HOW FEAR AND STRESS SHAPE THE MIND

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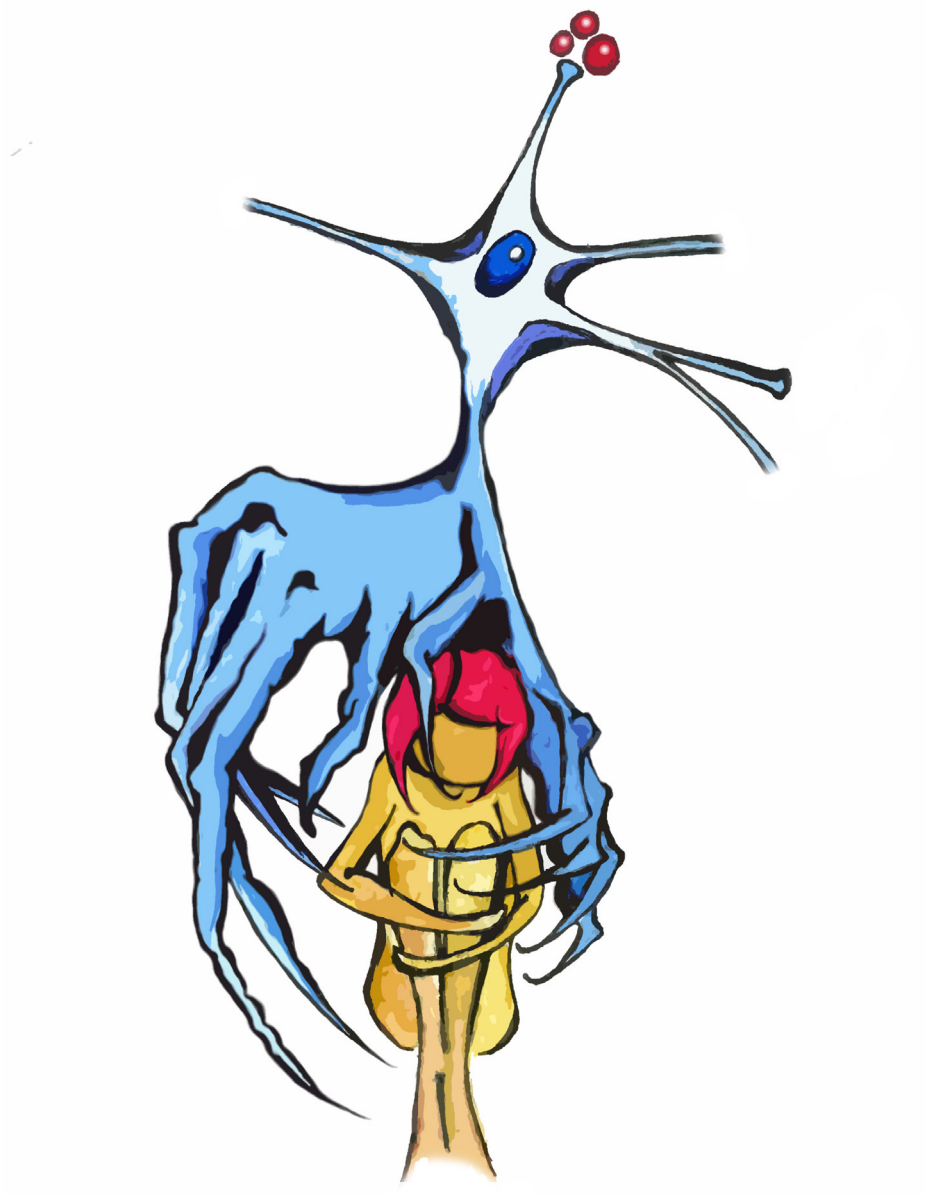

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HOW FEAR AND STRESS SHAPE THE MIND

Topic Editor:
Luke R. Johnson, Uniform Services University of the Health Sciences, USA; Queensland University of Technology, Australia

Artwork by Rachel Lazarus, 2010

The experience of fear and stress leaves an indelible trace on the brain. This indelible trace is observed as both changes in behavior and changes in neuronal structure and function. Fear and stress interact on many levels. The experience of stress may lead to the formation of a fearful memory trace of a place or reminder cue, and fearful memory formation is regulated by the extent of concurrent stress. The concurrent experience of fear and stress may amplify fear and slow fear extinction which may lead to pathology. Fear memory formation involves changes in synaptic plasticity while stress and glucocorticoids change neuronal structure. Thus, both neurons and synapses are changed. These changes can be identified, visualised and mapped within focused microcircuits. In this Research Topic we focus on current advances in both the neurobiology and behavioral consequences of fear and stress.

