a main effect of extinction/test days, $F(1, 54) = 21.7$, $p < 0.001$ (**Figure 7**). Even though there were no significant interaction effects, we conducted a priori planned comparisons to confirm that the retrieval-extinction paradigm was still effective at keeping the food-cup

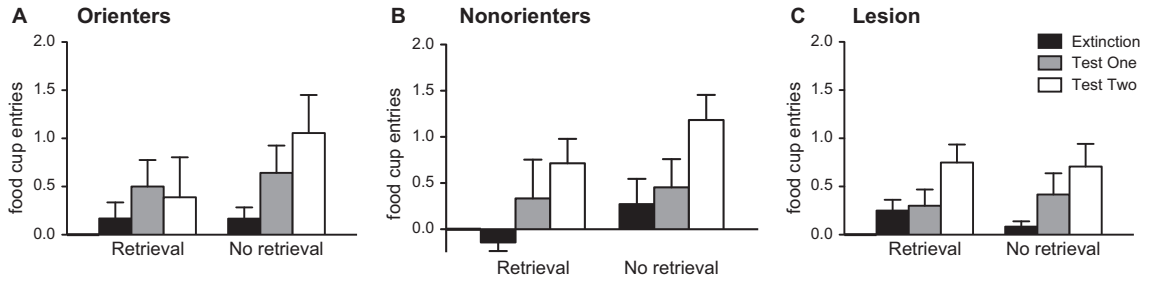

FIGURE 7 | Mean (±SEM) food-cup responding during extinction and tests both 24-h (Test 1) and 21 days (Test 2) after extinction. The values are responses during the last two CS alone presentations of the extinction session, and the first two CS alone presentations during Test 1 and Test 2. Orienters in the retrieval condition are the only animals not showing spontaneous recovery of conditioned food-cup response.

response low for Orienters when tested 3 weeks after extinction. Paired *t*-tests between extinction and Test 2 for the Retrieval condition in each orienting/lesion classified groups confirmed no significant effect among Orienters, $t(8) = 0.61$, $p > 0.5$, but significant effects among Nonorienters, $t(6) = 3.29$, $p = 0.0167$, and Lesion rats, $t(10) = 2.95$, $p = 0.014$ after correcting for multiple comparisons (significant p value at 0.0167).

DISCUSSION

The current studies highlight the role of conditioned OR in cue processing, specifically in cue-associated memory retrieval and updating. Experiment 1 showed that extinction within the reconsolidation window was effective at persistently reducing conditioned food-cup approach only in those rats that showed robust conditioned OR during the acquisition phase. In addition, results from Experiment 2 suggest that fear conditioning introduced during an appetitive memory reconsolidation window altered the original CS-associated appetitive memory—Orienters in the retrieval group showed slower reacquisition of conditioned food-cup behavior when tested for savings of appetitive memory. Together these results suggest that the differences in the display of conditioned OR reflect fundamental differences in stimulus encoding, memory retrieval and updating. Finally, Experiment 3 suggests that the CeA, known to be necessary for the acquisition of conditioned OR, is critical for the retrieval-extinction paradigm to effectively block return of conditioned food-cup behavior.

ROBUST EFFECTS OF THE RETRIEVAL-EXTINCTION PARADIGM IN DIVERSE PROCEDURES

It should be noted that the attenuation of conditioned food-cup response following the retrieval-extinction paradigm was replicated in Experiments 1 and 3 despite several major differences between the original Monfils et al. (2009) work and the current study. The differences included valence of the US (shock vs. food pellet), modality of the CS (tone vs. light), number of CS-US pairings (3 vs. 56), rat strain (Sprague-Dawley vs. Long-Evans), and circadian rhythm (testing in light vs. dark cycle). Indeed, within the current studies, differences existed in rat strain (Long-Evans in Experiments 1 and 3 vs. Sprague-Dawley in Experiment 2), light cycle (dark in Experiment 1 and 3 vs. light in Experiment 2), and number of appetitive CS-US pairings (56 in Experiment 1 and 3 vs. 72 in Experiment 2). Furthermore, in Experiment 2, fear conditioning rather than extinction during the reconsolidation window was used and was still effective in updating a previously acquired appetitive memory. As was the case in Monfils et al. (2009), the current study also showed that the retrieval-extinction paradigm relied on exposure to the specific CS and not on general exposure to the context. The context exposure effect was directly tested in Experiment 3 among animals in the No Retrieval group; one subgroup was exposed to the context without CS presentation while the other group remained in the home cage. Equivalent spontaneous recovery was observed in both groups. Thus, the current study suggests that the retrieval-extinction paradigm can be effective in updating appetitive memory. In fact, other recent studies have reported that the retrieval-extinction paradigm was effective in a variety of appetitive settings. For example, extinction after drug-associated cue presentation prevented drug-seeking behaviors in rats and drug craving in humans (Xue et al., 2012). In another study, rats did not acquire conditioned reinforcement with a food-associated light cue that was subjected to the retrieval-extinction paradigm (Flavell et al., 2011). However, unlike earlier findings, our results showed that the retrieval-extinction paradigm worked only in a subset of animals (Orienters). Similarly, the effectiveness of fear conditioning within the reconsolidation window in Experiment 2 was also dependent upon propensity of OR. Moreover, unlike conditioned food-cup approach behavior, conditioned OR was not affected by the retrieval-extinction/new learning paradigm in which conditioned OR was still seen during the tests (in Experiments 1 and 3) and reacquisition (in Experiment 2) among Orienters.

SPECIFIC EFFECTS OF THE RETRIEVAL-EXTINCTION PARADIGM ON FOOD-CUP RESPONSE

Although both OR and food-cup approach behavior are reflective of CS-US associative strength, conditioned OR is thought to reflect attentional processing in particular (Holland, 1977; Holland and Gallagher, 1999). In support, various studies have shown independent neural processing of these two conditioned responses. Conditioned OR, but not conditioned food-cup response, relies on the CeA-nigral dopamine system (Han et al., 1997; Lee et al., 2005; El-Amamy and Holland, 2006), which has also been implicated in several behavioral tasks designed

to measure attentional processing (Lee et al., 2006, 2007, 2008, 2009). Interestingly, the CeA is only required during the acquisition of conditioned OR and is unnecessary for the expression of fully acquired conditioned OR (McDannald et al., 2004). In contrast, the nigro-dorsolateral striatal circuitry is needed to express conditioned OR (Han et al., 1997; El-Amamy and Holland, 2006), suggesting a habit-like process of fully conditioned OR. Thus, extinction during the reconsolidation window may not target fully conditioned OR that relies on the dorsolateral striatum for expression. The neural circuitry underlying the conditioned food-cup response is unknown; however, the BLA, but not the CeA, is known to play an important role in encoding and representing reinforcement value of the CS (Hatfield et al., 1996). In particular, the BLA and its connections with the orbitofrontal cortex are important for updating the current value of a specific CS (Gallagher et al., 1999; Schoenbaum et al., 1999, 2003a,b). Thus, different neural circuitries contribute to different processes engaged in appetitive conditioning (Holland and Gallagher, 1999). The retrieval-extinction and retrieval-novel training paradigms, which aim to update the original CS-US association to a CS-no US and CS-new US association, respectively, might be more effective at targeting the neural process for encoding and updating CS value rather than the process important for regulating attention to CS. Interestingly, in Xue et al. (2012), the retrieval-extinction paradigm influenced protein kinase Mζ expression in the BLA, but not in the CeA.

It should be pointed out that both Nonorienters and rats with CeA lesions showed ORs comparable to Orienters during the test days (see **Table 1**). Both at the end of acquisition phase (**Figure 6C**) and at the beginning of extinction session (**Figure 6D**), Nonorienters and CeA lesioned rats showed significantly fewer ORs as compared to Orienters, as expected. However, during the habituation period when the light CS is presented without food, all three groups of rats displayed comparable unconditioned ORs: overall OR counts over eight trials were 2.4 (Orienters), 2.5 (Nonorienters), and 2.2 (CeA lesion). In accord, previous work (and the current study) has repeatedly shown neural and behavioral dissociations between unconditioned and conditioned orienting (Gallagher et al., 1990; Lee et al., 2005, 2011). Thus, one possibility is that the return of orienting seen during the tests might partly reflect unconditioned orienting. Our interpretation of this finding is limited in the current form and further investigation is needed.

INDIVIDUAL VARIATIONS IN THE DISPLAY OF CONDITIONED ORIENTING AND MEMORY UPDATING

Even though the retrieval-extinction/new learning did not influence conditioned OR, the effectiveness of this paradigm at persistently reducing conditioned food-cup behavior was influenced by the animals' propensity to display conditioned OR. Others have shown individual differences in the display of cue-approach behavior, also termed sign-tracking (see Flagel et al., 2009 for review) and reported behavioral and physiological differences seen in sign-trackers. For example, different monoamine activities in mesolimbic system (Tomie et al., 2000; Flagel et al., 2007, 2010, 2011), elevated corticosterone levels (Tomie et al., 2000), enhanced cocaine-induced psychomotor sensitization (Flagel et al., 2008), and high impulsivity (Tomie et al., 1998; but see Lovic et al., 2011) have been reported in sign-trackers. Our unpublished work also suggests that Orienters make more impulsive decisions and show enhanced 50-kHz ultrasonic vocalization in response to amphetamine. While some specific circuitries remain unknown, dopamine neurotransmission appears to be involved in all forms of sign-tracking behaviors. In particular, Flagel et al. (2011) showed an interaction of dopamine and cue-approach behavior: dopamine release in the nucleus accumbens following the CS was associated with animals showing prepotent sign-tracking behavior and intact dopamine function was necessary for the acquisition of sign-tracking. These data suggest that animals with a natural tendency to develop cue-approach behavior encode and process stimulus information differently from animals that do not show robust cue-approach behavior.

In the current studies, presumably enhanced attention to the CS (as measured by heightened conditioned OR) may allow for complete retrieval of the original CS-US memory, subsequently making that memory more apt for updating during extinction or new learning. Given that the CeA-nigral dopamine circuitry is essential for the acquisition of conditioned OR (Lee et al., 2005; El-Amamy and Holland, 2006), rats that show a natural tendency to develop a prepotent conditioned OR may have enhanced CeA-nigral dopamine function. Under normal extinction trials (or new learning), the CeA-nigral circuitry's role may not be as important, as typical extinction (or new learning) most likely does not rely upon retrieval of a previously acquired CS-US memory. However, enhanced CeA-nigral dopamine function may aid extinction (or new learning) during reconsolidation by enhancing cue-induced retrieval of CS-US associative memory and updating it to a CS-no US memory or, in the case of novel training following retrieval, a CS-new US memory. This view is supported by findings from Experiment 3, as rats with CeA lesion showed food-cup responding 3 weeks following retrieval-extinction, an indication that they were unable to permanently update the value of the CS. A future study will be needed to address whether the intact CeA function is necessary at the time of appetitive acquisition and/or during memory retrieval-extinction.

We also observed a trend in differences of food-cup approach between Orienters and Nonorienters when they were tested a day after extinction: Orienters showed substantial conditioned food-cup approach, which was not evident among Nonorienters (Test 1 data of No Retrieval group in **Figure 1B**). The observed conditioned food-cup approach in Orienters-No Retrieval group during Test 1 was only marginally significant compared to its own food-cup behavior seen at the end of extinction ($p = 0.094$), but was significantly different (without correcting for multiple comparisons) from the food-cup behavior seen in Nonorienters-No Retrieval group at Test 1 ($p = 0.047$). However, this observation was not replicated in Experiment 3, questioning the consistency of this particular phenomenon observed between Orienters and Nonorienters. Nonetheless, the retrieval-extinction paradigm was effective at keeping the conditioned food-cup approach low at both Test 1 and 2 for Orienters. More work is needed to examine the potential orienting phenotypic differences in maintenance of extinguished food-cup behavior, which can have implications

in the interpretations of how the retrieval-extinction paradigm reduces food-cup behavior persistently.

CONDITIONED ORIENTING AND FEAR LEARNING

In Experiment 2, Orienters in No Retrieval group displayed higher conditioned freezing levels generally. They showed rapid acquisition rate of fear conditioning, better long-term memory (seen in the first block of fear extinction), and reduced extinction learning. It is interesting that the enhanced conditioned freezing is not seen among Orienters that were fear conditioned after memory retrieval (i.e., receiving a single presentation of the CS previously paired with food). Because rats in the Retrieval group were exposed to an additional presentation of the light, we cannot rule out the possibility that exposure to a single unreinforced CS itself (independent of the retrieval effect) had an impact on subsequent fear conditioning and memory updating. Interestingly, the enhanced freezing in No Retrieval group compared to Retrieval group was not observed among Nonorienters. What should be noted though is that despite slightly lower conditioned fear in Retrieval group compared to No Retrieval group among Orienters, fear learning in the Retrieval group had a more profound effect on the original appetitive memory. Appetitive reacquisition was significantly lower in Orienters-Retrieval group, suggesting successful updating of CS associative memory in this group.

Rats in the No Retrieval condition that received light-food pairings first and then light-footshock pairings are likely to form two separate appetitive and aversive memory for the same light CS. Perhaps, Orienters with already enhanced attention to the light CS are better at forming parallel associations for the same CS. A recent study also reported that sign-tracking animals showed enhanced conditioned fear to a discrete tone cue (Morrow et al., 2011). Interestingly, the same study showed that sign-tracking animals were worse than goal-tracking animals in contextual fear conditioning. Unlike our study, in which the same light CS was used for appetitive and aversive conditioning, Morrow et al. (2011) used two different CSs for appetitive and aversive conditionings (i.e., insertion of a lever paired with food and tone/context paired with footshock). However, in our other work published in the same issue (Olshavsky et al., 2013), we saw no difference in conditioned freezing between Orienters and Nonorienters when a different tone CS was used for fear conditioning with 0.7 mA footshock. Interestingly, when 1.0 mA footshock was used in the same study (Olshavsky et al., 2013), Nonorienters displayed more post-shock freezing. The discrepant results could partially be due to procedural differences and deserve further investigation. For example, our work used three presentations of 500 ms 0.7 mA (or 1 mA) footshock while the work by Morrow et al. (2011) used five presentations of 2 s 1.0 mA footshock. It is also plausible that the two forms of sign-tracking behaviors, conditioned orienting and lever-approach, rely on different neural mechanisms (as discussed earlier) and therefore reflect different phenotypes.

MECHANISMS OF THE RETRIEVAL-EXTINCTION PARADIGM

Even though the current study is limited in providing mechanistic explanation, it contributes to our understanding of the retrieval-extinction paradigm on memory maintenance and opens the door for many follow-up experiments to be conducted, in the appetitive as well as fear fields. One possible explanation of the current results is that the retrieval-extinction manipulation works via memory updating mechanism. In Monfils' 2009 work, GluR1 phosphorylation in the lateral nucleus of the amygdala was increased following a single CS presentation, but returned to baseline levels after the administration of a second CS 1 h, but not 3 min, after the first. Other studies (Clem and Huganir, 2010; Rao-Ruiz et al., 2011) also provided evidence consistent with the results and mechanistic explanation Monfils provided in 2009 and 2010 in the follow up study in humans (Schiller et al., 2010).

Recently, Baker et al. (2013) showed that a single CS presentation either before or after a standard extinction session (i.e., retrieval + extinction or extinction + retrieval) essentially produced the same effect. They suggested that these two manipulations were driven by the same mechanism; that is some form of facilitation and/or strengthening of extinction would be occurring due to the spacing of the stimuli. We believe that the retrieval + extinction and extinction + retrieval, though they yield similar behavioral outcomes, are likely to operate through different mechanisms—the retrieval-extinction is due to an updating during reconsolidation, and the extinction + retrieval is due to extinction facilitation/strengthening. The study by Baker et al. (2013) does not allow for a distinction in mechanisms, since they only tested behavior (freezing). Published data from our lab as well as others generally point to the latter interpretation of memory updating (Monfils et al., 2009; Clem and Huganir, 2010; Rao-Ruiz et al., 2011). Nevertheless, Baker et al.'s approach is an interesting one and contributes to the field by introducing potential factors that can influence extinction and memory updating. For example, the Baker et al. study study found the retrieval-extinction effect in young adolescent rats while their earlier study did not find the retrieval-extinction effect in adult rats (Chan et al., 2010). Our current study tried to address whether the retrieval + extinction effect on fear conditioning was generalizable to another form of learning, but also aimed to understand some of the boundary conditions that may be contributing to the variability in reported effects from various groups.

IMPLICATIONS

Work investigating how CSs elicit and maintain certain conditioned responses is important in delineating the psychological processes and neural mechanisms that contribute to drug addiction. Accumulating evidence suggests an important role of associative learning processes in drug addiction, in which the environmental cues become associated with reinforcing effects of a drug and later induce a vulnerable state of drug craving and elicit drug-seeking behaviors (Everitt et al., 1999; Weiss et al., 2001; Wise, 2004; Hyman et al., 2006; Robbins et al., 2008; Robinson and Berridge, 2008; Belin et al., 2009). Thus, weakening or undoing the cue-drug association can potentially prevent drug relapse (Taylor et al., 2009). In fact, Xue et al. (2012) showed that the retrieval-extinction paradigm was effective in reducing drug craving and relapse. However, they reported that the drug seeking behavior was only reduced, and not completely blocked, in some cases. Our study suggests that individual differences in cue-directed behavior may affect memory retrieval and updating

of CS-associated memory differently. Thus, treatments for drug addiction based on the retrieval-extinction paradigm might work more effectively in a subset of populations. Further studies will be necessary to understand if individual differences in processing discrete CS-associated memory can be used effectively to target drug-associated memory.

AUTHOR CONTRIBUTIONS

Megan E. Olshavsky designed, conducted and wrote the work. Bryan J. Song designed and conducted the work. Daniel J. Powell and Carolyn E. Jones helped with the experiments and data analyses. Marie-H. Monfils and Hongjoo J. Lee designed and wrote the work.

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Mechanisms governing the reactivation-dependent destabilization of memories and their role in extinction

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The extinction of learned associations has traditionally been considered to involve new learning, which competes with the original memory for control over behavior. However, a recent resurgence of interest in reactivation-dependent amnesia has revealed that the retrieval of fear-related memory (with what is essentially a brief extinction session) can result in its destabilization. This review discusses some of the cellular and molecular mechanisms that are involved in the destabilization of a memory following its reactivation and/or extinction, and investigates the evidence that extinction may involve both new learning as well as a partial destabilization-induced erasure of the original memory trace.

Keywords: extinction, reconsolidation, destabilization, reactivation, memory, fear

INTRODUCTION

Under certain conditions, memories can be rendered temporarily labile and sensitive to modification, after which they must be re-stabilized through a process called reconsolidation (Lewis et al., 1972; Przybyslawski et al., 1999; Nader et al., 2000; Lee, 2009; Finnie and Nader, 2012). Once memories are destabilized it is also possible to enhance (Tronson et al., 2006; Lee, 2008; Debiec et al., 2011; Tian et al., 2011) and even incorporate new information into existing memories (Winters et al., 2009; Choi et al., 2010; Lee, 2010; Winters et al., 2011), which has led to the suggestion that reactivation-induced destabilization of memory is an important updating mechanism that is required for new learning (Lee, 2009). In models of associative learning, destabilization of previously acquired memory traces can be achieved by re-exposure to a conditioned stimulus (CS; e.g., tone) in the absence of the unconditioned stimulus (US; e.g., a foot shock). This can promote either reconsolidation of the original memory or extinction depending on several boundary conditions, including stimulus intensity, training to test interval, or the duration of the reminder cue (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Pedreira et al., 2004; Suzuki et al., 2004; Lee et al., 2006; Power et al., 2006; Tronson et al., 2006; Nader and Hardt, 2009; Wang et al., 2009; Reichelt and Lee, 2012; Flavell and Lee, 2013). If reconsolidation is interrupted, memories may also be prevented from returning to a stable state, which can lead to amnesia (Lewis et al., 1972; Przybyslawski and Sara, 1997; Nader et al., 2000; Lee et al., 2006). Conversely, if processes related to extinction are interrupted, the relative strength of the memory will remain unchanged and presentation of the CS will continue to elicit conditioned behavior (Eisenberg et al., 2003; Pedreira et al., 2004; Suzuki et al., 2004; Flavell and Lee, 2013).

Retrieval refers to a process whereby the activation of neuronal networks leads to the recall of a memory, allowing the expression of an appropriate behavioral response. However, this does not necessarily imply that when a memory is retrieved it is also reactivated. Retrieval and reactivation are independent processes and there are accounts of memories being retrieved but not susceptible to change (Cammarota et al., 2004). Reconsolidation refers to the molecular mechanisms required to return a memory to a stable state, therefore, for a memory to be reconsolidated it must have first entered a reactivated state and become destabilized.