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EDITORIAL
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Editorial: How Fear and Stress Shape the Mind

Luke R. Johnson [1, 2]*

[1] School of Psychology and Counselling, Translational Research Institute, Institute of Health and Biomedical Innervation, Queensland University of Technology, Brisbane, QLD, Australia, [2] Department of Psychiatry, Center for the Study of Traumatic Stress, Uniformed Services University School of Medicine, Bethesda, MD, USA

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The Editorial on the Research Topic

How Fear and Stress Shape the Mind

How do fear and stress systems interact and how do they shape ongoing and future behavioral responses? In a classical definition of fear and stress, we think of threatening stimuli activating a species-specific defensive threat reaction. This defensive reaction triggers physiological stress responses including adrenal hormone release (for review see LeDoux, 2003, 2012; Johnson et al., 2012). Knowledge of the microanatomy of conditioned threat memory is developing however, knowledge of its interaction with stress mediated adrenal steroid systems is still emerging (LeDoux, 2003, 2012; Johnson and LeDoux, 2004; Prager and Johnson, 2009; Prager et al., 2010; Bergstrom et al., 2011, 2013a,b; Bergstrom and Johnson, 2014; Krugers et al.). Studies have identified the key role of the lateral amygdala and within this nucleus the microanatomy of Pavlovian fear/threat memory consolidation, reconsolidation, and extinction has begun to be revealed (Bergstrom et al., 2011, 2013a,b; Bergstrom and Johnson, 2014). This Frontiers Research Topic builds on previous research by addressing key questions that reveal unique aspects and mechanisms of how fear and stress shape the mind.

The fear neural circuitry includes; amygdala output circuits that directly activate the sympathetic nervous system and also the hypothalamic pituitary adrenal (HPA) axis, thereby including stress hormones in the negative emotional response (Radley). It is generally accepted that negative emotion involves a stress response, however what stress is and how it manifests in the body has been, and continues to be, vigorously investigated and debated. Radley summarizes detailed circuit tracing and connectivity approaches to understand the interaction between stress and fear systems in the brain. Proposing that the anterior bed nuclei of the stria terminalis (aBST) is the central point for regulation of chronic stress induced hyperactivity of the HPA axis. This GABA projecting nucleus, upstream of the PVH, receives convergent input from amygdala, prelimbic cortex, and other fear related nuclei. Aspects of amygdala anatomy and its control of HPA responding may underlie differences in mental responding to fear and stress (Johnson and LeDoux, 2004; Johnson et al., 2012; McGuire et al., 2013).

Krugers et al. describe a series of studies in animals and humans that highlight the key time course and mechanisms of stress hormones norepinephrine and glucocorticoids in facilitating fear memories. They describe short-term rapid activation of NE Beta and Mineralocorticoid receptors (MR) in the postsynaptic space leads to rapid insertion of AMPA receptors in the postsynaptic membrane. Over a longer period (hours), Glucocorticoid receptors (GR) acting through genomic mechanisms also drive insertion of AMPA receptors into the postsynaptic membrane. These authors found that these multiple complementary cellular mechanisms facilitate and strengthen memories of stressful events.

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Nuno Sousa,
University of Minho, Portugal

***Correspondence:**
Luke R. Johnson
LukeJohnsonPhD@gmail.com

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By identifying the fundamental mechanisms underlying structural changes in the fear system in response to threatening stimulus associations, Lamprecht describes changes to the actin cytoskeleton and suggests, that it may be essential for pre- and post- synaptic changes that occur in the dendrite spines (particularly in lateral amygdala and hippocampus) following fear conditioning. It was found that inhibitors of the actin cytoskeleton modify neuron structure and dampen long-term memory (Lamprecht).

Starting from the assumption that age is a risk factor for anxiety disorders (Pardon and Rattray, 2008; Shoji and Mizoguchi, 2011), Beracochea et al. used stressed middle-aged and non-stressed young adult mice to understand the interaction between the fear circuitry and its link with anxiety disorder, memory, and pharmacology. When administered benzodiazepines in specific dose range, stressed middle-aged mice became like young adult non-stressed mice, on a hippocampal memory task. This provides the first evidence of a dynamic interaction between benzodiazepines and corticosterone levels, indicating a reduced stress effect and improved memory performance.

Potential overlapping pathways between fear, stress, suicide, anxiety, and aging are identified by Choi et al., who found kinase gene expression levels increased in the prefrontal cortex of suicide victims compared to controls. Postnatal disruption of (kinase) genes by environmental factors may increase later pathophysiology increasing the risk of suicide. In addition to Kinase genes, other regulators of stress may be important indicators and pharmacological regulators of the amygdala-prefrontal cortex stress axis. McGuire et al. report that Neuropeptide Y (NPY) plays a role in integrating stress and emotion in part through regulation of CRH, and, that a dysregulation of NPY may leave an individual more exposed to the negative aspects of subsequent stress.

Nolte et al. summarize important work on how attachment experiences during development influence the development of anxiety and HPA axis sensitivity. They propose, that stress sensitivity characteristics that an infant is born with could represent in utero adaptation of stress regulation style of the mother. Thus, anxiety in the mother can be transferred from mother to child through dysregulation of the HPA axis. A person's sensitivity to developing post-traumatic stress disorder (PTSD) may be influenced by their genetic, development and environmental experiences.

PTSD is associated with dysregulated fear and stress systems. In an elegant article by Jovanovic and Norrholm, fear inhibition models are suggested to be possible translational tools for studying fear reduction in animals and humans. Facilitation of fear extinction mechanisms both, behaviorally, and pharmacologically, may produce therapeutic modification to underlying neural circuitry. They identify that decreased ability to reduce fear is a risk factor for the development of PSTD. Reduction of fear is context and time dependent. Huff et al. developed a sophisticated virtual reality procedure for context and cued fear in humans. They identified a time dependency and memory consolidation of context fear develops quickly. In contrast, memory consolidation of differential cued fear (CS+/CS−), develops slowly. These finding have important implications for understanding anxiety and testing anxiety in humans.

In a fresh and novel perspective for PTSD research in wild animals Clinchy et al. propose, that we need to know how real animals deal with real stress. They investigate the "predator model of PTSD" in which exposure to odor of the predator leads to long lasting changes in the brain and body, including to CRH and corticosterone, and to dendrite morphology. Predator exposure to wild prey animals has been shown to lead to 40% less offspring production and it is linked to glucocorticoid elevation in the parents. Multi-generational stress has been demonstrated in snowshoe hares which may increase an adaptive predator response in future offspring. Clinchy et al. propose, that trans-generation stress responses may be personally maladaptive but evolutionarily adaptive. If stress is maladaptive why does it persist? It may be a struggle to live with but not necessarily maladaptive to survival, thus maladaptive stress responses may make sense.

Throughout human history, every generation has arguably faced an epidemic of fear and stress associated mental trauma which frequently manifests as PTSD (Ursano et al., 2010). This epidemic afflicts past, present and future generations. The 11 studies presented provide a fresh perspective into how fear and stress systems interact and how they may influence the development of emotional and pathological states. How bodily stress systems interact with the neurobiology of fear and mental health continues to be an important question in neuroscience (Prager et al.). Future studies will need to revisit and solve fundamental mechanisms of emotion in order to effectively understand and treat pathologies of fear, stress, and trauma.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Toward a limbic cortical inhibitory network: implications for hypothalamic-pituitary-adrenal responses following chronic stress

Jason J. Radley

Program in Neuroscience, Department of Psychology, University of Iowa, Iowa City, IA, USA

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Jennifer McGuire, Uniformed Services University of the Health Sciences, USA
Jay F. Muller, University of South Carolina School of Medicine, USA

Correspondence:
Jason J. Radley, Program in Neuroscience, Department of Psychology, University of Iowa, E11 Seashore Hall, Iowa City, IA 52242, USA.
e-mail: jason-radley@uiowa.edu