Extinction training is commonly used to reduce aberrant emotional responses associated with phobias or post-traumatic stress disorder (PTSD; Rauch et al., 2012) and results in a reduction in the strength of a given memory, due to repeated exposure to the CS in the absence of the US. The general consensus is that extinction does not affect the original memory trace as it is prone to spontaneous recovery (return of fear over time), reinstatement (return of fear following unsignaled presentation of the US) and renewal (return of fear following the presentation of contextual cues) (Myers and Davis, 2007). However, a reconsolidation-extinction paradigm has recently been introduced, whereby extinction training applied within the reconsolidation window, i.e., when the memory is in a destabilized state, leads to a permanent reduction in the expression of fear behavior. This reduction is resistant to renewal, reinstatement and spontaneous recovery (Monfils et al., 2009; Schiller et al., 2010; but see Chan et al., 2010; Kindt and Soeter, 2013), indicating that the original memory trace can indeed be modified. A reactivation session and an extinction session differ only in terms of their duration and/or frequency, yet they can lead to very different outcomes. Reactivation followed by reconsolidation can serve to strengthen or update a memory, extinction leads to the formation of a new competing memory, and a reactivation-extinction session appears to permanently affect a previously stable memory trace. Therefore, it is likely that the molecular mechanisms invoked at the time of reactivation are critical for determining the consequences of what are procedurally very similar behavioral

manipulations. An understanding of these mechanisms is likely to have great therapeutic relevance for the treatment of PTSD, phobia and addiction. The purpose of this review is to discuss some of the cellular and molecular mechanisms involved when a memory is retrieved and subsequently extinguished, and what impact this process is likely to have on the original memory trace.

DENDRITIC SPINE REMODELING

In its simplest form, memory can be viewed as the strengthening of synaptic connections, which occurs through the experience-dependent structural remodeling of dendritic spines. Dendritic spine formation and elimination have both been associated with the formation of new memories (for review see Yuste and Bonhoeffer, 2001; Lamprecht and Ledoux, 2004; Bailey and Kandel, 2008; Caroni et al., 2012). Structural modification to dendritic spines occurs *in vivo* in both invertebrates (Bailey and Chen, 1983, 1988a,b, 1989a,b) and mammals (Geinisman et al., 2001; Knafo et al., 2001; Kleim et al., 2002; Leuner et al., 2003; Restivo et al., 2009; Xu et al., 2009; Yang et al., 2009; Fu et al., 2012; Lai et al., 2012).

In an emotional learning paradigm, the changes in dendritic spines that occurr following fear acquisition are opposed to those that occur after extinction, For example, fear conditioning induces spine elimination within the prefrontal cortex (PFC), whereas fear extinction increases the rate of spine generation (Vetere et al., 2011; Lai et al., 2012). Additionally, it has been demonstrated that spine formation induced by extinction occurs within very close proximity to the original position of spines that were previously eliminated by acquisition, thereby illustrating that increases in spine density following extinction training may compete with reductions that were induced by acquisition (Lai et al., 2012). Synaptic plasticity is differentially modulated across different areas of the brain. Instead of fear conditioning resulting in the elimination of spines as observed by Lai et al. (2012), training correlates with an increase in both the size and number of spines in the anterior cingulate cortex (aCC) and the infralimbic (IL) portion of the PFC (Vetere et al., 2011). Extinction was again found to have opposing effects on the morphological changes brought about by fear conditioning, but they differed according to the region studied: the number of spines in the aCC decreased but they remained enlarged, whereas the number of spines in the IL-PFC remained constant, but diminished in size (Vetere et al., 2011). These examples illustrate that extinction training is able to reverse morphological changes induced by acquisition and implies that, at least to some extent, extinction may mediate a partial erasure of the original memory trace. However, these findings may be restricted to regions of the brain that are critically involved in cognitive flexibility such as the PFC, as it has been shown that in the amygdala, networks originally associated with fear conditioning are left intact and merely silenced by extinction (Repa et al., 2001; Herry et al., 2008). Furthermore, the neuronal circuits activated in the amygdala during fear acquisition are distinct to those activated during fear extinction (Herry et al., 2008).

Finally, an elegant study recently demonstrated that a subset of amygdala neurons which fire during fear conditioning (and which subsequently also fire upon retrieval) are not activated following fear extinction, due to structural remodeling within inhibitory perisomatic synapses (Trouche et al., 2013). This illustrates that extinction activity directly influences the structure of neurons that code for the original memory. In summary, the evidence indicates that extinction training interacts with the original fear circuit (which is unsurprising given that an extinction memory without reference to the original fear memory is essentially meaningless), and that structurally, extinction appears to oppose acquisition. However, this interaction may only result in partial suppression of the original memory trace due to the regional specificity associated with fear and extinction.

RECEPTOR SIGNALING MECHANISMS

A number of neurotransmitters and their cognate receptors are important for the reconsolidation and extinction of retrieved memories, and pharmacological manipulation of the glutamatergic NMDA and AMPA receptors (NMDAR and AMPAR, respectively) has revealed that both have crucial roles in these processes (Baker and Azorlosa, 1996; Suzuki et al., 2004; Winters and Bussey, 2005; Ben Mamou et al., 2006; Yamada et al., 2009; Nikitin and Solntseva, 2013). Systemic administration of NMDAR antagonists can prevent both the reconsolidation of the original memory and the consolidation of an extinction memory (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004; Lee et al., 2006; Flavell and Lee, 2013). Interestingly, reconsolidation and extinction mechanisms do not appear to occur at the same time, with one process being preferred over the other. This has been interpreted as a trace dominance effect, in that only the dominant trace will be impaired (Eisenberg et al., 2003). In terms of destabilization, this implies that despite a reactivation session being procedurally the same as a short extinction session, destabilization cannot occur at the beginning of session and simultaneously destabilize both the original CS-US while promoting the consolidation of a new extinction memory. This is illustrated by the observation that administration of NMDA receptor antagonists or protein synthesis inhibitors during extinction impairs the consolidation of extinction, but not the reconsolidation of the original trace, and by the fact that reconsolidation can be preferentially targeted by varying the duration of exposure to the CS (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004; Flavell and Lee, 2013).

On the surface, the role of NMDARs in fear extinction and reconsolidation appears to be relatively well understood with activation potentiating both processes (Walker et al., 2002; Lee et al., 2006, 2009) and inhibition leading to an impairment (Cox and Westbrook, 1994; Baker and Azorlosa, 1996; Lee et al., 2006); however, there have been conflicting reports. The NMDAR antagonist ifenprodil prevents anisomycin-induced amnesia when injected into the basolateral amygdala (BLA) before, but not after, retrieval, suggesting that NMDARs are crucial for destabilization but not reconsolidation (Ben Mamou et al., 2006). This is at odds, however, with the many reports of NMDAR antagonists, in particular MK-801, preventing reconsolidation (Przybyslawski and Sara, 1997; Pedreira et al., 2002; Lee et al., 2006; Brown et al., 2008; Lee and Everitt, 2008; Winters et al., 2009; Flavell and Lee, 2013). MK-801 is a non-selective NMDAR antagonist, whereas ifenprodil specifically targets the NR2B subunit of the NMDAR complex (Williams, 1993). NR2B-containing NMDARs have been

shown to suppress CREB, promote long-term depression (LTD) and activate protein degradation pathways (Hardingham et al., 2002), while GluN2A-containing NMDARs promote CREB phosphorylation and long-term potentiation (LTP; Liu et al., 2004). Recently, a double dissociation for the roles of NMDAR subtypes has emerged, with NR2A-containing NMDARs being required for reconsolidation, whereas NR2B-containing NMDARs are required for their destabilization (Milton et al., 2013), perhaps explaining what were previously paradoxical results and illustrating that the destabilization step may be entirely separate to reconsolidation.

Pharmacological blockade of AMPA receptors (AMPARs) has been shown to impair both the consolidation and retrieval of memories (Liang et al., 1994; Bast et al., 2005; Winters and Bussey, 2005), as well as their extinction (Walker and Davis, 2002; Zushida et al., 2007; Yamada et al., 2009) so it is somewhat surprising that AMPAR antagonists have been reported to have no effect on either destabilization or reconsolidation of fear memories (Ben Mamou et al., 2006; Milton et al., 2013). As is the case with NMDARs, different sub-populations of AMPARs are likely to be important for different mechanisms. Calcium-permeable AMPARs (CP-AMPARs) generally lack the GluA2 subunit, are less stable at synapses and have been associated with LTD, while calcium-impermeable AMPARs (CI-AMPARs), which do contain the GluA2 subunit are more stable, have been associated with LTP and make up the majority of basal AMPA activity (Isaac et al., 2007). Phosphorylation of AMPARs regulates receptor trafficking (Blackstone et al., 1994; Esteban et al., 2003) and there is an increase in phosphorylation of the GluR1 subunit at serine 845 associated with memory retrieval (Monfils et al., 2009; Jarome et al., 2012). Memory consolidation is associated with the increased expression of CI-AMPARs at synaptic sites but memory retrieval results in an abrupt exchange of CI-AMPARs for CP-AMPARS (Clem and Huganir, 2010; Rao-Ruiz et al., 2011; Hong et al., 2013a). Over a period of hours, the CP-AMPARs are gradually replaced with CI-AMPARs, an event that correlates with the "reconsolidation window" (Clem and Huganir, 2010; Rao-Ruiz et al., 2011; Hong et al., 2013a). Finally, preventing the exchange of AMPARs blocks destabilization and protects from anisomycin-induced amnesia, as does blockade of the newly inserted CP-AMPARs (Hong et al., 2013a). Thus, glutamate receptor trafficking mechanisms are crucial for the determination of whether a memory will undergo reconsolidation or extinction.

In addition to glutamate receptors, the roles of several other neurotransmitters have been investigated. The endogenous cannabinoid receptor, CB1, has been shown to prevent reconsolidation by blocking destabilization, along with L-type voltage-gated calcium channels, as they are able to protect memories from the effects of protein synthesis inhibitors applied during reactivation (Suzuki et al., 2008). Enhancing cannabinoid activity potentiates fear extinction (Marsicano et al., 2002; Chhatwal et al., 2005; Pamplona et al., 2006; de Oliveira Alvares et al., 2008), so it is possible that destabilization could also be required for extinction. A recent study has shown that CB1Rs were increased in neurons that are activated by both fear conditioning and subsequent fear extinction. This was thought to represent an attempt to preserve the original CS-US trace through the ability of CB1R activity to prevent the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA; Trouche et al., 2013). However, this seems to be at odds with its established role in the potentiation of fear extinction (Marsicano et al., 2002; Pamplona et al., 2006), so it is possible that it is having a different effect via modulation of protein-kinase and phosphatase pathways during extinction (Cannich et al., 2004) perhaps through a destabilization mechanism.

Given their established roles in varied cognitive functions linked with memory, it is very likely that the receptors for catecholamines and/or acetylcholine act as upstream triggers for the molecular mechanisms subserving memory destabilization. Reichelt et al. (2013) recently demonstrated the important role that dopaminergic transmission appears to play in appetitive memory destabilization. Dopaminergic activity mediated by the ventral tegmental area (VTA) is an important component of prediction error signaling in an appetitive Pavlovian goal-tracking task in rats; unexpected changes in the nature of reward are associated with a negative prediction error signal, which depends on VTA dopaminergic tone (Takahashi et al., 2009). Reasoning that prediction error, indicative of a potential memory updating situation, may be necessary for memory destabilization in such tasks, Reichelt et al. (2013) assessed the effects of VTA dopamine dysregulation on the memory reconsolidation process. Manipulation of VTA dopaminergic signaling, achieved via microinfusions of either the GABAergic agonists baclofen and muscimol or the D2 receptor antagonist sulpiride, prevented destabilization of the appetitive goal-tracking memory as evidenced by the failure of post-retrieval systemic injections of the NMDAR antagonist MK-801 to disrupt subsequent goal tracking. A follow-up experiment suggested that the VTA is not the site of memory storage for this task; rather, the dopaminergic signal from the VTA likely regulates destabilization of the memory in the nucleus accumbens or amygdala (Reichelt et al., 2013). Whether dopamine plays a similar role in destabilization of aversively motivated memories remains a question for future research.

Winters and colleagues have investigated the involvement of acetylcholine in object memory destabilization. In a previous study using the spontaneous object recognition paradigm for rats, the boundary conditions of memory age and encoding strength were shown to influence the likelihood of object memories becoming labile upon reactivation (Winters et al., 2009). Specifically, memories that were more strongly encoded at the time of initial learning or relatively more remote at the time of memory reactivation did not readily destabilize, such that post-reactivation MK-801 failed to disrupt object memory reconsolidation. However, when similar object memories were reactivated in the presence of an explicit novel cue—a salient floor insert with a novel texture placed in the testing apparatus during the reactivation phase—the memories were destabilized, and systemic post-reactivation MK-801 disrupted object recognition performance when assessed in a test phase 24 h later. This finding highlights the importance of novel information in rendering consolidated memories labile upon reactivation and is consistent with an updating role for the reconsolidation process.

Building on this interpretation, cholinergic transmission could contribute to this novelty-induced memory destabilization

process, given the established roles for acetylcholine in various cognitive functions linked with new learning, such as attention, arousal, and novel memory encoding (Furey et al., 2000; Sarter et al., 2003; Hasselmo, 2006; Winters et al., 2006, 2007). Systemic administration of the muscarinic ACh receptor (mAChR) antagonist scopolamine blocks the reconsolidation impairment typically caused by systemic post-reactivation MK-801 when object memories are reactivated in the presence of a novel floor texture, thereby directly implicating mAChRs in the novelty-induced reactivation of strongly encoded and relatively remote object memories. Moreover, a highly similar result is seen when scopolamine is administered into the perirhinal cortex (PRh), a brain region commonly implicated in mammalian object recognition (Winters et al., 2011). Intra-PRh scopolamine administered before the reactivation phase appears to block memory destabilization, as it prevents the object memory reconsolidation deficit that is otherwise observed when intra-PRh anisomycin is infused immediately following the reactivation phase. Finally, enhancing cholinergic transmission with the mAChR agonist oxotremorine appears to mimic the memory destabilizing effects of novel information during reactivation. When object memories are strongly encoded or relatively remote, systemic co-administration of MK-801 and oxotremorine prior to reactivation leads to a significant impairment in reconsolidation. As noted above, MK-801 does not normally disrupt reconsolidation of such object memories under these conditions, and additional experiments also indicated that oxotremorine alone does not affect reconsolidation using these parameters. Thus, activating mAChRs with an exogenously administered drug appears to trigger the same cellular signaling cascade prompted by the presence of a salient novel cue in the reactivation phase, resulting in memory destabilization.

The exact nature of the intracellular mechanisms underlying the role of mAChRs in memory destabilization remains uncertain. However, mAChRs can influence both NMDAR- and AMPAR-mediated glutamatergic signaling (Segal and Auerbach, 1997; Fernandez de Sevilla et al., 2008; Fernandez de Sevilla and Buno, 2010). Indeed, activation of M1 mAChRs can produce post-synaptic insertion of AMPARs (Fernandez de Sevilla et al., 2008), which may provide a mechanistic link between the effects of cholinergic transmission on object memory destabilization and the previously reported requirement of AMPAR exchange for destabilization of fear memories (Hong et al., 2013b). Moreover, the effects of mAChR activation on AMPAR and NMDAR function are related to mAChR-induced stimulation of the inositol 1,4,5-triphosphate (IP_3) second messenger cascade and may partly rely on activation of CaMKII activity (Fernandez de Sevilla et al., 2008; Fernandez de Sevilla and Buno, 2010). These findings suggest a potential connection between the effects of mAChR activation on object memory destabilization and the previously reported reliance of fear and object-in-place memory destabilization processes on protein degradation (Lee et al., 2008a; Choi et al., 2010). Like NMDAR, mAChRs may recruit the UPS via CaMKII activation (Bingol et al., 2010). There is a demonstrable link between M1 receptor activation and UPS-mediated protein degradation (Jiang et al., 2012); in this study, *in vitro* M1 receptor overexpression enhanced the degradation of β-site amyloid precursor protein cleaving protein 1 (BACE1), a protein that is elevated in sporadic Alzheimer's disease. Importantly, the muscarinic effect on BACE1 degradation was blocked by the proteasome inhibitor β-lac, suggesting that M1 receptors regulate BACE1 degradation via the UPS pathway (Jiang et al., 2012).

The bidirectional effects of cholinergic manipulations on object memory destabilization provide particularly strong evidence for a key role of acetylcholine transmission in this process. It will be important for future studies to assess whether these effects can be generalized to other forms of memory and in other brain regions known to demonstrate reconsolidation effects. A reliable role for acetylcholine in memory destabilization would have important implications for understanding age- and disease-related deficits in cognitive flexibility and could influence new thinking about remediation strategies for such conditions, as well as cases characterized by pervasive maladaptive memories, such as PTSD and phobias.

PROTEIN DEGRADATION

De novo protein synthesis is required for both memory consolidation (e.g., Flexner et al., 1963; Bourtchouladze et al., 1998; Hernandez et al., 2002; Bekinschtein et al., 2007; Duvarci et al., 2008; reviewed in Hernandez and Abel, 2008) and reconsolidation (e.g., Nader et al., 2000; Debiec et al., 2002; Morris et al., 2006; Duvarci et al., 2008; for a review see Alberini et al., 2006). However, a growing number of studies indicate that protein degradation also plays a key role in memory (e.g., Merlo and Romano, 2007; Artinian et al., 2008; Lee, 2008, 2010; Lee et al., 2008b, 2012; for reviews see Kaang et al., 2009; Fioravante and Byrne, 2011; Kaang and Choi, 2012; Jarome and Helmstetter, 2013). Protein degradation is mediated, in large part, by the ubiquitin-proteasome system (UPS), in which proteins are marked for degradation by polyubiquitination (Nandi et al., 2006). This process occurs in almost every mammalian cell; however, within the brain it can be modulated by neuronal activity. Depolarization of cultured hippocampal neurons leads to a rapid redistribution of the proteasome complex into dendritic spines (Bingol and Schuman, 2006), and changes in synaptic activity can increase the ubiquitination and turnover of plasticity-related proteins within the post-synaptic density (Ehlers, 2003). UPS activity is modulated by calcium ion entry via N-methyl-D-aspartate receptors (NMDARs) and by L-type voltage gated calcium channels (LVGCCs), which in turn activate calcium/calmodulin-dependent protein kinase II or CaMKII (Djakovic et al., 2009). Furthermore, impairment of UPS activity affects long-term potentiation (Fonseca et al., 2006; Karpova et al., 2006; Dong et al., 2008; Cai et al., 2010; Pick et al., 2013), indicating that it has a key role in synaptic plasticity.

There are several reports of UPS blockade leading to deficits in learning and memory, fear conditioning leads to an increase in polyubiquitination in the amygdala, and infusion of clasto-lactacystin-beta-lactone (β-lac), a proteasome inhibitor, immediately after training, results in a deficit in both contextual and cued fear (Jarome et al., 2011). However, this is somewhat controversial as others have observed no effect on contextual fear following the disruption of protein degradation (Lee, 2008, 2010; Lee et al., 2008b; Pick et al., 2013; Ren et al., 2013). There is evidence that

UPS disruption impairs the consolidation of spatial memory tasks (Lopez-Salon et al., 2001; Merlo and Romano, 2007; Artinian et al., 2008), which may indicate that spatial but not emotional memories require protein degradation for consolidation.

While its role in memory consolidation is unclear, several studies have demonstrated that synaptic protein degradation is a critical step in the destabilization that occurs prior to reconsolidation (Lee, 2008, 2010; Lee et al., 2008b, 2012; Jarome et al., 2011; Ren et al., 2013). Following the reactivation of a contextual fear memory, an increase in polyubiquitination can be observed, in a selective manner, within the hippocampus (Lee et al., 2008b). The ubiquitination of proteins leads to their degradation, and this decrease can be blocked with β-lac (Lee et al., 2008b), indicating that the reactivation of previously acquired memories leads to a specific pattern of protein degradation at the synapse.

Inhibition of protein synthesis prevents reconsolidation and leads to profound amnesia (Nader et al., 2000; Debiec et al., 2002; Morris et al., 2006); however, infusion of a proteasome inhibitor in conjunction with the protein synthesis inhibitor prevents reactivation-dependent amnesia (Lee, 2008, 2010; Lee et al., 2008b; Jarome et al., 2011; Ren et al., 2013). Infusion of a proteasome inhibitor alone during reactivationl has no effect on the original memory (Lee, 2008, 2010; Lee et al., 2008b; Jarome et al., 2011; Ren et al., 2013; but see Artinian et al., 2008 who do report an effect), suggesting that protein degradation is required for destabilization of the reactivated memory, while protein synthesis is required for its reconsolidation (re-stabilization). Furthermore, it implies that reconsolidation cannot occur without first destabilizing the original memory. Protein degradation has been shown to be important for the strengthening of previously acquired contextual fear memories (Lee, 2008). An infusion of β-lac prior to a second training session prevented further learning observed in vehicle-treated animals, indicating that inhibition of protein degradation blocked strengthening, while leaving the original memory intact (Lee, 2008). Similarly, inhibiting protein synthesis blocks memory updating, as β-lac infusion after foot shock prevented the association of this aversive stimulus with a previously neutral context (Lee, 2010).