A network of interconnected cell groups in the limbic forebrain regulates hypothalamic-pituitary-adrenal (HPA) axis activation during emotionally stressful experiences, and disruption of these systems is broadly implicated in the onset of psychiatric illnesses. A significant challenge has been to unravel the circuitry and mechanisms providing for regulation of HPA output, as these limbic forebrain regions do not provide any direct innervation of HPA effector cell groups in the paraventricular hypothalamus (PVH). Recent evidence will be highlighted that endorses a discrete region within the bed nuclei of the stria terminalis serving as a neural hub for integrating and relaying HPA-inhibitory influences to the PVH during emotional stress, whereas the prevailing view has involved a more complex organization of mulitple cell groups arranged in parallel between the forebrain and PVH. A hypothesis will be advanced that accounts for the capacity of this network to constrain the magnitude and/or duration of HPA axis output in response to emotionally stressful experiences, and for how chronic stress-induced synaptic reorganization in key cell groups may lead to an attrition of these influences, resulting in HPA axis hyperactivity.

Keywords: bed nuclei of the stria terminalis, prefrontal cortex, hippocampus, ventral subiculum, HPA axis, paraventricular nucleus of the hypothalamus, plasticity, dendritic spine

INTRODUCTION

Stress may be broadly defined as the constellation of physiological and behavioral responses to any challenge that overwhelms, or is perceived to overwhelm, selective homeostatic systems of the individual (Selye, 1980; Day, 2005). A hallmark feature of stress entails activation of the hypothalamic-pituitary-adrenal (HPA) axis. This neuroendocrine cascade is initiated when visceromotor neurons in the paraventricular nucleus of the hypothalamus (PVH) stimulate the release of pituitary adrenocorticotropic hormone (ACTH) into the bloodstream, which, in turn, activates glucocorticoid (GC; cortisol in humans, corticosterone in rodents) secretion from the adrenal gland (Antoni, 1986). GCs are the end-products of HPA axis activation, and facilitate catabolic processes throughout the body during stress by increasing energy metabolism and utilization. GCs also have activating effects on cardiovascular output, and inhibit non-essential processes, such as immune and reproductive functions. Finally, HPA axis activation during stress alters cognitive and emotional processes relevant for behavioral adaptation (e.g., Shors et al., 1992; McIntyre et al., 2003).

Despite the critical role that stress plays for adaptive coping and survival of the individual, it is widely implicated in the onset of psychiatric disease, most notably depression and post-traumatic stress disorder (Kessler, 1997; Yehuda, 2002). Initial studies revealed that patients hospitalized for major depressive illness commonly manifested hypercortisolemia and HPA axis insensitivity to GC receptor agonist treatment (i.e., dexamethasone supression test; Carroll et al., 1976). A wealth of research implicates elevated GCs in compromised brain function, disruptions in the neural circuits imparting negative feedback control over the HPA axis, and further endangerment of brain regions targeted by GCs (for reviews, see Sapolsky et al., 1986; Conrad, 2008). Since the neural substrates providing restraining influences over the stress axis are also regions that play important roles in cognition and emotion, elevated GC levels and HPA axis dysregulation may be key steps in producing the disordered thought and affect that characterize stress-related mental illnesses.

Animal models of repeated stress (e.g., chronic variable stress, chronic intermittent stress, chronic social defeat stress) have proven useful for modeling HPA axis hyperactivity and depression-like behaviors, and would appear to provide the appropriate setting for teasing apart the role of the HPA axis in the pathogenesis of depression. However, progress has been hampered by the fact that the neural circuitry and mechanisms accounting for limbic forebrain control over the HPA axis have proven difficult to unravel. While a number of these candidate regions have been implicated in HPA axis inhibition during emotional stress (Herman et al., 2003; Radley and Sawchenko, 2011), none of these cell groups provide any appreciable direct innervation of the PVH. Combined pathway tracing and immediate-early gene mapping studies have helped to identify a number of candidate cell groups that could serve as disynaptic relays to interface between forebrain regulators and the PVH. The picture that emerges is one involving a complex network of higher-order

structures interconnected in a parallel and multisynaptic manner with the PVH (Cullinan et al., 1993; Roland and Sawchenko, 1993; van de Kar and Blair, 1999; Herman et al., 2003).