Preventing the destabilization of a previously acquired memory with a proteasome inhibitor has been reported in invertebrates at both a behavioral and a cellular level (Lee et al., 2012) and in addition to the aversive paradigms discussed above, has also been observed during the recall of appetitive (Ren et al., 2013) and spatial memories (Da Silva et al., 2013) in vertebrates. The conclusion that protein degradation is a crucial step in the destabilization of memories before reconsolidation is supported by the fact that blockade of LVGCCs (known to be upstream activators of the UPS) will also block anisomycin-mediated amnesia at retrieval, while leaving the original memory intact (Suzuki et al., 2008).

Finally, there is emerging evidence that preventing synaptic protein degradation can disrupt extinction learning (Lee et al., 2008b; Mao et al., 2008; Pick et al., 2013; Ren et al., 2013). Proteasome inhibitors impair extinction (Lee et al., 2008b; Ren et al., 2013) and block D-cycloserine-mediated enhancement of extinction (Mao et al., 2008). Moreover, a Cdh1 (a subunit of ubiquitin E3 ligase and a crucial enzyme in the UPS) knock-out mouse exhibits profound extinction deficits (Pick et al., 2013). In summary, the current evidence indicates that protein degradation is required by both reconsolidation and extinction mechanisms, and it has been proposed that degradation is a key component of destabilization. Some authors have gone on further to suggest that the requirement of protein degradation during extinction represents a destabilization of the original fear memory allowing a partial erasure or "unlearning" (Lee et al., 2008b). This explanation seems unlikely, because as was described above, if the original CS-US memory is destabilized during extinction then protein synthesis inhibitors would block reconsolidation of the original memory rather than disrupting extinction. Given that protein degradation does not appear to be a pre-requisite for the consolidation of new fear memories (Lee, 2008, 2010; Lee et al., 2008b; Pick et al., 2013; Ren et al., 2013), it is possible that it is specific to the process of extinction and could represent an interaction with the original memory through a mechanism other than reconsolidation.

EPIGENETIC MECHANISMS AND GENE REGULATION

Dynamic changes in chromatin structure play a vital role in altering gene expression and are necessary for memory formation (Graff and Tsai, 2013). For example, distinct patterns of histone acetylation at the site of the brain-derived neurotrophic factor (BDNF) gene have been associated with both the acquisition of fear and its extinction. (Bredy et al., 2007). Histone modifications also play a role in the reconsolidation of a fear memory, as reconsolidation is associated with an increase in histone 3 (H3) acetylation (Maddox and Schafe, 2011) and blockade of histone deacetylation prevents the reconsolidation of strong memories (Federman et al., 2012). As such, it has been suggested that histone modification and the enzymes that mediate these changes in chromatin state may contribute to memory reconsolidation by destabilizing fear memory (Bredy and Barad, 2008; Maddox et al., 2013a,b). For example, the histone acetyltransferase (HAT) p300/CBP-associated factor (PCAF) has also recently been implicated in memory formation. PCAF knock-out mice exhibit impaired spatial learning and difficulty in adapting to reversal of an operant conditioning task (Maurice et al., 2008). Nuclear expression of PCAF within the IL-PFC is increased following extinction training and there is evidence that PCAF is vital for LTP within this region (Wei et al., 2012). Following administration of the PCAF activator SPV106, a marked reduction in renewal of conditioned fear has been observed (Wei et al., 2012). Thus, this finding supports the role of PCAF in promoting the formation of extinction memory.

MicroRNAs (miRNAs) are a distinct class of small non-coding RNAs, which have important roles in epigenetic regulatory mechanisms. They belong to a family of endogenously expressed small regulatory RNAs that post-transcriptionally regulate gene silencing in plants, invertebrates, and mammals by inhibiting the function of their target mRNAs through complementary binding (Bartel, 2004). A unique feature of these non-coding RNAs is their ability to bind and regulate many genes, and in some cases multiple miRNAs target similar families of genes (Krichevsky et al., 2003; John et al., 2004; Lim et al., 2005; Friedman et al., 2009; Hendrickson et al., 2009), thereby enhancing their ability

to regulate plasticity in the brain. The transient nature of miRNAs, their localized expression in dendrites, their capacity to respond in an activity-dependent manner, and the fact that a single miRNA can simultaneously regulate many genes, make brain-specific miRNAs, together with other non-coding regulatory RNAs, ideal candidates for the fine-tuning of gene expression associated with neural plasticity and memory formation.

Across three different learning paradigms, Nudelman et al. (2010) found that hippocampal expression of miR-132 consistently peaks 45 min after training and returns to baseline within 90 min, perhaps indicating a general role for miR-132 in learning processes such as encoding or the initial phase of memory consolidation. Similarly, we have recently observed that another brain-specific miRNA, miR-128b, is induced within the PFC 2 h after fear extinction training but returns to baseline within 6 h of training (Lin et al., 2011). This transient, learning-induced increase in miRNA expression in the adult brain bears a striking resemblance to the pattern of expression typically reserved for plasticity-related immediate early genes such as c-fos, Arc or zif268, further suggesting a regulatory function for miRNA activity in learning and memory. There are regional and cell type-specific miRNAs that participate in the regulation of gene function in a learning-dependent manner. In the case of miR-128b, our early evidence indicates that this miRNA, expressed within neurons innervated by dopamine in the PFC, may be intimately related to the formation of fear extinction memory. Given that the initial phase of fear extinction learning involves competition with a previously acquired fear memory trace, this transient increase in miR-128b expression may serve to temporarily inhibit the expression of plasticity-related genes associated with retrieval of the original fear, in order to allow the formation of fear extinction memory to proceed.

It has been reported that miR-128b targets a protein called regulator of calmodulin (RCS), which is a competitive inhibitor of the protein phosphatase calcineurin (CaN) (Rakhilin et al., 2004). CaN has been shown to regulate the strength of aversive memory (Baumgartel et al., 2008) and an increase in CaN activity is essential for the formation of extinction memory (Lin et al., 2003; Havekes et al., 2008). Recent evidence also suggests that this protein phosphatase may exert its effect on fear extinction memory by destabilizing the original fear-related memory at the time of retrieval (de la Fuente et al., 2011; Shaw et al., 2012). Thus, given the labile state of the fear memory during extinction and inhibition of the reconsolidation by CaN, the evidence suggests that there is at least some capacity for degradation of the strength of the original fear memory, which can be mediated indirectly by miR-128b through increased CaN activity at the time of retrieval and lead to enhanced extinction (Lin et al., 2011).

OUTLOOK

It is evident that the relationship between reconsolidation and extinction is intimately related to cellular and molecular mechanisms engaged at the time of retrieval. Factors influencing this process include structural modifications to dendrites, the stability of synaptic proteins, membrane-bound receptor signaling, intracellular signal transduction and dynamic changes in chromatin states within the nucleus that are mediated by epigenetic regulatory mechanisms. There is evidence that promotion of reconsolidation or extinction is dependent on the activity of the transcription factor nuclear factor kappa B (NF-κB). Reactivation of fear memories leads to a number of protein phosphorylation cascades, including the ERK-MAPK (extracellular signal-regulated kinase—mitogen-activated protein kinase) pathway (Duvarci et al., 2005) and the IKK (inhibitor kappa B kinase) pathway (Lubin and Sweatt, 2007). Recently, it has been demonstrated that reconsolidation is specifically associated with the activation of the IKK/NF-κB pathway (Lee and Hynds, 2013) and a consequent increase in NF-κB (Merlo et al., 2005), while extinction is associated with an active suppression of NF-κB (Merlo and Romano, 2008). These studies provide a convincing explanation as to how procedurally similar experiences can result in a markedly different outcome.

Since it is presumed that the parameters of the reactivation process, in particular duration or frequency of exposures to the CS are critical in determining whether reconsolidation or extinction will occur (Pedreira and Maldonado, 2003), it seems that the reactivation of a memory describes a process in which initially, protein phosphorylation pathways leads to the levels of transcription factors such as NF-κB to initially rise, thus promoting reconsolidation, but the prolonged presentation of the CS (extinction) will lead to an inhibition of reconsolidation apparatus, thereby promoting extinction. This requirement for transcription factor activity to be altered over the course of the reactivation/extinction session further implies that destabilization is distinct to the process of reactivation and that destabilization may occur at the end of the session, once the reconsolidation or extinction pathway has been determined. This would suggest that reconsolidation and extinction cannot occur at the same time and, therefore, that extinction does not involve a concurrent suppression of the original fear memory through reconsolidation. It does not, however, exclude the possibility that the original memory is being suppressed through another mechanism that does not require protein synthesis.

Future studies should also consider other factors that influencing reactivation, which are likely to be important for memory destabilization and extinction. These include electrical signaling mediated by voltage-gated calcium channels (Suzuki et al., 2008) and gap junctions (Bissiere et al., 2011), and other classes of non-coding RNAs. The expansion of transcriptionally active long non-coding sequences (lncRNA) in the mammalian genome, in particular, appears to have occurred primarily in species with higher-order cognitive function (McLean et al., 2011), and brain-enriched lncRNAs are expressed in both a spatiotemporal- and cell-type-specific manner (Mercer et al., 2008). Conservative annotations estimate that there are at least 9500 independent lncRNA genes, several of which have been implicated in neocortical development (Bond and Fox, 2009), neurogenesis (Ng et al., 2012), and synaptogenesis (Bernard et al., 2010). Importantly, these enigmatic non-coding RNAs function as decoys for transcription-related factors, as modular scaffolds or as guides to direct chromatin-modifying complexes to their genomic sites of action (Rinn and Chang, 2012; Spadaro and Bredy, 2012; Mercer and Mattick, 2013). Thus, given their rapid rate of turnover, brain-specific lncRNAs are uniquely positioned

to mediate rapid genomic responses to external stimuli in a manner distinct from, and more complex than, the much slower acting protein-coding genes (Clark et al., 2012), they are therefore likely to be involved in the rapid cellular and molecular responses required for memory destabilization at the time of reactivation leading to either reconsolidation or extinction of various forms of memory.

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A phosphodiesterase 4-controlled switch between memory extinction and strengthening in the hippocampus

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Established fear-related memories can undergo phenomena such as extinction or reconsolidation when recalled. Extinction probably involves the creation of a new, competing memory trace that decreases fear expression, whereas reconsolidation can mediate memory maintenance, updating, or strengthening. The factors determining whether retrieval will initiate extinction, reconsolidation, or neither of these two processes include training intensity, duration of the retrieval session, and age of the memory. However, previous studies have not shown that the same behavioral protocol can be used to induce either extinction or reconsolidation and strengthening, depending on the pharmacological intervention used. Here we show that, within an experiment that leads to extinction in control rats, memory can be strengthened if rolipram, a selective inhibitor of phosphodiesterase type 4 (PDE4), is administered into the dorsal hippocampus immediately after retrieval. The memory-enhancing effect of rolipram lasted for at least 1 week, was blocked by the protein synthesis inhibitor anisomycin, and did not occur when drug administration was not paired with retrieval. These findings indicate that the behavioral outcome of memory retrieval can be pharmacologically switched from extinction to strengthening. The cAMP/protein kinase A (PKA) signaling pathway might be a crucial mechanism determining the fate of memories after recall.

Keywords: phosphodiesterase 4, rolipram, extinction, reconsolidation, hippocampus, inhibitory avoidance, fear memory

INTRODUCTION

Newly formed memory traces become increasingly resistant to disruption or enhancement by different types of interference, through the process known as *consolidation* (McGaugh, 2000). However, the retrieval of a previously consolidated memory can lead to phenomena such as *extinction*, which is likely based on the formation of a new memory that weakens the expression of the original learning (Bouton and Bolles, 1979; Quirk and Mueller, 2008), and *reconsolidation*, a process involving labilization followed by a new phase of stabilization, that may serve to maintain, update, or strengthen the memory trace (Nader et al., 2000; Sara, 2000a; Alberini, 2011). Extinction and reconsolidation are usually viewed as two opposing and possibly competing processes triggered by retrieval, resulting in long-lasting modifications of the original memory trace, or at least of its behavioral expression.

The factors determining whether extinction, reconsolidation accompanied by strengthening, or neither of these processes will be initiated by retrieval remain poorly understood. Studies have found that manipulations of training intensity, retrieval duration, and age of the memory can be used to guide memory retrieval towards extinction or reconsolidation. For example, the use of longer retrieval sessions led to extinction, while a shorter exposure to the learning environment during retrieval induces labilization and sensitivity to drug interference (Pedreira and Maldonado, 2003; Suzuki et al., 2004; Lee et al., 2006). In addition, retrieval more likely results in reconsolidation-mediated strengthening when the original memory is younger or more robust (Eisenberg et al., 2003; Inda et al., 2011). Thus, some of the behavioral training and testing conditions that allow for the discrimination between extinction and reconsolidation have been characterized.

However, previous studies have not shown whether purely pharmacological, biochemical, or molecular factors can act as switches determining the occurrence of extinction or reconsolidation upon retrieval. Here we investigated the effect of post-retrieval phosphodiesterase type 4 (PDE4) inhibition in the dorsal hippocampus on memory retention. The original aim of this study was to examine the role of PDE4 in extinction, and our initial hypothesis was that rolipram could accelerate extinction of inhibitory avoidance (IA). We chose rolipram as a selective PDE4 inhibitor known to enhance hippocampal long-term potentiation (LTP) and memory in different models (Barad et al., 1998; Tully et al., 2003). Surprisingly, we found that, under experimental conditions in which retrieval normally leads to extinction, this outcome can be switched to memory strengthening by a single intrahippocampal infusion of rolipram. To our knowledge, this finding provides the first evidence that whether retrieval will lead to extinction or strengthening (possibly mediated by reconsolidation) can be influenced by manipulating cell signaling mechanisms in the brain.

METHODS

ANIMALS

Adult male Wistar rats (310–400 g of weight, around 90 days of age at time of surgery) were obtained from the institutional breeding facility (CREAL, ICBS, UFRGS, Porto Alegre, Brazil) and the State Health Science Research Foundation (FEPPS-RS, Porto Alegre, Brazil). Animals were housed five per cage in plastic cages with sawdust bedding, and maintained on a 12 h light/dark cycle at a room temperature of 22 ± 1 C. The rats were allowed *ad libitum* access to standardized pellet food and water. All experiments took place between 9 AM and 6 PM. All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care committee (CEUA-HCPA 05-519).

SURGERY

Animals were implanted under anesthesia with ketamine (75 mg/kg) and xylazine (25 mg/kg) with bilateral 14-mm or 9.0-mm, 23-gauge guide cannulae aimed 1.0 mm above the CA1 area of the dorsal hippocampus, as described in previous studies (Roesler et al., 2006; Jobim et al., 2012). Coordinates antero-posterior, −4.3 mm from bregma; mediolateral, ±3.0 mm from bregma; ventral, −2.0 mm from skull surface) were obtained from the atlas of Paxinos and Watson (2007). Animals were allowed to recover for at least 7 days after surgery.

DRUGS AND INFUSION PROCEDURES

The general procedures for intra-hippocampal infusions were as described in previous reports (Quevedo et al., 1999; Luft et al., 2006; Roesler et al., 2006). At the time of infusion, a 30-gauge infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the CA1 area of the dorsal hippocampus. The animals received, via the infusion cannula, a bilateral 0.8 μl infusion of vehicle (20% dimethylsulfoxide, DMSO, in saline), the PDE4 inhibitor rolipram (7.5 μg /side dissolved in vehicle; Sigma-Aldrich, St. Louis, USA), the protein synthesis inhibitor anisomycin (80.0 μg/side dissolved in vehicle; Sigma-Aldrich, St. Louis, USA), or rolipram combined with anisomycin at the doses described above. Drug doses were chosen on the basis of previous studies (Quevedo et al., 1999; Vianna et al., 2001; Luft et al., 2006; Werenicz et al., 2012). Drug or vehicle was infused over a 30-s period. Solutions were freshly prepared before each experiment.

In different experiments, intra-hippocampal infusions were given immediately after the first retrieval session (which also served as extinction training), 1 h after retrieval (delayed infusion controls), 24 h after training in the absence of retrieval (no retrieval controls), or immediately after training.

INHIBITORY AVOIDANCE

We used the single-trial step-down IA task as an established model of fear memory. In step-down IA training, animals learn to associate a location in the training apparatus (a grid floor) with an aversive stimulus (footshock). The general procedures for IA behavioral training and retention tests have been described in previous reports (Quevedo et al., 1999; Luft et al., 2006; Jobim et al., 2012). The IA apparatus was a 50 × 25 × 25-cm acrylic box (Albarsch, Porto Alegre, Brazil) whose floor consisted of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against the left wall.

On training trials, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with a digital chronometer connected to the box control unit. Immediately after stepping down on the grid, rats received a mild footshock (0.5-mA, 2.0-s) and were removed from the apparatus immediately afterwards. Retention test trials (retrieval sessions also serving as extinction training trials) took place at different time points after training by placing the rats on the platform and recording their latencies to step down. No footshock was presented during retention test trials. In trials in which post-retrieval drug infusions were given, rats that did not step down to the grid floor within 180 s were led by the experimenter to step down on the grid floor for 3 s. Step-down latencies on the retention test trial (maximum 180 s) were used as a measure of IA memory retention. In some of the experiments, rats showing extinction were given a 0.2-mA, 2.0-s reminder footshock at the end of the series of testing sessions (Tronel and Alberini, 2007), followed by an additional retention test 24 h later. It should be mentioned that this is a collaborative experiment in which two identical IA training apparatuses in two different laboratories were used for different experiments.

HISTOLOGY

Twenty-four to 72 h after behavioral testing, a 0.5-μl infusion of a 4% methylene blue solution was given into the dorsal hippocampus. Rats were sacrificed by decapitation 15 min later, and their brains were removed and stored in 10% formalin for at least 72 h. The brains were sectioned and examined for cannulae placement in the hippocampus. The extension of the methylene blue dye was taken as indicative of diffusion of the drugs given to each rat.

Animals included in the final analysis (146 rats) had bilaterally placed cannula in the intended sites. Infusion placements into the dorsal hippocampus, as revealed by the diffusion of methylene blue, was similar to those described in previous reports (Quevedo et al., 1999; Roesler et al., 2006, 2009; Jobim et al., 2012; data not shown).

STATISTICS

Data are shown as mean ± S.E.M. retention test latencies to step-down (s). Comparisons of training and retention test step-down latencies between groups were performed using Kruskal-Wallis analysis of variance followed by Mann-Whitney *U*-tests, two-tailed, when appropriate. Comparisons between behavioral sessions within the same group were made using Friedman tests. Nonparametric tests were chosen because of the ceiling cutoff imposed to retention test latencies (Quevedo et al., 1999; Vianna et al., 2001; Luft et al., 2006; Roesler et al., 2006; Jobim et al., 2012). In all comparisons, $P < 0.05$ was considered to indicate statistical significance.

RESULTS

ADMINISTRATION OF ROLIPRAM INTO THE DORSAL HIPPOCAMPUS AFTER RETRIEVAL SWITCHES MEMORY FROM EXTINCTION TO STRENGTHENING

In the first experiment, we examined the effect of an intrahippocampal administration of rolipram immediately after IA memory retrieval, using a protocol that induces extinction in control rats (Vianna et al., 2001). The experimental design is shown in **Figure 1A**. Rats were trained in IA and underwent a retrieval session (Test 1, which also acted as extinction training) 24 h later. Immediately after retrieval, animals were infused with vehicle ($N = 9$), rolipram ($N = 10$), anisomycin ($N = 9$), or rolipram combined with anisomycin ($N = 10$). Animals were tested again 48 h (Test 2) and 72 h (Test 3) after Test 1. Rats infused with vehicle also received a mild 2.0-s reminder footshock (0.2 mA) immediately upon stepping down on Test 3, and were given an additional test trial 24 h after Test 3 ("Reinstatement"), as a procedure used to confirm that the decrease in latencies across trials was due to extinction (Tronel and Alberini, 2007).

Results are shown in **Figure 1B**. A Kruskal-Wallis analysis of variance showed significant differences among groups in Test 3 ($H = 17.3$, df = 3, $P < 0.01$), but not in any other behavioral session (Training, $H = 2.3$, df = 3, $P = 0.52$; Test 1, $H = 2.60$, df = 3, $P = 0.46$; Test 2, $H = 4.3$, df = 3, $P = 0.23$). Further analysis with Mann-Whitney tests showed that rats given rolipram or anisomycin (*P*s < 0.01), or rolipram combined with anisomycin ($P < 0.05$) had latencies in Test 3 that were significantly higher than those in control rats given vehicle. Rats infused with rolipram alone had higher Test 3 latencies compared to rats given anisomycin or rolipram combined with anisomycin (*P*s < 0.05). In control rats given vehicle, retention test latencies progressively declined across test trials, indicating memory extinction. A Friedman test showed a significant decrease in latencies across test trials ($H = 13.8$, df = 2, $P < 0.01$). Step-down latencies in this group went back to the levels observed in Test 1 in the "Reinstatement" test trial following a reminder shock, consistent with what would be expected for memory extinction. In contrast, rats infused with rolipram showed a progressive *enhancement* of IA retention across test trials (comparison among all three test trials using a Friedman test, $H = 9.5$, df = 2, $P < 0.01$). There were no differences between test trials within the groups treated with either anisomycin or rolipram combined with anisomycin (comparison among all three test trials using a Friedman test, anisomycin, $H = 1.4$, df = 2, $P = 0.49$; rolipram plus anisomycin, $H = 4.2$, df = 2, $P = 0.12$).

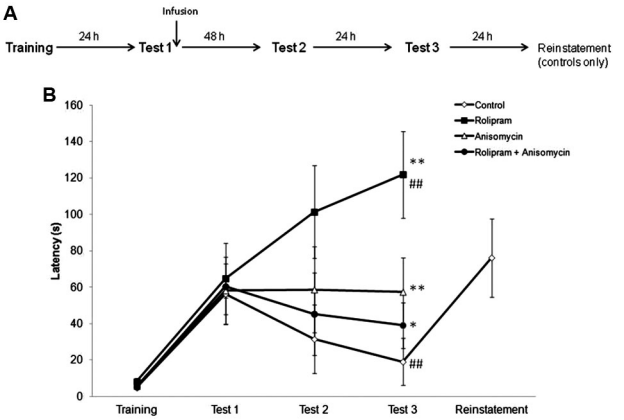

FIGURE 1 | The PDE4 inhibitor rolipram switches memory for IA from extinction to strengthening when given into the hippocampus immediately after retrieval. Rats were given an IA training trial followed 24 h later by a retrieval session (Test 1), which also served as an extinction training trial. Immediately after Test 1, animals were infused into the dorsal hippocampus with vehicle ($N = 9$), rolipram (7.5 μg/side, $N = 10$), anisomycin (80.0 μg/side, $N = 9$), or rolipram combined with anisomycin ($N = 10$). Retention test trials were carried out 48 (Test 2) and 72 h (Test 3) after Test 1. Vehicle-treated rats received a mild reminder footshock (0.2 mA) immediately upon stepping down on Test 3, and were given an additional test trial 24 h later ("Reinstatement"). **(A)** Schematic diagram showing the experimental design. **(B)** Rats given vehicle showed extinction of IA memory, whereas rolipram-treated rats showed memory strengthening, across test trials. The protein synthesis inhibitor anisomycin prevented both extinction and rolipram-induced strengthening; * $P < 0.05$, ** $P < 0.01$ compared to the vehicle group within the same test trial; ## $P < 0.01$ compared to Test 1 within the same group.

These results indicate that (1) in rats trained and tested in a protocol that induces extinction, intrahippocampal rolipram caused memory strengthening rather than extinction to occur after retrieval, and (2) blocking protein synthesis in the dorsal hippocampus prevented both extinction in vehicle-treated rats and the rolipram-induced retention strengthening in animals receiving the drug.

MEMORY ENHANCEMENT BY POST-RETRIEVAL ADMINISTRATION OF ROLIPRAM REQUIRES RECALL

The second experiment was a "no retrieval control" in which we verified whether retrieval was necessary for the memory facilitation induced by rolipram. Rats were infused with vehicle ($N = 13$), rolipram ($N = 12$), anisomycin ($N = 7$), or anisomycin plus rolipram ($N = 9$), 24 h after training in the absence of a retrieval

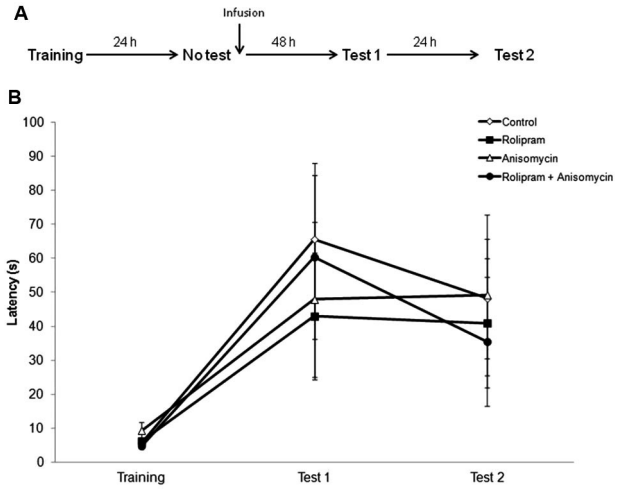

FIGURE 2 | No retrieval control. Rats were trained in IA and 24 h later received an intrahippocampal infusion of vehicle (N = 13), rolipram (7.5 μg/side, N = 12), anisomycin (80.0 μg/side N = 7), or anisomycin plus rolipram (N = 9), in the absence of a retrieval session. Retention test trials were carried out at both 48 (Test 1) and 72 h (Test 2) after infusion. **(A)** Schematic diagram showing the experimental design. **(B)** Latencies to step-down during IA behavioral trials. There were no significant differences among groups.

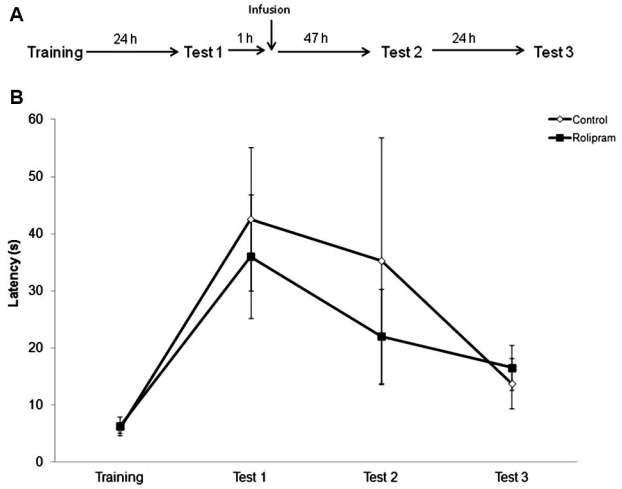

FIGURE 3 | Delayed infusion control. Rats were given an IA training trial followed 24 h later by a retrieval/extinction session (Test 1). 1 h after Test 1, animals were infused into the dorsal hippocampus with vehicle or rolipram (7.5 μg/side, N = 8 rats per group). Retention test trials were carried out 48 (Test 2) and 72 h (Test 3) later. **(A)** Schematic diagram showing the experimental design. **(B)** Latencies to step-down during IA behavioral trials. There were no significant differences among groups.

trial (**Figure 2A**). Rats were tested for retention at both 48 h (Test 1) and 72 h (Test 2) after infusion. There were no significant differences among groups (Kruskal-Wallis test, Training, H = 2.9, df = 3, P = 0.41; Test 1, H = 1.0, df = 3, P = 0.80; Test 2, H = 2.0, df = 3, P = 0.56; **Figure 2B**). These results confirm that the drug infusion needs to be paired with retrieval in order for rolipram to enhance memory.

DELAYED POST-RETRIEVAL ADMINISTRATION OF ROLIPRAM INTO THE HIPPOCAMPUS DOES NOT AFFECT MEMORY

Rolipram had no effect when the intrahippocampal infusion was given 1 h after retrieval measured at Test 1 ("delayed infusion control"), indicating that PDE4 inhibition can modulate memory strengthening specifically at an early time period after retrieval (**Figure 3**). Rats were trained and tested as in the first experiment. Mann-Whitney tests showed no significant differences between groups (Training, P = 0.88; Test 1, P = 0.72; Test 2, P = 0.72; Test 3, P = 0.42; N = 8 rats per group).

THE MEMORY-ENHANCING EFFECT OF INTRAHIPPOCAMPAL ROLIPRAM GIVEN AFTER RETRIEVAL LASTS FOR AT LEAST 1 WEEK

In order to examine the persistence of the memory-enhancing effect of post-retrieval rolipram, rats were trained as before and tested at 24 h (Test 1), 72 h (Test 2), 96 h (Test 3), and 7 days (Test 4) later. Vehicle (N = 10) or rolipram (N = 9) was infused immediately after Test 1 (**Figure 4A**). Mann-Whitney tests showed significant differences between groups in Test 3 ($P < 0.05$) and Test 4 ($P < 0.01$), but not in Training (P = 0.97), Test 1 (P = 0.55), or Test 2 (P = 0.13). Control rats, but not rats given rolipram, showed a significant decrease in latencies across test trials (Friedman test, comparison across tests trials, H = 8.4, df = 3, $P < 0.05$) (**Figure 4B**). The results indicate that the enhancing effect of intrahippocampal rolipram given immediately after retrieval can last for at least 1 week.

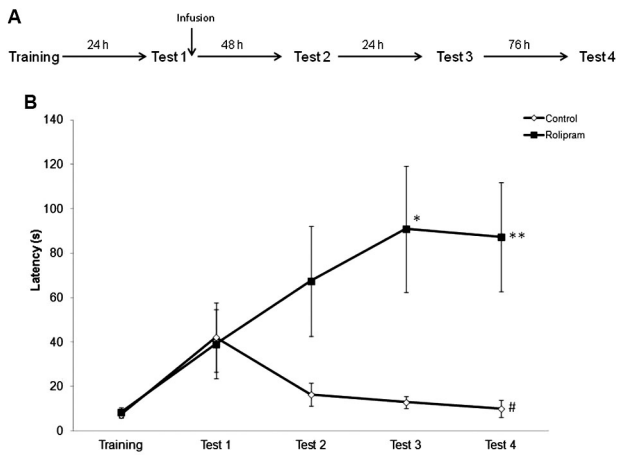

FIGURE 4 | Persistence of the memory enhancement induced by rolipram administration after retrieval. Rats were given an IA training trial followed by retention test trials at 24 h (Test 1), 72 h (Test 2), 96 h (Test 3), and 7 days (Test 4) later. Vehicle (N = 10) or rolipram (7.5 μg/side, N = 9) was infused immediately after Test 1. **(A)** Schematic diagram showing the experimental design. **(B)** Vehicle-treated rats showed extinction of IA memory, whereas rats infused with rolipram showed memory strengthening, across test trials; * $P < 0.05$, ** $P < 0.01$ compared to the vehicle group within the same behavioral trial; # $P < 0.05$ compared to Test 1 within the same group.

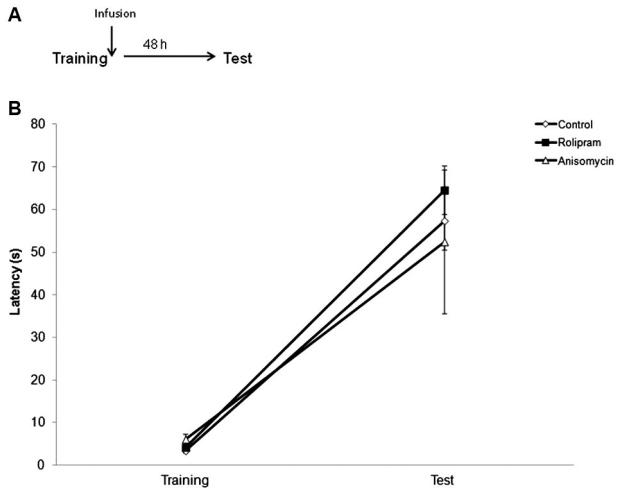

FIGURE 5 | Neither rolipram nor anisomycin affect IA memory formation when infused into the hippocampus immediately after training. Rats were given an IA training trial followed 48 h later by a test trial. An intrahippocampal infusion of vehicle (N = 11), rolipram (7.5 μg/side, N = 12) or anisomycin (80.0 μg/side, N = 9) was given immediately after training. **(A)** Schematic diagram showing the experimental design. **(B)** Latencies to step-down during IA behavioral trials. There were no significant differences among groups.

ADMINISTRATION OF ROLIPRAM INTO THE HIPPOCAMPUS IMMEDIATELY AFTER TRAINING DOES NOT AFFECT MEMORY CONSOLIDATION

In the last experiment, we verified whether rolipram and anisomycin could also affect IA memory consolidation. Vehicle (N = 11), rolipram (N = 12) or anisomycin (N = 9) was infused into the hippocampus immediately after training, and retention was tested 48 h later. Results are shown in **Figure 5**. There were no differences between groups in Training (Kruskal Wallis test, Training, $H = 3.8$, df = 2, $P = 0.15$; Test, $H = 4.2$, df = 2, $P = 0.12$). The result suggests that neither rolipram nor anisomycin affected the consolidation phase of memory when given early after training.

DISCUSSION

Recall of a fear-motivated memory can lead to extinction, which likely involves the creation of a second memory trace that decreases fear expression (Quirk and Mueller, 2008). Alternatively, retrieval can induce the labilization of the original memory, which again becomes sensitive to interference, a process usually referred to as reconsolidation. It has been proposed that reconsolidation can serve to maintain, update, or alter the strength of memories (Sara, 2000a,b; Amaral et al., 2008; Lee, 2008, 2010; Alberini, 2011; Alberini and Ledoux, 2013; de Oliveira Alvares et al., 2013; Reichelt and Lee, 2013).

Several studies have shown that whether a retrieved memory will undergo extinction or reconsolidation depends on the conditions under which the memory is learned and reactivated, factors that are generally manipulated experimentally by altering the training intensity, retrieval session duration, or intervals between behavioral trials (Eisenberg et al., 2003; Pedreira and Maldonado, 2003; Suzuki et al., 2004; Lee et al., 2006; Inda et al., 2011; Flavell and Lee, 2013). Here, we show that, within an experimental condition that promotes extinction in control rats, inhibiting PDE4 in the dorsal hippocampus can alter the fate of the memory towards strengthening. Both extinction and rolipram-induced strengthening depend on protein synthesis, since infusion with anisomycin blocked both processes. Control experiments omitting the first retrieval trial or using delayed and posttraining infusions indicate that the effects were not due to long-lasting drug-induced alterations in locomotion, motivation, anxiety, sensory function, or other nonspecific factors. To our knowledge, this is the first direct demonstration of a pharmacologically-inducible "switch" between memory extinction and reconsolidation.

Although most studies on reconsolidation have focused on the *disruption* of recalled memories by administration of amnestic agents, there is previous evidence that some drug treatments can *enhance* retention when paired with retrieval. Early studies showed that systemic injections of strychnine after retrieval could enhance IA memory in rats (Gordon, 1977). More recently, memories for fear conditioning in rats have been shown to be enhanced by post-retrieval administration of drugs including the protein kinase A (PKA) activator 6-BNZ-cAMP infused into the basolateral amygdala (BLA; Tronson et al., 2006), the partial *N*-methyl-D-aspartate (NMDA) receptor agonist D-cycloserine injected systemically (Lee et al., 2006), or the CB1 cannabinoid receptor antagonist AM251 infused into the dorsal hippocampus (de Oliveira Alvares et al., 2008). However, in all previous studies, the experimental conditions used were such that control rats did not show significant extinction across test trials.

Memory strengthening has been observed after either reinforced (i.e., with additional training) or non-reinforced (retrieval alone in the absence of a reinforcing stimulus) re-exposure to the learning context (Roesler et al., 1998, 2000; Quevedo et al., 1999; Lee, 2008; Roesler and Quevedo, 2009; Inda et al., 2011; Pedroso et al., 2013; Reichelt and Lee, 2013). Since strengthening depends critically on retrieval of the original memory (Roesler and Quevedo, 2009), and requires molecular mechanisms in the hippocampus that specifically underlie reconsolidation (Lee, 2008), is has been proposed that reconsolidation is the mechanism mediating strengthening (Lee, 2008; Alberini and Ledoux, 2013). Memory enhancement by repeated retrieval has been seen as a possible adaptive function of reconsolidation, since it allows relevant fear memories to be strengthened without requiring re-exposure to the original aversive learning experience (Alberini and Ledoux, 2013). It should be noted, however, that our findings do not clearly allow us to exclude the possibility that mechanisms other than reconsolidation mediate memory strengthening. One argument against the possibility of reconsolidation in this case is the fact that the latencies of animals treated with rolipram combined with anisomycin were similar between Test 2 and Test 1, suggesting that anisomycin selectively blocked the rolipram-induced enhancement without affecting the original memory. Thus, rolipram could be inducing a condition in which memory reinforcement occurs without labilization of the original memory (Osan et al., 2011; Pedroso et al., 2013). However, it should be noted that reconsolidation blockade with post-retrieval intrahippocampal anisomycin has not been consistently demonstrated in

the step-down IA task (Vianna et al., 2001). Moreover, a slight trend for decreased latencies across the 3 test trials was observed in rats receiving rolipram and anisomycin, although this did not reach statistical significance. Thus, our data do not exclude the possibility that reconsolidation-like mechanisms are involved in the memory strengthening effect observed.

It has been hypothesized that high levels of attention or arousal during retrieval could reinforce the memory trace through endogenous mechanisms that might involve increased release of modulators such as catecholamines (Sara, 2000b). This possibility is consistent with the studies mentioned above showing that drugs that stimulate modulatory pathways can enhance memory when given shortly after retrieval. The findings reported by Tronson et al. (2006) showing strengthening of fear conditioning memory by a PKA activator after retrieval are particularly relevant for comparison with our present results, since PDE4 inhibitors such as rolipram enhance memory by increasing neuronal levels of cAMP, thus ultimately activating the cAMP/PKA/cAMP response-element binding protein (CREB) pathway (Barad et al., 1998; Bach et al., 1999; Bourtchouladze et al., 2003; Tully et al., 2003; Gong et al., 2004; de Lima et al., 2008). Further support for a crucial role of cAMP/PKA/CREB signaling in promoting memory strengthening upon retrieval has been provided by recent evidence that the experimentally-induced activation of amygdalar neurons expressing elevated CREB was sufficient to induce the recall of an established fear memory and promote a reconsolidation-like reorganization process leading to memory strengthening (Kim et al., 2014). The cAMP/PKA/CREB pathway is a particularly promising candidate mechanism regulating the fate of memories during retrieval, since it is crucially involved in memory formation and mediates the actions of many endogenous modulators of emotional memory, including dopamine and norepinephrine (Abel et al., 1997; Bevilaqua et al., 1997; Bach et al., 1999; Tully et al., 2003; Quevedo et al., 2004; Roesler and Schröder, 2011).

In previous studies using IA, we found that similar retrieval conditions could result in memory extinction (Luft et al., 2006), reconsolidation sensitive to impairment by mTOR inhibition (Jobim et al., 2012), or protein-synthesis dependent, retrieval-induced, memory strentgthening (Pedroso et al., 2013). However, the behavioral and neurochemical factors determining these different outcomes of retrieval remain elusive. According to the "trace dominance" model, the result of a retrieval session/extinction trial involves the sum of multiple and conflicting processes, including a competition between the original excitatory memory trace and a new inhibitory extinction trace, for the control of behavior (Eisenberg et al., 2003). More recent computational work has proposed that network dynamics can lead to strengthening without labilization, reconsolidation or extinction depending on the degree of mismatch between the original memory and the retrieval session (Osan et al., 2011). Nevertheless, the current results indicate that the definition of the dominant process during retrieval can be altered by pharmacological manipulation of the hippocampus.

The present findings indicate that PDE4 inhibition, presumably by enhancing cAMP signaling, can shift the balance between the processes occurring during retrieval, directing a recalled memory in a way that favors strengthening rather than extinction. In this sense, it is interesting to note that, in fear conditioning, some data suggest that hippocampal and prefrontal inputs converge on the amygdala, with the former driving fear expression and reconsolidation and the latter favoring extinction (Herry et al., 2008; Mamiya et al., 2009). It is possible that stimulating neuronal populations responsible for the representation of the fear memory in the hippocampus through manipulation of the AMPc/PKA/CREB cascade could shift this balance in favor of hippocampal inputs driving maintenance and strengthening of the original memory. This hypothesis should be further examined by future experiments.

Although both protein synthesis and PKA activity in the dorsal hippocampus are required for memory formation, we did not find effects of rolipram or anisomycin when infused after learning. However, previous reports have indicated that intra-hippocampal anisomycin can impair IA memory consolidation when given before or 3 h after, but not immediately after training (Quevedo et al., 1999). Also, drugs acting on the PKA pathway have been shown to influence IA memory consolidation only when infused into the hippocampus 3 h posttraining (Bevilaqua et al., 1997). Thus, the reason for the lack of effect of rolipram and anisomycin in this case is likely to be related to temporal factors, and does not imply that IA consolidation is independent from protein synthesis.