Here we highlight recent advances in our research suggesting an entirely different organization for limbic forebrain control over the stress axis: one involving convergence onto a circumscribed cluster of GABAergic neurons within the anterior bed nuclei of the stria terminalis (aBST), that, in turn, directly inhibits the PVH and HPA activation. This model has several implications for neural circuits and mechanisms underlying HPA axis control and GC-dependent negative feedback. An unforeseen but not incidental feature is that this model helps to clarify the sequelae of chronic stress-induced HPA axis hyperactivity, whereby structural reorganization within limbic forebrain cell groups (i.e., synapse loss/gain) throughout the network leads to an attrition of HPA axis control.

EMOTIONAL STRESS CIRCUITRY: A SEARCH FOR THE MISSING LINK

Over the years, attempts to organize stressors into a taxonomical framework have resulted in two major groupings, physiological (a.k.a., systemic), and emotional (a.k.a., neurogenic, psychogenic) (Fortier, 1951; Allen et al., 1971). More recent immediate-early gene mapping as a generic index of cellular activation in stress-related circuits has helped to provide a considerable degree of face validity for these distinctions (Cullinan et al., 1995; Li and Sawchenko, 1998; Dayas et al., 2001). Physiological stressors are generally considered to involve more targeted challenges that overwhelm selective homeostatic systems, such as hemorrhage, hypoxia, or immunogenic stimuli. Emotional stressors require interpretation by exteroceptive sensory modalities and integration with distinct cognitive (comparison with past experience) and affective information processing systems in the brain (Herman and Cullinan, 1997; Sawchenko et al., 2000; Dayas et al., 2001). Commonly employed animal models of emotional stress are restraint, immobilization, and footshock. Whereas each class of stressor enlists brainstem and hypothalamic effectors for activation of the sympathoadrenal and HPA axis output, emotional stressors manifest widespread activation in the limbic forebrain, and correspond to a broad array of behavioral changes (e.g., vigilance, fear, anxiety) that help to facilitate adaptive coping as required by the specific environmental demand (Cullinan et al., 1995; Campeau et al., 1997; Li and Sawchenko, 1998; Dayas et al., 2001).

Functional and lesion studies implicate a network of limbic forebrain cell groups in the inhibitory control of HPA activation during emotional stress (Cullinan et al., 1995; Herman and Cullinan, 1997; Akana et al., 2001; Jaferi and Bhatnagar, 2006). Noteworthy examples of regions implicated in HPA axis inhibition are the septum (Feldman and Conforti, 1980b), posterior paraventricular nucleus of the thalamus (PVTp; Jaferi and Bhatnagar, 2006), ventral subiculum (vSUB, the region issuing extrinsic projections of hippocampal formation involved in stress regulation; Herman et al., 1995), and mPFC (Diorio et al., 1993). These cell groups are conspicuously lacking in any direct innervation of HPA effector neurons within the PVH, instead issuing projections throughout numerous basal forebrain and hypothalamic structures (Sesack et al., 1989; Cullinan et al., 1993; Herman et al., 2003). Many of these regions (notably, mPFC and hippocampal outputs) give rise to predominantly excitatory projections, utilizing the neurotransmitter glutamate (Malthe-Sorenssen et al., 1980; Walaas and Fonnum, 1980; Ottersen et al., 1995), implicating a hitherto unknown, GABAergic relay. Previous work has identified candidate GABAergic cell groups (i.e., preoptic area, aBST, posterior BST, dorsomedial hypothalamic nucleus, PVH-surround regions) between vSUB and PVH (Cullinan et al., 1993), laying a foundation for understanding how controls over the axis may be organized. Nonetheless, whether influences from vSUB, and other HPA-inhibitory cell groups, are mediated via several disynaptic relays arranged in parallel to each other, and which relays are capable of integrating inhibitory signals from the limbic forebrain during emotional stress, has remained elusive.