In conclusion, we provide evidence suggesting that the behavioral outcome of the recall of an established memory can be pharmacologically switched from extinction towards strengthening through a purely pharmacological intervention, by pairing retrieval with PDE4 inhibition in the dorsal hippocampus. These findings may contribute to our understanding of the factors governing memory modifications induced by recall.

ACKNOWLEDGMENTS

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Memory formation for trace fear conditioning requires ubiquitin-proteasome mediated protein degradation in the prefrontal cortex

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The cellular mechanisms supporting plasticity during memory consolidation have been a subject of considerable interest. *De novo* protein and mRNA synthesis in several brain areas are critical, and more recently protein degradation, mediated by the ubiquitin-proteasome system (UPS), has been shown to be important. Previous work clearly establishes a relationship between protein synthesis and protein degradation in the amygdala, but it is unclear whether cortical mechanisms of memory consolidation are similar to those in the amygdala. Recent work demonstrating a critical role for prefrontal cortex (PFC) in the acquisition and consolidation of fear memory allows us to address this question. Here we use a PFC-dependent fear conditioning protocol to determine whether UPS mediated protein degradation is necessary for memory consolidation in PFC. Groups of rats were trained with auditory delay or trace fear conditioning and sacrificed 60 min after training. PFC tissue was then analyzed to quantify the amount of polyubiquibated protein. Other animals were trained with similar procedures but were infused with either a proteasome inhibitor (clasto-lactacystin β-lactone) or a translation inhibitor (anisomycin) in the PFC immediately after training. Our results show increased UPS-mediated protein degradation in the PFC following trace but not delay fear conditioning. Additionally, post-training proteasome or translation inhibition significantly impaired trace but not delay fear memory when tested the next day. Our results further support the idea that the PFC is critical for trace but not delay fear conditioning and highlight the role of UPS-mediated degradation as critical for synaptic plasticity.

Keywords: memory, ubiquitin, protein degradation, protein synthesis inhibitors, fear conditioning, trace conditioning, prefrontal cortex

INTRODUCTION

Pavlovian fear conditioning has proven to be exceptionally useful in elucidating the molecular mechanisms underlying learning and memory. This procedure involves the association of a conditional stimulus (CS) with an aversive unconditional stimulus (UCS). Through repeated pairings of these two stimuli the CS becomes a predictor of the UCS and the subject will emit a fear response to the CS alone. In the most commonly used version of Pavlovian fear conditioning, "delay" fear conditioning, the UCS normally occurs at CS offset. The acquisition and storage of this association requires the amygdala (Fanselow and LeDoux, 1999; Wilensky et al., 2006; Helmstetter et al., 2008; Pape and Pare, 2010). Several studies have shown that the consolidation of fear memory depends on mRNA transcription and translation of new protein in the amygdala and that inhibiting these processes prevents the formation of a stable fear memory (Bailey et al., 1999; Parsons et al., 2006; Helmstetter et al., 2008; Kwapis et al., 2011). In addition, protein degradation, mediated by the ubiquitin-proteasome system (UPS), is another critical regulatory mechanism in synaptic plasticity required for memory (Hegde et al., 1997; Jarome et al., 2011). Protein degradation triggered by neural activity may be a key factor in making synapses labile, which is crucial for both memory consolidation and "reconsolidation" (Jarome and Helmstetter, 2013).

Proteins are marked for degradation through the covalent attachment of ubiquitin tags. The ubiquitin proteins are attached through the action of an enzymatic pathway consisting of 3 enzymes, termed E1, E2, and E3 (Hershko and Ciechanover, 1998). This pathway is able to add additional ubiquitin molecules to an already substrate-bound ubiquitin at different lysine (K) residues, thus creating a polyubiquitin chain. These chains act as molecular signals for a variety of cellular processes, depending upon the lysine residue at which they are linked (Deng et al., 2000; Ye and Rape, 2009). Polyubiquitin chains linked together at the lysine-48 (K48) residue of ubiquitin are likely to be degradation specific (Hegde, 2010). Proteins tagged with K48 polyubiquitin chains are targeted by the 26S proteasome and subsequently degraded.

Ubiquitin-proteasome mediated protein degradation is critical for memory consolidation and reconsolidation in several forms of learning. For example, an infusion of the proteasome inhibitor, clasto-lactacystin- β-lactone (β-lac) into the CA1 region of hippocampus after retrieval prevents anisomycin-induced memory deficits and extinction of a context fear memory (Lee et al.,

2008). Infusion of a different proteasome inhibitor (lactacystin) into the CA3 region of the hippocampus impairs the consolidation and reconsolidation of a spatial memory (Artinian et al., 2008). Recently, Jarome et al. (2011) showed that the consolidation of fear conditioning requires UPS-mediated protein degradation in the amygdala. Post-training infusions of β-lac into the amygdala of rats immediately following training in delay fear conditioning (DFC) impaired the formation of conditional responses. Thus, UPS-mediated protein degradation may represent a common mechanism supporting synaptic plasticity and memory consolidation in multiple brain areas. As mentioned above, *de novo* protein synthesis is critical for the formation of trace fear memory in the amygdala (Kwapis et al., 2011) but there have been very few studies that have investigated a similar role for protein synthesis in the prefrontal cortex. One study infused the protein synthesis inhibitor anisomycin into the PFC of rats immediately following trace fear training. When tested for fear to the CS 30 days later, it was found that post-training inhibition of protein synthesis impaired memory (Blum et al., 2006). However, since this study only addressed the role of protein synthesis on memory tested remotely, it remains unknown whether protein synthesis in the PFC is necessary for initial consolidation of trace fear memory. Furthermore, no study has investigated the role of UPS-mediated protein degradation in the formation and consolidation of trace fear memory.

Despite a wealth of information regarding the mechanisms underlying delay fear memory, much less is known about those supporting the consolidation of memory for more complex variations of Pavlovian fear conditioning, such as trace fear conditioning. Unlike DFC, the CS and UCS in trace fear conditioning are not temporally contiguous. Instead, they are separated by a brief stimulus free interval during training. Associating the CS and UCS across this trace interval requires structures in addition to the hippocampus and amygdala (McEchron et al., 1998; Esclassan et al., 2009; Gilmartin and Helmstetter, 2010; Czerniawski et al., 2011; Guimarais et al., 2011; Kwapis et al., 2011; Gilmartin et al., 2012). One structure that has gained significant attention in this regard is the prefrontal cortex (PFC). Importantly, the PFC has been shown to be necessary for auditory trace but not DFC. Gilmartin and Helmstetter (2010) demonstrated that inactivation of the prelimbic region of PFC (PL), as well as the blockade of NMDAR-mediated synaptic transmission in PFC, significantly attenuated the acquisition of fear to a trace CS further supporting the importance of the PFC to trace but not DFC. Additionally, the PFC is involved in the long-term storage of trace fear memories suggesting that the PFC is necessary not only for the acquisition of trace fear memory but also for the storage of trace fear memory (Runyan et al., 2004).

Our study focused on the role of UPS-mediated protein degradation and protein synthesis in the PFC following trace vs. DFC in rats. Specifically, we examined if (1) degradation specific polyubiquitin tagging was selectively increased following trace but not DFC and if (2) post-training inhibition of the 26S proteasome or *de novo* protein synthesis in the PFC impaired the consolidation of trace but not delay fear memory.

MATERIALS AND METHODS

ANIMALS AND SURGERY

The experiments used 87 male Long Evans rats (∼300–400 g; Harlan, Madison, WI). The rats were individually housed with *ad libitum* access to food and water. The colony room was maintained under a 14:10-h light/dark cycle, and all behavioral tests were conducted during the light portion of the cycle. All procedures were approved by the Institutional Animal Care and Use Committee (University of Wisconsin-Milwaukee) and were in compliance with the NIH ethical guidelines for the Care and Use of Experimental Animals. All animals were handled for 3 days prior to surgery. On the day of surgery, rats were anesthetized with isoflurane in 100% O_2 (4% induction, 2% maintenance). Stainless steel guide cannulae (26 ga; Plastics One, Inc) were implanted bilaterally into the prelimbic cortex of the mPFC at a 15° angle to vertical (AP +2.9; ML ± 1.6; DV −3.2 from bregma). Coordinates were based on a rat brain atlas (Paxinos and Watson, 2007). Cannulae were secured to the skull with a stainless steel screw, ethyl cyanoacrylate, and acrylic cement. All animals were given a recovery period of at least 7 days before subsequent behavioral training.

BEHAVIORAL PROCEDURES

In all behavioral experiments, rats received immediate post-training bilateral infusions of clasto-Lactacystin β-lactone (βlac; 32 ng/μl; Sigma), anisomycin (ANI; 125 μg/μl; Sigma), or vehicle into the PL mPFC. Both β-lac and ANI were dissolved in 20% DMSO in HCL and diluted in artificial cerebral spinal fluid (aCSF). Control rats were given infusions of 20% DMSO diluted in aCSF. Each infusion was given at a rate of 0.3 μl/min with a total volume of 0.3 μl/side. Concentration of β-lac and ANI as well as total infusion volume were taken from previous work on memory consolidation in the amygdala and PFC (Gilmartin and Helmstetter, 2010; Jarome et al., 2011; Kwapis et al., 2011). The injectors remained in place for an additional 90 s to ensure sufficient diffusion of the drug. After infusion, the obdurators were re-inserted into the cannulae and the animal was returned to its home cage.

All conditioning sessions occurred in a set of four identical Plexiglas and stainless-steel chambers housed inside sound-attenuating boxes. Each outer box was illuminated by a 7.5 watt house light and contained a ventilation fan with a background noise level of 62–64 dB. The floors of the Plexiglas chambers (Context A) were made of evenly spaced stainless steel rods through which the foot-shock was delivered. Additionally, each chamber was cleaned and wiped down with 5% ammonium hydroxide between each set of rats.

After the 7 day recovery period, all animals received 3 days of transport and handling in which they were habituated to the infusion procedure. During transport handling, each rat was lightly restrained in a towel and the infusion pump was activated to habituate the animal to the noise. On the day of training, rats were placed into the conditioning chambers and were given a 6 min baseline period followed by either 4 trials of DFC or 6 trials of trace fear conditioning. These protocols typically result in similar conditional responding to the CS

(Kwapis et al., 2011). Each DFC trial consisted of a 10-s, 72-dB white noise CS and a 1-s, 1-mA foot-shock UCS. Each trial of DFC was separated by an inter-trial interval (ITI) of 110 ± 20 s. Each trace fear conditioning (TFC) trial consisted of the same CS and UCS separated by a stimulus-free 20-s trace interval (ITI 240 ± 20 s). To analyze the percent freezing during training, the training session was divided into 3 distinct phases. The baseline phase represents the first 6 min of training wherein neither stimulus is presented. This is followed by the CS-UCS phase in which the CS-UCS pairings are given. The last 3 min of training represent a post training phase after which the animal was removed from the training context and returned to its homecage. Immediately after the training session, each rat was injected with ANI, β-LAC, or vehicle. In this experiment, there were 6 total groups (TFC β-lac, $n = 11$; TFC ANI, $n = 10$; TFC VEH, $n = 9$; DFC β-lac, $n = 10$; DFC ANI, $n = 7$; DFC VEH, $n = 10$).

Approximately 24 h after training, each rat was tested for fear to the auditory CS in a novel context (Context B). Context B was illuminated with an infrared light and had opaque white floor panels. Before testing each rat, the walls of the context B were wiped with 5% acetic acid solution. After a 1-min baseline, rats were given 8, 30-s presentations of the CS (ITI 60 s). Rats were removed from the chamber immediately following the final CS presentation. To test for context memory, rats were placed back into the training context (Context A) for 12 min with no CS or US presentations. The percent of time spent freezing during the entire period was used as the dependent measure. The CS test and context test were counterbalanced and occurred 4 h apart. Fear to the auditory CS and to the training context were tested a second time, 48 h after training, using the same test procedures.

After testing, animals were overdosed with isoflurane and transcardially perfused with saline followed by 10% buffered formalin. The heads were placed in formalin for 24 h. The brains were then removed from the skull and cryo-protected in 20% sucrose formalin. Each brain was then sectioned through the prelimbic region of the PFC (40 μm). The sections were mounted on slides and stained with cresyl violet. The infusion sites were then verified using a rat brain atlas (Paxinos and Watson, 2007).

The behavior of each rat during training and testing was recorded on digital video. The percent time freezing was determined through frame-by-frame analysis of pixel changes using FreezeScan 2.0 software (Clever Sys, Inc.). The automatic scoring parameters were chosen to match hand-scoring parameters previously used in our laboratory to measure freezing.

WESTERN IMMUNOBLOTTING

For western blot experiments, rats were trained using the same delay ($n = 11$) and trace ($n = 9$) fear conditioning procedures described above but were sacrificed 60 min following the training session. Home cage control (HC; $n = 10$) animals were sacrificed throughout the day. Brains were immediately removed and placed on dry ice and then stored at −80°C until dissected. Prefrontal cortical tissue was dissected out, homogenized in buffer (in 100 ml DDH_2O;.605 g Tris Base, 0.25 g sodium deoxycholate, 0.876 g NaCl,.038 g EDTA, 0.0042 g sodium fluoride, 1 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 ml 10% SDS, 1 mM sodium orthovanadate), and stored at −80°C. The samples were thawed and centrifuged at 4000 rpm for 20 min. A Bradford protein assay kit (BioRad) was then used to measure protein concentration.

Each sample was then loaded into a 7.5% SDS-PAGE gel. The separated proteins were transferred onto PVDF membranes using a Turbo Transfer system (BioRad). Membranes were incubated in blocking buffer for 1 h before being incubated at 4°C overnight in primary antibody for K48 polyubiquitin (Millipore) and actin (Cell Signaling). The next day, the membranes were incubated in secondary antibody (dilution 1:30,000; Upstate Biotechnology anti-rabbit) for 1 h. Membranes were then washed and soaked in a chemiluminescence solution for 5 min (Supersignal West Dura, Thermo).

Images were captured using the G-BOX Chemi XT-4 camera system (Syngene). The mean optical density for each sample was analyzed with GeneSYS analysis software (Syngene). The optical density of K48 polyubiquitination for each sample was normalized to the optical density of the loading control, actin, for each sample. A percentage of home cage control value was then derived for each animal by dividing the percent optical density of K48 relative to actin by the percent K48 optical density relative to actin of the home cage. Values were then analyzed with SPSS, using a One-Way ANOVA and Fisher's least significant difference (LSD) *post-hoc* tests.

RESULTS

We first determined whether UPS-mediated protein degradation was up-regulated in the PFC as a result of training with delay vs. trace fear conditioning (**Figure 1A**). Western blot analysis revealed an increase in degradation specific polyubiquitinated proteins in PFC following trace but not DFC (**Figures 1C,D**). ANOVA revealed a significant main effect of training [$F_{(2, 32)} = 4.124$, $p = 0.008$]. Fisher LSD *post-hoc* analysis showed that the TFC group was significantly different from home cage controls ($p = 0.009$). The TFC group trended toward a significant increase compared with DFC ($p = 0.066$). Importantly, a mixed model ANOVA revealed no effect of training group [$F_{(1, 19)} = 0.408$, $p = 0.531$], a significant effect of phase within the session [$F_{(1, 19)} = 928.684$, $p = 0.001$] and a significant phase by group interaction [$F_{(1, 19)} = 6.860$, $p = 0.017$] on the acquisition of freezing (**Figure 1B**). A subsequent student's *t*-test confirmed a significant difference in post CS-UCS freezing between DFC and TFC animals [$t_{(19)} = 2.746$, $p = 0.013$] in which animals trained with TFC showed less freezing in the post training phase than those trained with DFC.

Next we tested whether the observed increase in degradation after training is necessary for memory consolidation. This experiment also tested whether protein synthesis in PFC is necessary for the consolidation of memory at 24 h. Immediately following trace or delay conditioning rats were injected with inhibitors of protein synthesis or degradation or vehicle (**Figure 2A**). Rats were tested for memory the following day. All animals showed increased freezing as a result of CS-UCS pairings

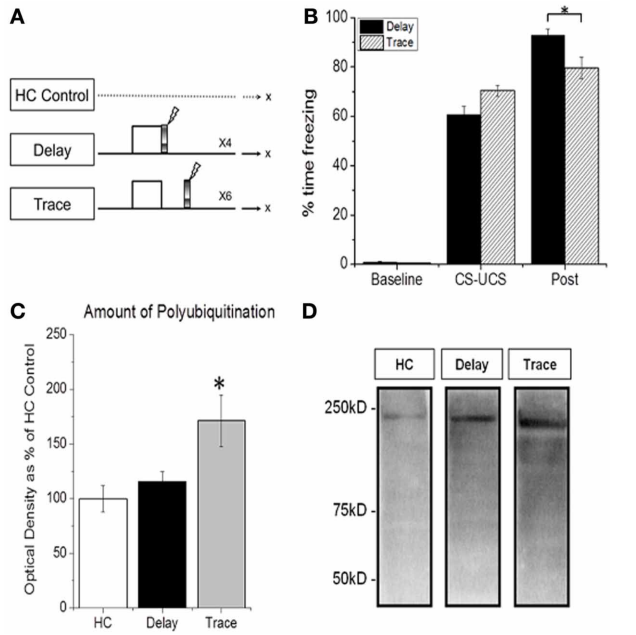

FIGURE 1 | TFC-specific increase in prefrontal protein degradation. (A) Training procedure for **(B–D)**. Animals were trained with delay or trace fear conditioning and PFC tissue was collected 60 min later. Home cage (HC) control animals were not trained and PFC tissue was collected throughout the day. **(B)** All animals show normal acquisition of fear conditioning. **(C,D)** Degradation specific polyubiquitination is increased in PFC 60 min after TFC ($n = 9$) but not DFC ($n = 11$), relative to HC animals ($n = 10$). This further supports a selective role for the PFC in trace learning. *Indicates $p < 0.05$ from HC controls.

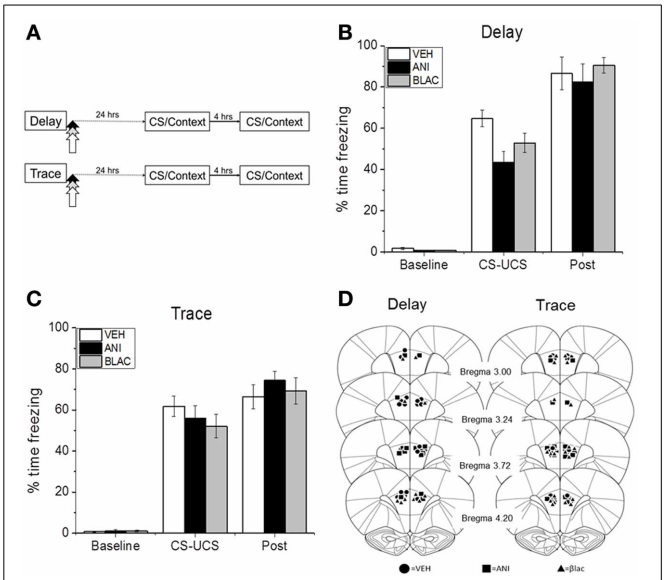

FIGURE 2 | Acquisition of DFC or TFC. (A) Training procedure for **(B–D)**. Animals were trained with DFC or TFC and infused with VEH, ANI, or β-Lac immediately after training. **(B)** Mean (±s.e.m) percent time freezing for DFC or TFC **(C)** animals during the baseline period (baseline), the CS-US pairing period (CS-US), and the post-shock period (post) of initial training. **(D)** Locations of injector tips in PL PFC for each group (adapted with permission from Paxinos and Watson, 2007). *$p < 0.05$.

(**Figures 2B,C**). Although injections occurred after the training sessions, DFC rats assigned to the ANI and BLAC groups exhibited slightly less freezing during the session. A mixed model ANOVA revealed that this decreased freezing was not statistically reliable as there was no effect of group [$F_{(2, 22)} = 1.796$, $p = 0.189$] and a non-significant group by phase interaction [$F_{(2, 22)} = 60.677$, $p = 0.287$]. As expected, there was a significant main effect of phase with all rats freezing more after training compared to pre-shock baseline [$F_{(2, 22)} = 423.907$, $p = 0.001$]. Immediately after training, rats were either infused with β-lac, the protein synthesis inhibitor ANI, or vehicle. **Figure 2D** shows location of injector tip for each animal included in the analysis.

Rats were tested for fear to the auditory CS and training context the next day. Blocking protein degradation or protein synthesis in PFC immediately after trace, but not delay training, impaired memory for the CS. ANOVA revealed a trend for a main effect of Group for animals trained with TFC [$F_{(2, 27)} = 2.785, p = 0.079$] but not with DFC [$F_{(2, 24)} = 1.535, p = 0.236$]. *Post-hoc* analysis using Fisher's Least Significant Difference test revealed no significant difference in CS freezing (**Figure 3A**) between the drug-treated groups trained with TFC ($p = 0.461$) or between the drug-treated groups trained with DFC ($p = 0.968$). Therefore, the drug-treated groups were collapsed and a planned comparison between the collapsed drug groups and the vehicle group revealed a significant reduction in CS freezing for drug-treated animals trained with TFC ($p = 0.032$) but not DFC ($p = 0.094$) compared to VEH animals trained with TFC or DFC, respectively. Prefrontal protein synthesis or degradation was not necessary for contextual fear memory in TFC animals [$F_{(2, 27)} = 0.117$, $p = 0.890$]. Blocking protein synthesis or degradation did impair background contextual fear conditioning in DFC trained animals with a non-significant trend toward reduced contextual fear [$F_{(2, 24)} = 2.773, p = 0.083$]. Again, *post-hoc* analysis revealed no differences in context freezing between DFC animals infused with ANI or β-lac ($p = 0.674$), so the drug-treated groups were collapsed. Planned comparisons revealed significantly lower context freezing in the drug-treated groups compared to the vehicle infused group for animals trained with delay ($p = 0.027$) but not trace ($p = 0.849$) fear conditioning (**Figure 3B**).

DISCUSSION

The present study may be the first to demonstrate the critical involvement of ubiquitin-proteasome mediated protein degradation in the consolidation of a memory that depends specifically on the PFC. We found an increase in degradation specific polyubiquitination in the PFC following trace but not DFC. We further demonstrate a functional role for prefrontal UPS-mediated degradation in the consolidation of memory. Inhibiting the proteolytic activity of the UPS in the PFC immediately after trace fear conditioning impairs auditory CS memory when tested the next day. In addition to protein degradation, *de novo* protein synthesis in PFC is also necessary for memory consolidation.

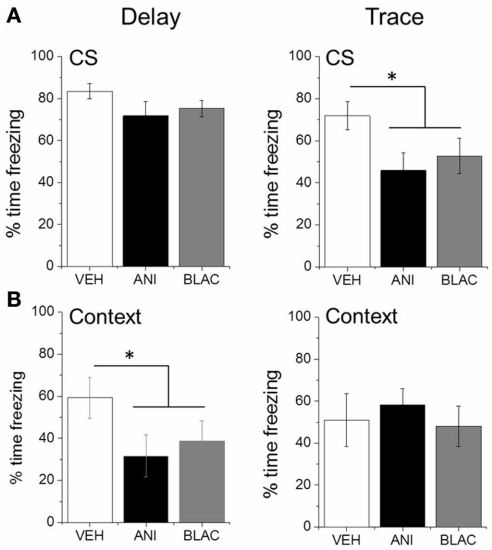

FIGURE 3 | Consolidation of trace fear memory is impaired by proteasome and protein synthesis inhibition. (A) Mean percent time freezing during CS presentations for DFC or TFC trained animals. Animals infused with ANI or β-lac and trained with TFC showed a significant reduction in freezing compared to VEH infused animals. Drug infusion did not significantly affect freezing in animals trained with DFC. **(B)** Mean percent time freezing during context test for DFC or TFC trained animals. Infusions of ANI or β-lac significantly reduced context freezing in DFC animals but not TFC animals. *Indicated $p < 0.05$ from VEH.

Together, our results suggest that both protein degradation via the UPS and *de novo* protein synthesis are critical for the initial consolidation of trace fear memory involving cells in the PFC.

The contribution of UPS-mediated proteolysis to learning and memory is gaining increasing support. In aplysia, the degradation of specific inhibitory proteins results in a facilitation of a signaling cascade involved in transcription and translation, ultimately leading to the consolidation of long-term facilitation (Hegde et al., 1997). In mammals, proteasome inhibition in the CA1 region of hippocampus resulted in a complete impairment in memory for a one-trial inhibitory avoidance task (Lopez-Salon et al., 2001). However, few studies have examined the role of the UPS in auditory fear memory consolidation and no studies thus far have examined its role in the consolidation of auditory trace fear memory. Here, we provide additional support for the PFC as a site of synaptic plasticity in TFC and further augment the role of the UPS in memory consolidation. Our results indicate that protein degradation in the PFC is critically involved in the initial consolidation of auditory trace fear memories.

While our findings further support the critical involvement of UPS mediated proteolysis in memory consolidation, the proteins targeted for activity-dependent degradation by the UPS remain relatively unknown. Jarome et al. (2011) provided some evidence for the learning related degradation of synaptic scaffolding proteins, such as SHANK, as well as a RNA helicase, known as MOV10, in the amygdala following DFC. The activity-dependent degradation of SHANK and MOV10 is believed to contribute to the destabilization of synapses after memory retrieval which is critical for the subsequent synaptic restabilization. The learning induced degradation of functionally disparate proteins illustrates the multi-faceted role of UPS-mediated protein degradation in learning and memory. Now that we have found that UPS-mediated degradation in PFC supports memory consolidation similarly to its role in amygdala and hippocampus, future work can investigate specific proteins being specifically targeted degradation in this brain structure.

Successful memory consolidation may require a balance between protein degradation and synthesis (Jarome and Helmstetter, 2013). We found that both are necessary in the PFC for trace fear conditioning. Blocking protein synthesis with ANI impaired TFC, consistent with previous work. Dash and colleagues showed that bilateral mPFC infusions of the protein synthesis inhibitor, anisomycin, immediately following TFC impaired memory tested at a remote time point 30 days later (Blum et al., 2006). We show that even recent memory requires protein synthesis. This is an important finding given that post-training lesions may not impair TFC (Quinn et al., 2008). Animals whose PFC is lesioned 2 days after training exhibit intact freezing at subsequent testing, suggesting PFC is not a site of permanent storage of TFC memory. It is likely that storage of this memory is distributed, but our results and those of Dash clearly demonstrate that the consolidation of memory requires protein synthesis and degradation following training.

Given previous work from our lab showing that contextual fear in both trace and delay conditioning are similarly affected by manipulation of prefrontal activity (Gilmartin and Helmstetter, 2010), it is somewhat surprising that we saw different patterns of context freezing between trace and delay conditioning in the present study. Our data show a significant effect of proteasome or protein synthesis inhibition on context freezing for animals trained with delay but not trace fear conditioning. However, one possible explanation for this discrepancy is that animals trained in DFCrecieved fewer footshocks (4) in the training context than animals trained in TFC (6).Furthermore, rats were tested for "background" context memory (i.e., the auditory CS was present during training). Together, this could make it difficult to make a conclusion about the impairment in contextual fear memory of DFC animals. Nevertheless, additional studies may be required to resolve this issue.

Protein synthesis is generally accepted as a mechanism of synaptic plasticity that is necessary for fear memory consolidation (Schafe and LeDoux, 2000; Maren et al., 2003; Parsons et al., 2006; Helmstetter et al., 2008). Additionally, UPS- mediated degradation has been shown to occur in parallel with protein synthesis in the amygdala to support memory consolidation following DFC (Jarome et al., 2011). We have demonstrated that both *de novo* protein synthesis and protein degradation in the PFC are necessary and critical to the formation of trace fear memories. The concurrence of these two mechanisms suggests

that they may act in concert and make up a larger regulatory mechanism of synaptic plasticity. There is some evidence that suggests that the UPS plays a role in regulating mechanisms involved in transcription or translation (Ehlers, 2003; Ghosh et al., 2008; Banarjee et al., 2009). Given the involvement of both mechanisms in memory formation, the idea of a reciprocal relationship between protein synthesis and protein degradation will certainly be of great interest in future studies.

While the specific protein-protein interactions may vary based on the learning paradigm, both protein degradation and *de novo* protein synthesis, in several brain structures, are critical for several types of learning. Our findings, taken together with previous work, may suggest the existence of a generalized and perhaps more unified mechanism of plasticity; one in which UPS-mediated proteolysis and protein synthesis function in a reciprocal fashion. Additional research should address the functional relationship between *de novo* protein synthesis and UPS-mediated proteolysis.

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An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD)

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The new-released Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines post-traumatic stress disorder (PTSD) as a "trauma and stressor-related disorder". PTSD pathogenesis relies on paradoxical changes of emotional memory processing induced by the trauma exposure and associated with emotional dysfunction. Several animal models of PTSD have been validated and are currently used. Each one mimics a particular subset of the disorder with particular emphasis, mainly driven by the past classification of PTSD in the DSM-4, on the emotional features. In view of the recent update in the DSM-5, our aim was to develop, by using well-validated paradigms, a modified model of PTSD able to mimic at the same time both the cognitive and emotional features of the disease. We exposed male rats to either a piece of worn cat collar or to a series of inescapable footshocks paired with a PTSD risk factor, i.e., social isolation. Animals were subsequently re-exposed to the conditioned contexts at different time intervals in order to test memory retention for the stressors. In addition, footshock-exposed rats were tested in the elevated-plus-maze and social interaction tests. We found that rats exposed to a cat collar exhibited an acute fear response that did not lead to enduring memory retention. Conversely, footshock-exposed rats expressed a successful retention of the stressful experience at 1, 7, 14, 21 and 56 post-exposure days. Footshock-exposed rats displayed an anxious behavioral profile in the social interaction test and a significantly reduced locomotor activity in the elevated-plus-maze test. These dysfunctions were not observed when animals were socially housed, thus highlighting a social buffering effect in the development of the pathology. Our results underline the good validity of a footshock-based paradigm paired with social isolation as a PTSD animal model, able to mimic at the same time both some of the enduring cognitive and emotional facets of the pathology.

Keywords: memory, predator odor, footshock, animal models of PTSD, social behavior, trauma, stress, rats

INTRODUCTION

Post-Traumatic Stress Disorder (PTSD) is a chronic psychiatric disorder triggered by a traumatic and/or life threatening event. Even if the majority of people experience at least one traumatic event during lifetime, only a subset ultimately develops PTSD (Breslau, 2009). According to the last edition of The Diagnostic and Statistical Manual of Mental Disorders (DSM-5), PTSD is a "trauma and stressor-related disorder" identified by eight diagnostic criteria (American Psychiatric Association, 2013): (a) stressor, i.e., the exposure to an intense source of stress; (b) intrusion symptoms (i.e., re-experiencing the trauma); (c) avoidance of trauma-related stimuli; (d) negative alterations in cognitions and mood; (e) alterations in arousal and reactivity; (f) duration of symptoms for at least 1 month; (g) functional significance (i.e., distress or functional impairments in different domains such as social or occupational); and (h) exclusion (of other possible causes for the symptomatology).

Consensus exists that dysregulation of emotional memory processes is a primary etiopathological factor for PTSD onset (Layton and Krikorian, 2002; Siegmund and Wotjak, 2006; de Quervain et al., 2009; Daskalakis et al., 2013; Parsons and Ressler, 2013). Many are the aberrant memory processes participating to PTSD development. Processes of over consolidation could take place right after any re-experiencing symptom, updating the traumatic memory and prolonging its persistence over time (de Quervain et al., 2009), thus leading to a failure of the extinction processes. This could ultimately account for the patient's inability to update the aversive nature of trauma-related reminders into a "no more harmful" representation (Charney et al., 1993; Milad et al., 2009).

Studying the neural mechanisms involved in the development of PTSD in humans would require prospective studies. Therefore, animal models are of crucial importance to study the neural underpinnings of the pathology and to develop innovative treatments (Pitman et al., 2012).

The alterations in arousal, reactivity, mood and social functioning, accompanied by the cognitive dysfunction, make PTSD a highly complex pathology not easy to model in preclinical research. Although there is no animal model that can capture, at the same time, all the molecular, cellular and behavioral features of the disorder, the development of animal models able to mimic some of the features of the pathology is of great help (Berardi et al., 2012; Daskalakis et al., 2013; Trezza and Campolongo, 2013). In recent years, many rodent models of PTSD have been described (see Berardi et al., 2012; Cohen et al., 2012a; Daskalakis et al., 2013; Goswami et al., 2013; for comprehensive review). All the models are based on the exposure to an acute stressor. Among the stressors frequently used, the exposure to predator threat (Adamec et al., 2004; Zoladz et al., 2008, 2013) or predator odor (Zohar et al., 2008; Mackenzie et al., 2010; Cohen et al., 2012b) has received considerable interest. Inescapable electric shocks represent another frequently used source of stress in PTSD studies (Yamamoto et al., 2009). In addition, the Single Prolonged Stress (SPS) model involves the combined exposure to multiple stressors (i.e., restraint stress, forced swim and exposure to ether) (Liberzon et al., 1997; Yamamoto et al., 2009; Ganon-Elazar and Akirav, 2012; Knox et al., 2012; Eagle et al., 2013). Although these models have good degrees of face and construct validity, they present some limitations with regards to the new additions present in the DSM-5. The use of predator stress is mainly limited by the difficulty in modulating the intensity of the evoked response (Siegmund and Wotjak, 2006). Moreover, the evidence of different behavioral outcomes caused by different stimuli, e.g., different cats (Muñoz-Abellán et al., 2010) or synthetic vs. natural odors (McGregor et al., 2002; Staples and McGregor, 2006; Staples et al., 2008; Hacquemand et al., 2013) are compelling and limits the possibility of standardized and replicable results. On the other hand, the footshock-based models (e.g., contextual fear conditioning paradigms), when not paired to any risk factor for PTSD development (Pitman et al., 1993), only furnish a measure of a physiological cognitive response in terms of memory retention of the emotional event (Siegmund and Wotjak, 2006). The SPS model has the limit to use not a unique source of stress but rather several stressors, thus not perfectly mimicking the common set of trauma experienced by PTSD patients (Yamamoto et al., 2009). Among these stressors, the loss of consciousness, obtained by means of different anesthetic agents (e.g., ether, isoflourane), presents a two-fold problem: (i) anesthetics are known *per se* to differentially influence cognitive processes with the effects depending on the specific drug, the dose, the type of memory, the experimental paradigm, the species and age of the experimental subject, thus making it difficult to have replicable results (Hauer et al., 2011; Hemmings and Mackie, 2011; Wang and Orser, 2011; Berardi et al., 2012; Goswami et al., 2013); and (ii) the use of anesthetics becomes an important confounding factor when evaluating potential new drugs, because of the large possibility of drug-drug interactions impossible to control.

The dissection of the mnestic content of the disease from its emotional consequence may pave the way to the discovery of pharmacological tools acting not only on PTSD symptoms but also on its causes (i.e., cognitive dysfunction). In this context, the ability of a PTSD animal model to evaluate at the same time both the cognitive and emotional aspects of the disease, by means of a combination of different already validated paradigms, has been proposed as a particularly attractive perspective (Siegmund and Wotjak, 2006; Berardi et al., 2012). Hence the objective of the present work was to develop an animal model useful to mimic and consequently study some of cognitive and emotional dysfunctions of PTSD at the same time in the same animal. To this aim we exposed male rats to a source of acute stress. In a first series of experiments we exposed animals to a cat collar. In a second set of experiments we exposed rats to a single exposure of multiple brief inescapable footshocks and we paired this stressor with a risk factor for PTSD development such as social isolation. More importantly, since PTSD is a chronic and persistent disorder, our second goal was to induce in the stress-exposed animals a form of measurable fear memory and emotional dysfunction persisting at very long retention intervals extending far beyond the standard timings usually considered.

MATERIALS AND METHODS

SUBJECTS

Adult male Sprague-Dawley rats (weighting 350–450 g and aging 2 months at the time of testing; Charles River Laboratories, Calco, Italy) were kept in an air-conditioned controlled colony room (temperature: 21° ± 1°C; lights on from 7:00 a.m to 7:00 p.m.) with food and water available *ab libitum*. All the experiments were run during the light phase of the cycle. Rats were handled for 1 min each once a day for 7 consecutive days before behavioral testing. All procedures were performed in compliance with guidelines from the Italian Ministry of Health (law D.L. 116/92) and the European Communities Council Directive (2010/63/EU).

BEHAVIORAL PROCEDURES

All the experimental sessions were video-recorded and subsequently scored by two experts and well trained researchers blind to the experimental conditions. For a graphical representation of the two models, see **Figure 1**.

Cat odor exposure model

All behavioral procedures took place in a sound-attenuated room with dim illumination (∼10 lux inside the test arena). The test arena consisted in a quadrangular box (40 × 40 × 60 cm, l × w × h) made of transparent Plexiglas. The floor was covered with 4 cm of clean sawdust. After each session, fecal boli were removed, sawdust was blended, and the arena's walls were cleaned with a 70% pure ethanol solution.

Housing. Rats were housed in groups of three per cage and were isolated from 3 days prior the habituation session until the end of the behavioral testing.

Habituation. Rats were individually taken from the home-cage and habituated for 20 min to the test arena in order to reduce

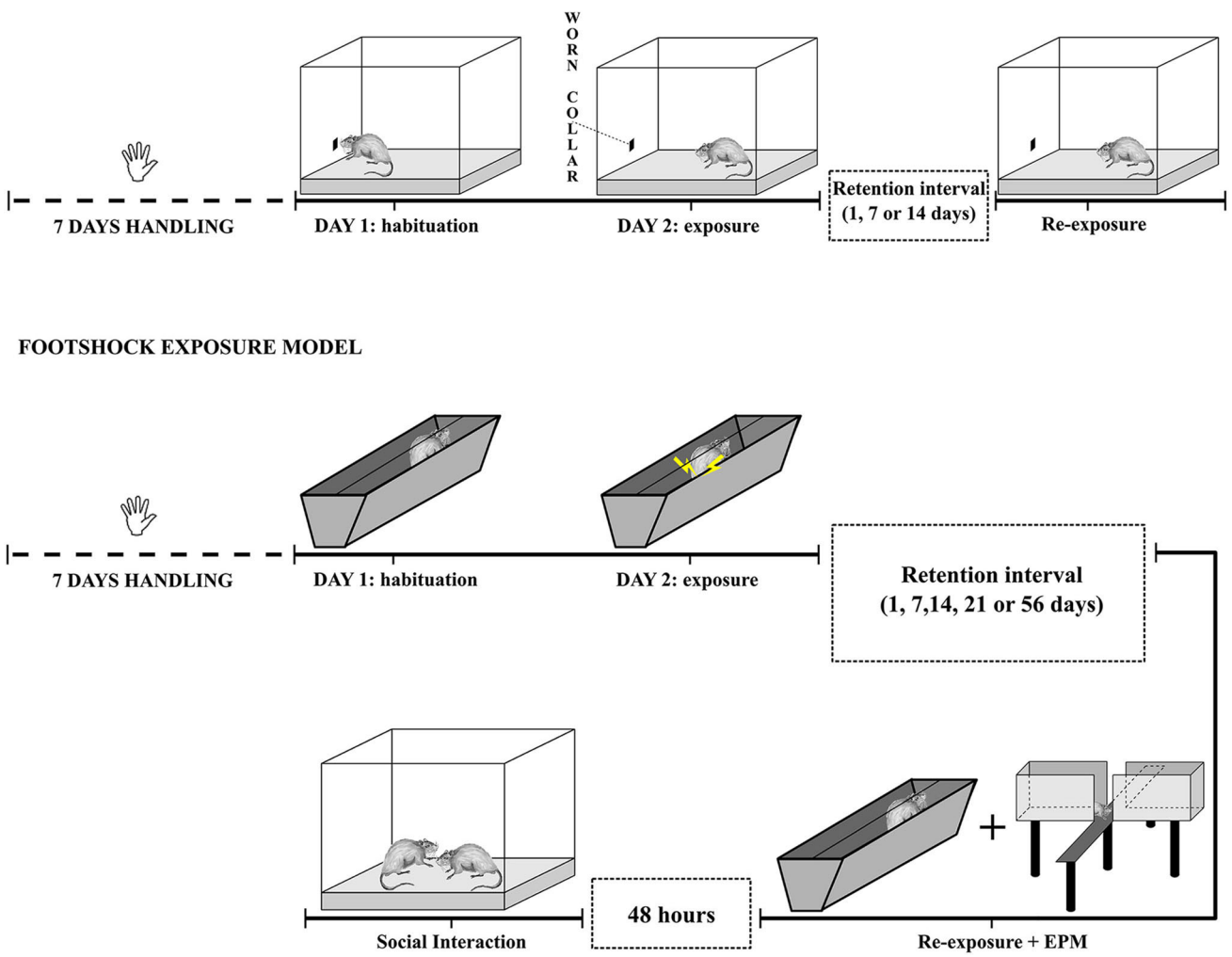

FIGURE 1 | Schematic representation of the cat odor and footshock exposure PTSD models.

novelty-induced stress. A small piece (2.5 cm) of unworn neoprene cat collar was attached with paper tape at the center of one of the arena's walls, approximately 4 cm above the sawdust level. At the end of the 20 min rats were returned to their home cage. The choice of the aversive stimuli was based on previous works (Dielenberg et al., 2001).

Exposure session. The day after, half of the rats were randomly assigned to the exposed group (EXP) while the other half were assigned to the unexposed control group (UNEXP). UNEXP rats received another 20 min session in the arena with an unworn cat collar, as in the habituation session. EXP rats received a 20 min session in the arena but with a piece of cat collar that was worn by a cat for 30 consecutive days. Worn cat collars were kept in polyethylene zipped bags at −20°C and put at room temperature 2 h prior the exposure session. To avoid any possible contamination of cat odor to UNEXP rats, different but identical arenas for the two conditions were used. Moreover, experimenters always changed their gloves after the placement of the worn collar into the arena.

Re-Exposure session. One, 7 or 14 days after the exposure rats from both EXP or UNEXP groups received another 20 min session in the arena with an unworn collar in it, as in the habituation session, in order to be tested for successful memory retention of the stressful experience.

Behavioral measures taken into account during both the exposure and the re-exposure sessions were: (i) percentage of freezing time; and (ii) crossing, wall rearing and rearing frequencies. Freezing was defined as the complete lack of movement except for those necessary for respiration (Fanselow, 1982) and was measured during the re-exposure session as a measure of memory retention. For crossing measurement, a grid drawn on a transparent sheet and dividing the arena into 16 identical sectors was

superimposed to the monitor during data collection, and a single crossing was defined as the rat passing from a square to another with all the four paws. Rearing was defined as the rat standing on the hind legs and wall rearing was defined as the rat standing on the hind legs and with the forepaws touching the inner side of the arena's walls.

Footshock exposure model

The footshock exposure procedure was conducted in a metal trough-shaped box 60 cm long, 15 cm deep, 20 cm wide at the top and 6.4 cm wide at the bottom made of two metal plates connected to an animal shocker. The apparatus was placed into a dimly illuminated and sound-attenuated room. After each session, fecal boli were removed and the apparatus was cleaned with a 70% pure ethanol solution. Illumination was provided by a 25W white light bulb to one corner of the room (∼0.40 lux inside the apparatus).

Housing. To evaluate whether social isolation could be a detrimental factor in the development of PTSD symptoms, rats were housed in two different conditions (isolation or social housing) and different cohorts of both conditions were tested in each experiment. Rats from the isolation condition were isolated 3 days prior the habituation session until the end of the behavioral testing. Rats from the social housing condition were always housed in groups of three and were isolated 24 h before the social interaction test in order to increase their motivation to interact.

Habituation. On the first day of testing, rats were individually taken from the home-cage and habituated for 5 min to the test apparatus. At the end of the 5 min, rats were returned to their home cage.

Exposure session. The day after, rats were divided in two different groups: exposed (EXP) and unexposed control group (UNEXP). The footshock exposure procedure consisted in a slightly modified version of the one used by Chen et al. (2012). EXP rats were individually placed in the apparatus and were left undisturbed for 2 min. After that, five footshocks (2 s, 0.8 mA) were randomly delivered with the last always given at the end of the fifth minute. Inter-shock intervals were randomized by a scrambler and were used in order to avoid any form of temporal conditioning. After the last shock, rats were kept in the apparatus for 60 additional seconds to facilitate the context association to the aversive stimuli. UNEXP rats received the same behavioral procedure except that no shock was delivered.

Re-Exposure session. Separate cohorts of rats of both the EXP and UNEXP groups were re-exposed to the apparatus 1, 7, 14, 21 or 56 days after the exposure session for the isolation condition, and 1, 7 and 14 days after the exposure session for the social housing condition (longer time points for the social housing conditions were not taken into consideration since no alterations were appreciated 14 days after the exposure). Memory retention was evaluated over a 10-min period by analyzing contextual freezing behavior (Chen et al., 2012).

To evaluate the level of emotional distress, both the EXP and UNEXP rats were tested in two well validated animal models used to assess emotionality in rats: the elevated plus maze (EPM) test, immediately after the re-exposure session, and the social interaction (SI) test 48 h after the re-exposure session.

ELEVATED PLUS MAZE TEST

The EPM was performed following the procedure used by Bortolato et al. (2006) and Trezza et al. (2008). The EPM comprised a central platform 10 × 10 cm, two opposed open arms 50 × 10 cm and two opposed closed arms 50 × 10 × 40 cm. The floor of the maze was made of black Plexiglas while the walls were made of transparent Plexiglas and was elevated 60 cm above the floor level. The EPM was conducted under red light illumination (∼5.5 lux on the apparatus). Immediately after the 10 min re-exposure session each rat was taken from the apparatus and placed in the central platform of an EPM facing one of the enclosed arms. The EPM session lasted 5 min after which the rat was returned to the home cage. After each session, fecal boli were removed, and the maze was cleaned with a 70% pure ethanol solution. The measured behavioral parameters were the percent of time spent in the open arms, the percentage of entries in the open arms, the number of entries in the closed arms, the number of stretched attend postures (SAP), i.e., when the rat stretches forward and retracts back afterward, and the number of head dips.

SOCIAL INTERACTION TEST

The SI test was performed following the procedure used by Segatto et al. (2014). Couples for the SI test were decided according to the following criteria: (1) belonging to the same experimental condition; (2) unfamiliarity, i.e., the two rats of each pair were not housed in the same cage; and (3) least weight difference. Each couple was put for 10 min in a quadrangular arena (40 × 40 × 60 cm; l × w × h) made of transparent Plexiglas with 4 cm of clean sawdust covering the floor, under red lights conditions (∼10 lux). After each session, rats were returned to their home-cage, fecal boli were removed, sawdust was blended, and the arena's walls were cleaned with a 70% pure ethanol solution. In this test, non-social behaviors i.e., wall rearing, rearing and crossing behaviors were scored as previously described and social behaviors were described as follows. Following was defined as one rats following the direction of the other, sniffing was defined as one rat sniffing the other in any part of the body, pouncing was defined as one rat nosing or rubbing the nape of the neck of the other, pinning was defined as the rotation of one rat to its dorsal surface after receiving a pounce from the other, boxing was defined as both rats standing on the hind legs in front of each other moving the forepaws, crawling over was defined as one rat passing over the back of the other. The social interaction time was obtained by summing together all the discrete durations of each social behavior.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS statistical software. Each measure is expressed as mean ± SEM. For each behavioral measure Student's *t*-test between UNEXP and EXP groups

was performed in cat exposure sessions. In all the other cases, two-way ANOVAs with condition and time interval as between-subjects factors were used and Tukey-Kramer *post-hoc* test was performed to control for significant differences between groups. Significance was considered for $p < 0.05$.

RESULTS

THE CAT COLLAR EXPOSURE INDUCES AN ACUTE FEAR RESPONSE ACCOMPANIED BY A RAPIDLY DECAYING CONTEXTUAL FEAR MEMORY

In this experiment we aimed to investigate the rats' memory retention for the stressful experience represented by the exposure to a worn cat collar at different time intervals. We found that rats exposed to worn cat collars displayed an acute intense and robust fear response during the exposure, as highlighted by longer freezing time in comparison to rats exposed to unworn piece of cat collars ($t_{22} = -2.169$, $P = 0.041$; **Figure 2A**). During the exposure session EXP rats, compared to UNEXP controls, showed a reduced motor activity in term of number of crossings ($t_{22} = 4.669$, $P < 0.001$; **Figure 2B**), rearings ($t_{22} = 3.386$, $P = 0.003$, mean EXP = 20.17 ± 3.65; mean UNEXP = 43.08 ± 5.70) and wall rearings ($t_{22} = 4.010$, $P = 0.001$, mean EXP = 30.50 ± 3.64; mean UNEXP = 57.83 ± 5.76). In the re-exposure sessions, the two-way ANOVA for freezing time with exposure condition and time intervals as between factors, revealed a significant main effect of exposure condition ($F_{1,44} = 9.593$, $P = 0.003$), a significant main effect of time intervals ($F_{2,44} = 5.566$, $P = 0.007$) and a significant condition × interval interaction ($F_{2,44} = 4.450$, $P = 0.017$). Two-way ANOVA for crossing, rearing and wall rearing frequencies showed no significant main effect of condition, a significant main effect of time intervals ($F_{2,44} = 7.097$, 8.515, 6.741; $P = 0.002$, 0.001, 0.003, respectively) and no significant condition × interval interaction. *Post-hoc* analysis indicated that rats re-exposed to the cat odor-paired context made significantly more freezing ($P < 0.05$; **Figure 2A**); less crossings ($P < 0.05$; **Figure 2B**), and less rearings (mean EXP = 13.25 ± 5.45; UNEXP = 26.00 ± 5.96, not shown in figure) when compared to their unexposed controls 1 day after the exposure. However, no such differences between EXP and UNEXP groups were detectable either 7 or 14 days after the exposure (freezing **Figure 2A**, crossings **Figure 2B**, rearings not shown in figure). The weak mnestic performance of exposed rats cannot be attributable to latent inhibition caused by prior habituation to the arena and collar stimuli. Indeed, the replication of the 7 days experiment without the habituation session, did not alter behavioral profile of exposed rats (data not shown). Taken together, these results demonstrated the inability of the predator stress model, at least in the experimental conditions used in the present work, to induce a long-lasting form of memory. Therefore, in accordance with the 3R principles of the European law for animal research, to reduce the number of animals used and their distress, rats socially housed were not tested and the emotional parameters were not evaluated.

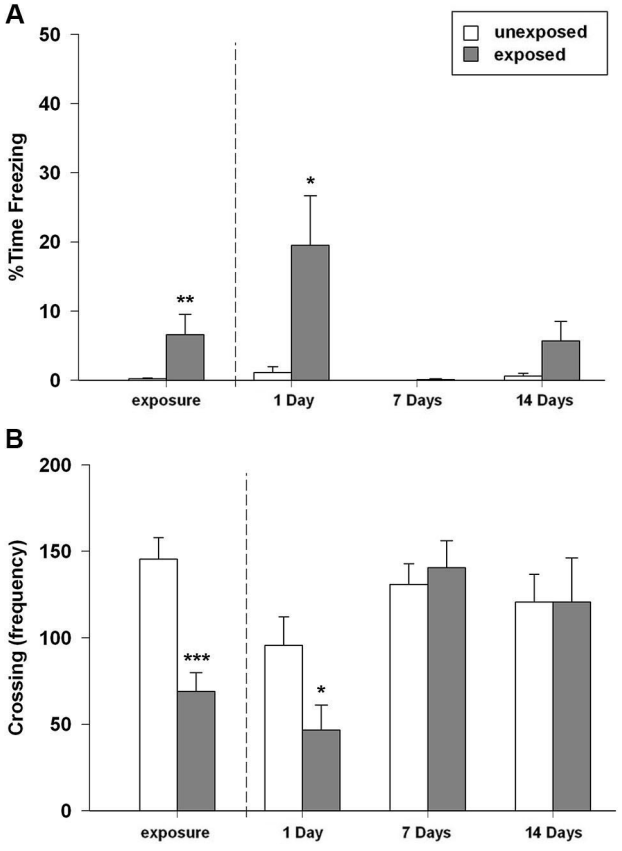

FIGURE 2 | (A) Percentage of time spent in freezing and **(B)** number of crossings of rats exposed to an unworn (unexposed) or to a worn (exposed) cat collar during the exposure session (left plot) (EXP, UNEXP: $n = 12$) and re-exposure sessions performed 1, 7, or 14 days after the exposure to the stressor (right plots) (1D EXP $n = 8$, UNEXP $n = 7$; 7D EXP, UNEXP $n = 9$; 14D EXP $n = 9$, UNEXP $n = 8$). Data are expressed as mean + SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; EXP vs. UNEXP).

THE EXPOSURE TO MULTIPLE INESCAPABLE FOOTSHOCKS, PAIRED WITH SOCIAL ISOLATION, INDUCES A LONG-LASTING CONTEXTUAL FEAR MEMORY AND EMOTIONAL DYSFUNCTION. SOCIAL HOUSING REDUCES THE ADVERSE CONSEQUENCES OF TRAUMA EXPOSURE ON EMOTIONAL DYSFUNCTION

In this experiment, we aimed to investigate memory retention for the stressful experience represented by the exposure to a series of five consequent footshocks. Freezing has been evaluated as a measure of memory retention.

In isolated animals, two-way ANOVA for freezing revealed a significant main effect of exposure condition ($F_{1,89} = 158.548$, $P < 0.001$), of re-exposure time intervals ($F_{4,89} = 6.773$, $P < 0.001$), and condition × intervals ($F_{4,86} = 7.092$, $P < 0.001$). *Post-hoc* analysis showed that EXP rats spent significantly longer time in freezing at any re-exposure interval than the UNEXP control group did ($P < 0.01$ for all the tested intervals; **Figure 3A**).

With regard to group-housed rats, ANOVA revealed a significant effect of exposure condition ($F_{1,47} = 91.449$, $P < 0.001$), time intervals ($F_{2,47} = 3.493$, $P = 0.039$), and condition × interval interaction ($F_{2,47} = 4.475$, $P = 0.017$). *Post-hoc* analysis revealed that EXP rats in the re-exposure session expressed significantly higher freezing rates than UNEXP animals ($P < 0.01$ for 1 and 14 days after exposure, $P < 0.05$ for 7 days after exposure; **Figure 3B**).

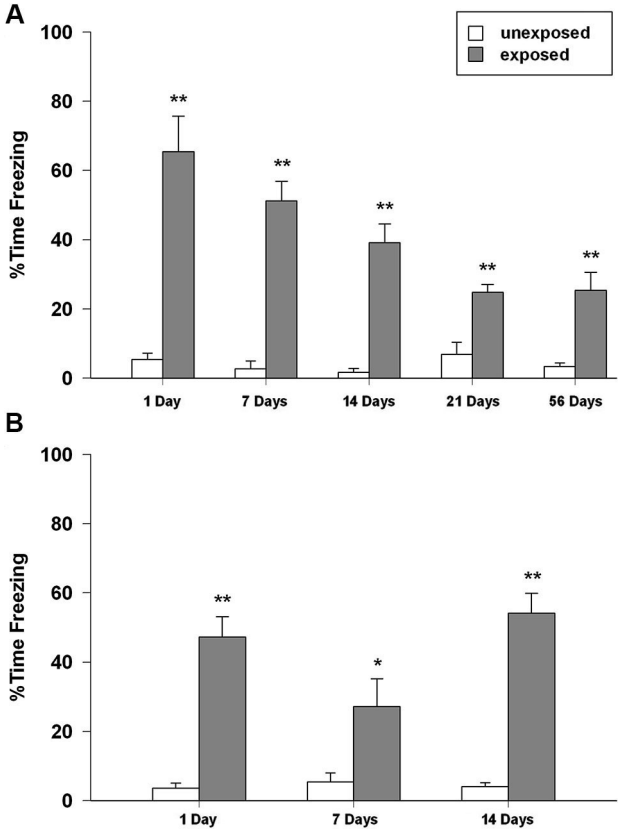

FIGURE 3 | Percentage of time spent in freezing of rats exposed to the context and not shocked (unexposed) or exposed to the context and given five inescapable footshocks (exposed) during the re-exposure sessions performed 1, 7, 14, 21 and 54 days after the exposure (n = 8–11). **(A)** Isolated, **(B)** social-housed animals. Data are expressed as mean + SEM (* $p < 0.05$; ** $p < 0.01$; EXP vs. UNEXP).

EMOTIONAL BEHAVIOR

We investigated the emotional profile of the footshock-exposed animals in the EPM and SI test, in order to assess whether the long-lasting memory retention was accompanied by any enduring change in emotional reactivity.

ELEVATED PLUS MAZE

Results from the EPM failed to demonstrate a classical anxious profile in the EXP animals at all tested intervals and for both housing conditions. Interestingly, socially isolated EXP rats always made significantly less entries into the closed arms, an index of locomotory activity. Similar results were obtained in isolated rats that underwent EPM procedure 7 days after footshock exposure but without being re-exposed to the footshock-paired context (data not shown). This indicates that the EPM performance was not altered by prior re-exposure to the footshock-paired context.

Isolated rats

ANOVA for percent time in the open arms revealed no significant main effect of exposure condition, a significant main effect of time intervals ($F_{4,89} = 2.775$, $P = 0.032$) and no significant condition × interval interaction (**Figure 4A**). With regard to the percentage of entries in the open arms, ANOVA revealed no significant effects of exposure condition, a main effect of time intervals ($F_{4,89} = 2.433$, $P = 0.053$) and no significant condition × interval interaction (**Figure 4B**). Regarding the number of entries in the closed arms, significant main effects of exposure condition ($F_{1,89} = 60.185$, $P < 0.001$) and time intervals were found ($F_{4,89} = 3.646$, $P = 0.008$) but no significant condition × interval interaction was observed. Tukey *post-hoc* tests showed that EXP rats made significantly less entries in the closed arms of the maze compared to unexposed control rats for all the tested intervals (1, 7, 14, 54 post-exposure days: $P < 0.01$; 21 post-exposure days interval $P < 0.05$; **Figure 4C**). With regard to the number of head dips, ANOVA showed a significant main effect of exposure condition ($F_{1,89} = 7.429$, $P = 0.008$), of time time intervals ($F_{4,89} = 4.615$, $P = 0.002$) but no significant condition × interval interaction. The ANOVA for SAP frequency revealed a trend toward significance for exposure condition ($F_{1,89} = 3.737$, $P = 0.056$), a significant main effect of time intervals ($F_{4,89} = 2.600$, $P = 0.041$) and no significant condition × interval interaction.

Social-housed rats

One-way ANOVAs for the percent time spent in the open arms of the EPM revealed no significant main effect of exposure condition or time intervals but a significant condition × interval interaction ($F_{2,47} = 3.229$, $P = 0.049$). *Post-hoc* analysis did not reveal any difference between EXP and UNEXP rats (**Figure 4D**). In the percentage of open arm entries, the ANOVA revealed no significant effects of condition, a significant main effect of time intervals ($F_{2,47} = 3.265$, $P = 0.047$) and a trend toward significance for condition × interval interaction ($F_{2,47} = 3.026$, $P = 0.058$). Tukey *post-hoc* tests showed that at 14 post-exposure days, EXP rats made a higher percentage of entries in the open arms than unexposed controls ($P < 0.05$; **Figure 4E**). ANOVA for the number of closed arm entries showed a significant main effect of condition ($F_{1,47} = 4.435$, $P = 0.041$) a significant main effect of time intervals ($F_{2,47} = 9.345$, $P < 0.001$) and no significant condition × interval interaction. *Post-hoc* analysis showed that at 14 post-exposure days, EXP rats made significantly less entries in the closed arms of the EPM than unexposed control rats ($P < 0.05$; **Figure 4F**). For frequencies of head dips ANOVA showed no significant main effects of exposure condition, time intervals or condition × interval interaction. For numbers of SAPs ANOVA showed no significant main effect for exposure condition a significant main effect for time intervals ($F_{2,47} = 11.889$, $P < 0.001$) and no significant condition × interval interaction.

SOCIAL INTERACTION TEST

Isolated rats

Stress-exposed animals displayed an enduring alteration in social behavior.

Rats previously exposed to the footshock spent less time interacting with the social partner than unexposed controls did. With regard to the total time of social interaction ANOVA revealed a significant main effect of exposure condition ($F_{1,89} = 63.501$,

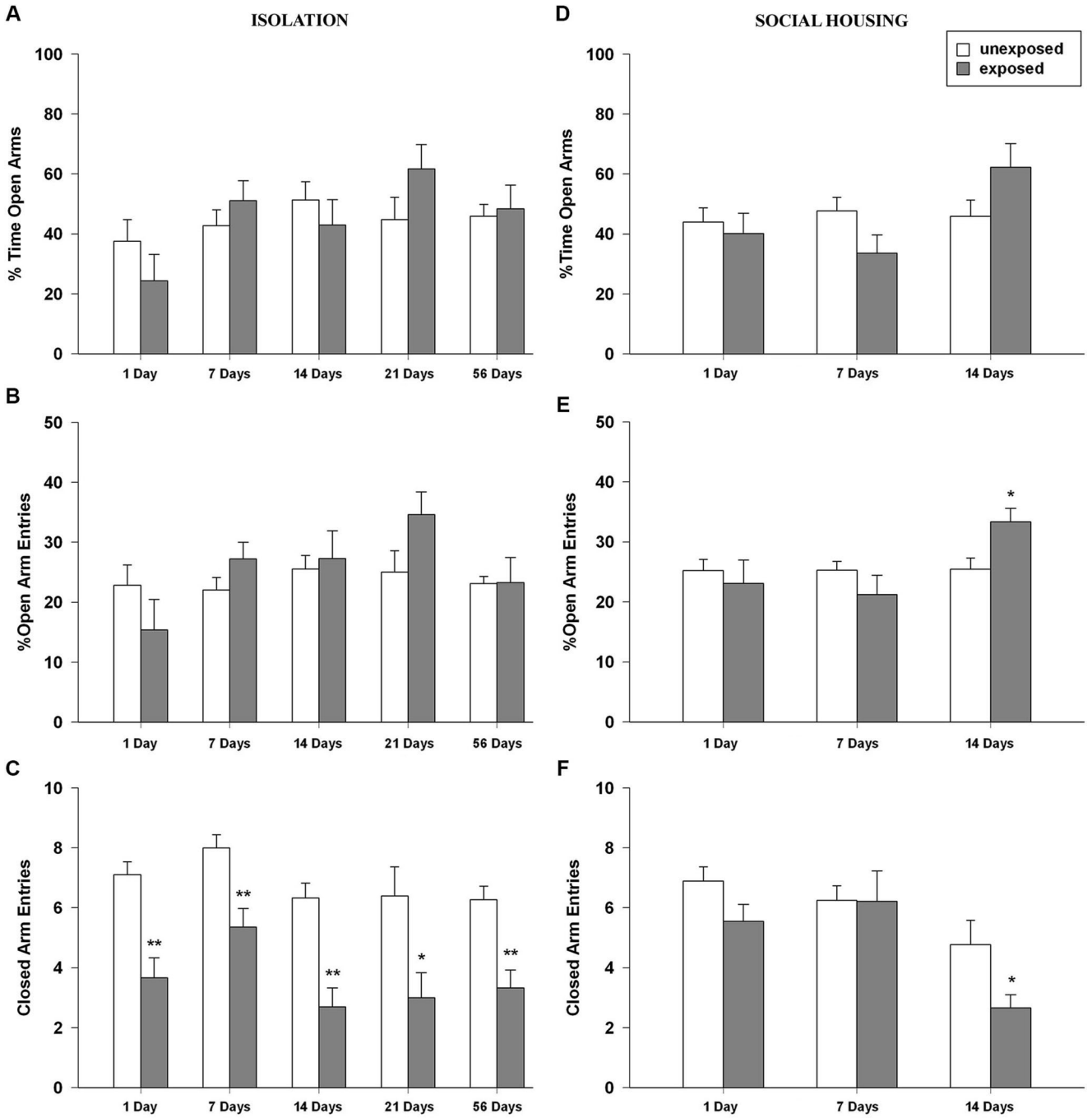

FIGURE 4 | (A,D) Percentage of time in the open arms, **(B,E)** percentage of open arm entries and **(C,F)** closed arms entries of rats unexposed or exposed to the footshock in the EPM 1, 7, 14, 21 days after exposure to the stressor (n = 8–11). Data are expressed as mean + SEM (* $p < 0.05$; ** $p < 0.01$).

$P < 0.001$) and time intervals ($F_{4,89} = 8.090$, $P < 0.001$), but no significance for the condition × interval. *Post-hoc* comparisons showed that exposed animals interacted less time with the social partner than unexposed animals did at all the tested intervals ($P < 0.01$ from 1 to 21 post-exposure days; $P < 0.05$ for 54 post-exposure days; **Figure 5A**). The ANOVA for the number of crossings revealed no significant effect of exposure condition, a significant effect of time intervals ($F_{4,89} = 5.098$, $P = 0.001$) and a significant condition × interval interaction ($F_{4,89} = 3.659$, $P = 0.008$). Tukey *post-hoc* tests showed that rat re-exposed to the footshock-paired context 1 day after the exposure made significantly more crossings than the respective unexposed control

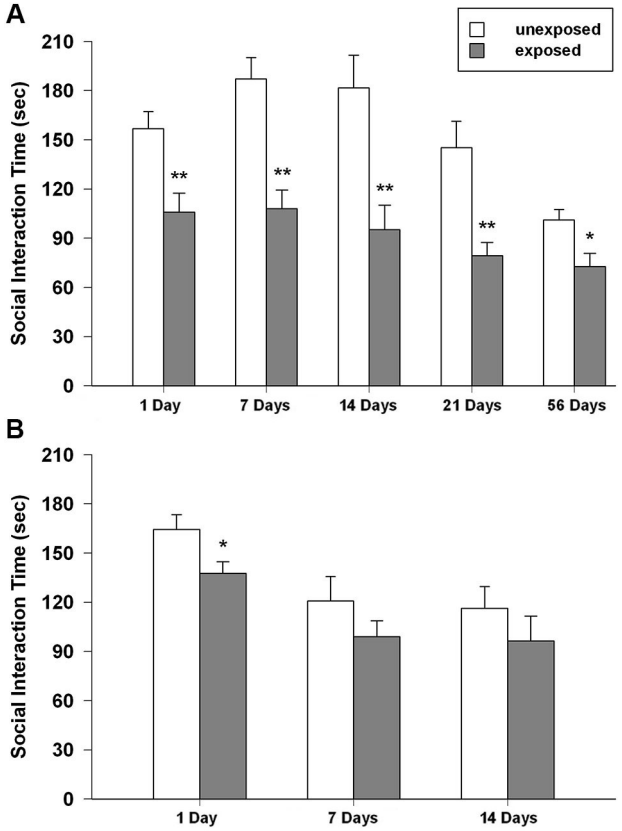

FIGURE 5 | Time spent in social interaction of unexposed and exposed rats 1, 7, 14, 21 and 54 days the exposure to the stressor (n = 8–11). **(A)** Isolated, **(B)** social-housed animals. Data are expressed as mean + SEM (* $p < 0.05$; ** $p < 0.01$).

group ($P < 0.05$; mean EXP = 421.89 ± 37.28; UNEXP = 321.56 ± 16.67; data not shown in figure).

Social-housed rats

In group-housed rats, the ANOVA for social interaction time showed a significant effect of exposure condition ($F_{1,47} = 5.514$, $P = 0.023$) and time intervals ($F_{2,47} = 8.862$, $P = 0.001$) but no significant condition × interval interaction. *Post-hoc* tests demonstrated that, EXP rats compared to UNEXP controls displayed less social interaction only 1 day after the exposure ($P < 0.05$, **Figure 5B**). The ANOVA for crossings showed a significant effect of exposure condition ($F_{1,47} = 19.303$, $P < 0.001$) and time intervals ($F_{2,47} = 48.588$, $P < 0.001$) but no significant condition × interval interaction was found. *Post-hoc* analysis revealed an augmented number of crossings for EXP rats compared to the UNEXP controls for all the tested interval ($P < 0.01$ for 1 post-exposure day, crossing mean EXP = 689.56 ± 25.26, UNEXP = 588.00 ± 11.46; $P < 0.05$ for 7 and 14 post-exposure days: 7 days crossing mean EXP = 423.44 ± 37.62, UNEXP = 306.25 ± 26.20, 14 days crossing mean EXP = 443.33 ± 49.78, UNEXP = 327.67 ± 18.99; data not shown in figure). Mean frequency scores ± SEM of the other behaviors from both the housing conditions are reported in the **Table 1**.

DISCUSSION

The present findings show that: (a) exposure to cat collar, with the protocol used in the experiments described herein, is able to induce a short-lasting/rapidly decaying form of fear memory in rats; (b) the exposure to a series of inescapable footshocks, paired with social isolation, is able to induce in rats a long-lasting memory trace for the traumatic event, accompanied by enduring changes in social behavior; and (c) the social buffering operated by a social housing condition is able to importantly attenuate the emotional dysfunction observed in the footshock-exposed animals, while keeping the trauma-related memory unaltered. The phenotype obtained in the footshock-exposed isolated animals mimics some of the main behavioral PTSD symptoms recently listed in the DSM-5, i.e., long-term memory of trauma-related cues and sensitized behaviors such as social withdrawal.

CAT ODOR EXPOSURE MODEL

Exposure to a predator is among the methods most commonly used to mimic some features of PTSD; it has been demonstrated that it is capable to induce fear and anxiety as well as avoidance and defensive behaviors (Dielenberg and McGregor, 2001; Apfelbach et al., 2005; Takahashi et al., 2005). Fear-related behaviors are generally measured during a period of several minutes of exposure to the predator odor (Mackenzie et al., 2010; Cohen et al., 2012b). At the conclusion of testing, the animal is returned to its home-cage and tested the next day for retention of contextual fear behavior in the conditioning apparatus without further exposure to predator odor; freezing and reduced locomotion are considered as the main form of fear-related behavioral responses (Takahashi et al., 2008). Only few studies have measured fear and anxiety 7 days after the trauma exposure (Takahashi et al., 2005; Mackenzie et al., 2010; Cohen et al., 2012b) and rarely longer time points have been taken into consideration. Although at longer time points Mackenzie et al. (2010) were able to partially reproduce long-lasting conditioned alterations (e.g., locomotor activity), sensitized alterations (e.g., social withdrawal and deficit in the acoustic startle) were not detectable. In view of the above-mentioned evidence, criticism on the validity of the model has been raised mainly because of the difficulties to mimic enduring alterations (Muñoz-Abellán et al., 2009; Mackenzie et al., 2010). In particular, inconsistencies in the long-term effects of cat odor exposure have been frequently reported in the anxiety domain. For example, a dissociation in cat odor ability to induce conditioning without modifying anxiety has been reported (Muñoz-Abellán et al., 2009). In addition, dissociating effects of cat odor exposure on endocrine and behavioral parameters were also observed (Muñoz-Abellán et al., 2008). Therefore, in the present study we first tested whether the predator odor model was able to induce detectable long-term behavioral alterations both in term of conditioned and sensitized alterations. In accordance with literature evidence (Takahashi et al., 2005; Mackenzie et al., 2010; Cohen et al., 2012b), we found that rats re-exposed to the cat odor-paired context 24 h after the

exposure session showed a robust freezing response and impaired locomotor activity, indicative of contextual fear. However, at longer retention intervals (i.e., 7 and 14 days after the exposure to the stressor) we did not appreciate any altered conditioned behavior indicative of augmented contextual fear memory. It has been argued that a valid animal model of PTSD should include a period of incubation after the stress exposure, after which the arising behavioral phenotype should persist unaltered or even worsen (Siegmund and Wotjak, 2006, 2007). Enduring alterations in fear conditioning, extinction learning, extinction retention and sensitization are involved in the development and/or maintenance of PTSD (Pitman et al., 2012). In this context, the predator odor undoubtedly remains a valuable model to test the behavioral dysfunction in the short-term. However, to appreciate long-term cognitive/emotional alterations different models are warranted.

FOOTSHOCK EXPOSURE MODEL

With regard to other commonly used PTSD animal models, the footshock model does not reproduce a pathological phenotype only permitting to measure a physiological (and functionally relevant) form of emotional memory (i.e., the conditioned freezing response to a fearful context) (Pitman et al., 1993). To increase its face validity, we used a footshock model paired to one risk factors for PTSD, such as social isolation (Pitman et al., 1993). We found that, when the traumatic event consisted of a brief session

Table 1 | Mean frequencies of behavioral responses of the social interaction test.

		ISOLATION HOUSING CONDITION							
		Exploratory behaviors		**Social behaviors**					
Interval	**Condition**	Wall rearing	Rearing	Following	Sniffing	Pouncing	Pinning	Crawling over	Boxing
1 DAY	**UNEXP (*n* = 9)**	40.4 ± 3.4	27.7 ± 3.5	3.4 ± 0.8	51.2 ± 2.7	11.8 ± 2.7	2.9 ± 1.1	3.4 ± 1.1	0.9 ± 0.4
	EXP (*n* = 9)	42.9 ± 4.2	26.2 ± 3.4	5.2 ± 1.2	53.0 ± 5.0	4.8 ± 2.0	2.6 ± 1.7	4.1 ± 1.1	1.0 ± 0.6
7 DAYS	**UNEXP (*n* = 9)**	43.4 ± 5.3	24.9 ± 2.7	5.0 ± 1.3	79.7 ± 6.2	9.4 ± 2.0	4.7 ± 1.7	4.0 ± 1.7	2.1 ± 0.9
	EXP (*n* = 11)	45.2 ± 4.1	33.0 ± 3.0	2.5 ± 0.5	62.6 ± 5.9	4.5 ± 1.3	2.0 ± 0.7	2.9 ± 1.0	1.0 ± 0.4
14 DAYS	**UNEXP (*n* = 9)**	37.7 ± 3.1	29.3 ± 4.3	5.1 ± 1.3	64.0 ± 5.0	9.1 ± 1.4	5.2 ± 1.2	0.1 ± 0.1	2.8 ± 0.4
	EXP (*n* = 10)	38.0 ± 3.7	42.8 ± 5.0	2.8 ± 1.0	52.0 ± 6.0	2.8 ± 1.3	1.6 ± 0.9	1.5 ± 0.8	0.1 ± 0.1
21 DAYS	**UNEXP (*n* = 10)**	26.5 ± 3.0	21.2 ± 5.6	3.9 ± 1.6	54.8 ± 6.7	4.8 ± 1.4	2.4 ± 1.0	1.0 ± 0.5	6.6 ± 2.0
	EXP (*n* = 10)	30.7 ± 3.6	29.4 ± 2.5	2.3 ± 0.7	44.7 ± 4.1	1.9 ± 0.5	1.1 ± 0.3	0.8 ± 0.5	0.7 ± 0.3
56 DAYS	**UNEXP (*n* = 11)**	39.7 ± 4.1	38.6 ± 3.6	5.4 ± 1.0	66.1 ± 4.3	3.3 ± 1.0	1.8 ± 0.9	0.3 ± 0.2	2.1 ± 0.7
	EXP (*n* = 11)	37.4 ± 3.6	42.7 ± 3.2	2.0 ± 0.7	54.2 ± 4.5	2.6 ± 0.8	1.6 ± 0.6	0.4 ± 0.2	1.2 ± 0.3
		SOCIAL HOUSING CONDITION							
1 DAY	**UNEXP (*n* = 9)**	50.1 ± 3.0	21.0 ± 4.5	6.7 ± 1.6	73.1 ± 6.1	13.2 ± 1.9	9.8 ± 1.6	1.7 ± 1.1	8.8 ± 2.0
	EXP (*n* = 9)	54.7 ± 4.0	25.4 ± 3.2	5.8 ± 1.0	63.1 ± 3.4	10.3 ± 1.4	9.3 ± 1.9	2.7 ± 0.8	2.9 ± 0.8
7 DAYS	**UNEXP (*n* = 8)**	47.0 ± 2.5	31.6 ± 6.5	4.0 ± 0.8	49.5 ± 3.1	5.9 ± 1.0	3.5 ± 1.0	0.4 ± 0.2	1.4 ± 0.6
	EXP (*n* = 9)	50.1 ± 5.7	37.8 ± 2.5	4.2 ± 1.2	54.7 ± 7.6	2.0 ± 0.8	1.3 ± 0.6	1.4 ± 0.8	1.0 ± 0.3
14 DAYS	**UNEXP (*n* = 9)**	44.6 ± 4.3	41.6 ± 4.0	4.7 ± 0.9	50.7 ± 5.7	7.6 ± 2.7	4.0 ± 2.1	1.4 ± 0.7	2.9 ± 0.8
	EXP (*n* = 9)	33.2 ± 2.4	39.8 ± 4.6	2.8 ± 0.8	45.7 ± 6.8	4.8 ± 2.6	3.6 ± 2.5	1.0 ± 0.4	2.6 ± 0.9

Data expressed as mean ± SEM.

of multiple footshocks, isolated animals displayed a contextual fear memory able to persist up to 8 weeks after exposure. This reveals the high stability of such learned response which may need a retention interval longer than 8 weeks or even of a lifetime to completely decay. To assess the presence and duration of both conditioned and sensitized behaviors, we additionally evaluated the emotional phenotype of the footshock-exposed rats in the EPM and SI tests. The EPM is a widely used paradigm for anxiety assessment and it is based on spontaneous rodent behavior involving the conflict between the exploration of a novel environment and its aversive characteristics (Pellow et al., 1985; File et al., 2004). Interestingly, in none of the tested intervals rats exposed to the footshocks showed a clear-cut anxious phenotype in this maze. Nevertheless, a significantly reduced locomotor activity (File et al., 2004) was systematically found in footshock-exposed rats when compared to unexposed animals, with exposed rats making less entries in the closed arms than controls did. This result is in line with literature data reporting hypoactivity in the EPM and other novel environments following contextual fear conditioning (Radulovic et al., 1998; Daviu et al., 2010). It is tentative to speculate that the footshock-induced trauma could sensitize the general responsiveness to stress thus reducing the rat activity in another stressful situation (i.e., EPM). This highlights the strong translational value in regard to the stress-sensitization occurring in PTSD patients (Siegmund and Wotjak, 2006; Brewin, 2011). An alternative specultaion for the reduction of entries in the closed arms of the EPM displayed by exposed rats could be represented by a generalization of fear; rats could systematically avoid the enlongated/narrow places with spatial configurations similar to the footshock-paired context. Noteworthy, the EPM may be not the ideal test to reveal the possible emotional dysfunction of footshock-exposed rats since the hypoactivity displayed by the exposed rats could mask a clear-cut anxiogenic-like profile. Therefore, the present results may help explaining the conflicting findings reported when the EPM is used as a measure of anxiety in PTSD animal models (Muñoz-Abellán et al., 2009). Conversely, here we show that the SI test (File et al., 2004) allows not only to evaluate the possible emotional alteration in traumatized animals but also to better mimic the human sensitized symptomatology. Animals exposed to the footshocks displayed reduced social interaction compared to unexposed control rats, thus showing augmented level of emotional distress during the social encounter or, with a different interpretation, less interest in social activities persisting up to 8 weeks after trauma exposure. These results are in line with previous studies reporting a reduction in social behavior in animals exposed to inescapable shocks (Maier and Minor, 1993; Short and Maier, 1993; Siegmund and Wotjak, 2007; Christianson et al., 2008).

EFFECTS OF SOCIAL HOUSING ON THE FOOTSHOCK EXPOSURE MODEL

To further examining the validity of our model we tested the hypothesis that lack of social support during the processing of the trauma might be an essential factor with respect to PTSD development. To this aim we subjected group-housed rats (social housing condition) to the above described footshock trauma model. Group-housed rats maintained a strong contextual fear memory at all the tested intervals as isolated animals did, but at the same time, they showed no enduring signs of emotional distress. In particular, the reduced time spent in social interaction by "traumatized" rats could only be detected 1 day after stressor exposure. This result could be attributed to a physiological emotional response to the acute stress and being not indicative of any pathological condition. If from one side it is tentative to speculate that the social support is able to reduce the adverse consequences of stress exposure and/or to enhance the ability to recover from stress, more appealing is the other side of the coin. The social isolation could be indeed considered a precipitating factor leading to an enhanced susceptibility to adverse emotional outcomes after trauma exposure. In both cases, our results are in line with previous findings showing long-term consequences of social defeat (and other stressors) on rodent behavior. Indeed, isolated rodents exposed to inescapable social defeat show long-lasting and adverse changes in behavior and physiology that are not observed, or are drastically reduced, in animals housed in groups (Ruis et al., 1999; Korte and de Boer, 2003; de Jong et al., 2005; Nakayasu and Ishii, 2008).

TRANSLATIONAL FEATURES OF THE FOOTSHOCK MODEL

The reduced social interaction displayed by isolated animals is of high translational value with respect to the human diagnostic criteria of "feeling of being alienated from others" and "reduction in social functioning" which are two symptoms included respectively in the criterion D (i.e., negative alterations in cognitions and mood) and G (i.e., functional significance) of the DSM-5 (American Psychiatric Association, 2013). Moreover, the "markedly reduced interest in significant activities" (i.e., social interaction) and the "persistent inability to experience positive emotions" represent two more symptoms of the DSM-5 criterion D, which can be easily attributed to a blunted positive emotionality. Considering the highly rewarding value of social interaction in rats (see Trezza et al., 2011 for review), the reduced social interaction observed in footshock-exposed animals may be also indicative of a general blunting of positive emotions. Collectively, these results show that rats exposed to footshocks and housed in isolation express a strong contextual memory for the traumatic experience accompanied by social withdrawal, anxiety, and blunted emotionality symptoms. Importantly, all symptoms are persistent and do not undergo towards spontaneous recovery, underlining the chronic nature of the observed phenotype.

The possibility to mimic in the same animal both the cognitive (contextual) and emotional (sensitized) features of PTSD could open new research paths not only in term of a better understanding of the neural underpinning of the disorder but also for the testing of innovative drugs which could act at the same time by reducing the cognitive disability and ameliorating the emotional dysfunction observed in PTSD patients.

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