Our starting point into this problem was to first address the nature of mPFC involvement in acute emotional stress-induced HPA activation, and we have shown that distinct subregions of mPFC differentially modulate the stress axis (Radley et al., 2006a). These studies were inspired from the idea that a variety of other functions subserved by mPFC are differentiated in a dorsal-to-ventral manner (Morgan and LeDoux, 1995; Heidbreder and Groenewegen, 2003). Indeed, previous reports in the stress literature tended to treat the mPFC as a homogeneous structure, and discrepancies remained concerning the nature of mPFC's influence (excitatory or inhibitory) on HPA output (Sullivan and Gratton, 1999; Akana et al., 2001; Figueiredo et al., 2003b; Spencer et al., 2005). Through a series of experiments employing discrete excitotoxin lesions in cortical subfields of mPFC, we found that lesions of dorsal mPFC (encompassing prelimbic cortex, PL, and portions of dorsal anterior cingulate cortex, ACd) enhanced, whereas ventral mPFC (infralimbic cortex, IL) lesions inhibited HPA activation in response to acute restraint stress (Radley et al., 2006a). Furthermore, dorsal mPFC lesions resulted in a prolonged elevation of plasma corticosterone after the cessation of restraint, which is consistent with its role as a target site for GC negative feedback under normal conditions (Diorio et al., 1993) (**Figure 1**).

Follow-up work has shown that a discrete cluster of GABAergic neurons in aBST forms the missing link in a circuit conveying HPA-inhibitory influences of PL during emotional stress (Radley et al., 2009). First, functional neuroanatomical experiments assayed for sources of GABAergic input to PVH whose sensitivity (i.e., as measured with Fos activation) to an acute stress or (restraint) was diminished by dorsal mPFC lesions. Of the stress-sensitive, GABAergic, PVH-projecting regions analyzed, a circumscribed region in the aBST (corresponding to the dorsomedial and fusiform subdivisions of Dong et al., 2001) was exclusive in showing a decrement in Fos activation following PL lesions (Radley et al., 2009). By contrast, IL lesions were noted to attenuate Fos activation in PVH-projecting neurons in the same region, albeit in a subpopulation of non-GABAergic neurons (**Figure 2**).

In a second series of experiments, functional ablation of GABAergic neurons in aBST recapitulated the effects of PL lesions on acute stress-induced HPA activation (Radley et al., 2006b, 2009). These studies were performed by focally administering

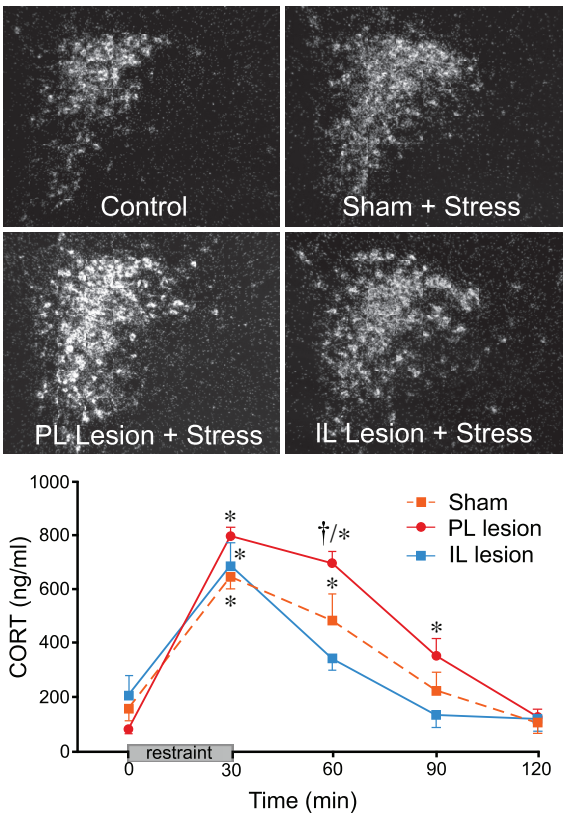

FIGURE 1 | Top: Darkfield photomicrographs showing corticotropin-releasing factor (CRF) mRNA expression in the paraventricular nucleus of the hypothalamus (PVH) as a function treatment condition. Restraint stress results in a marked increase in CRF mRNA expression in the PVH dorsal medial parvicellular subdivision of intact animals, which is enhanced in prelimbic (PL)-lesioned rats. No such enhancement of stress-induced CRF mRNA expression was seen in infralimbic (IL)-lesioned animals. **Bottom:** Stress exposure also significantly increases plasma corticosterone (CORT) levels in sham-lesioned animals. PL lesions result in a prolonged increase in stress-induced plasma CORT, while IL-lesioned animals show a more rapid recovery to pre-stress levels. *Differs significantly from basal (0 min) values from within each group, $p < 0.05$; †Differs significantly from sham-lesioned animals for a given time point, $p < 0.01$. Data are from Radley et al. (2006a).

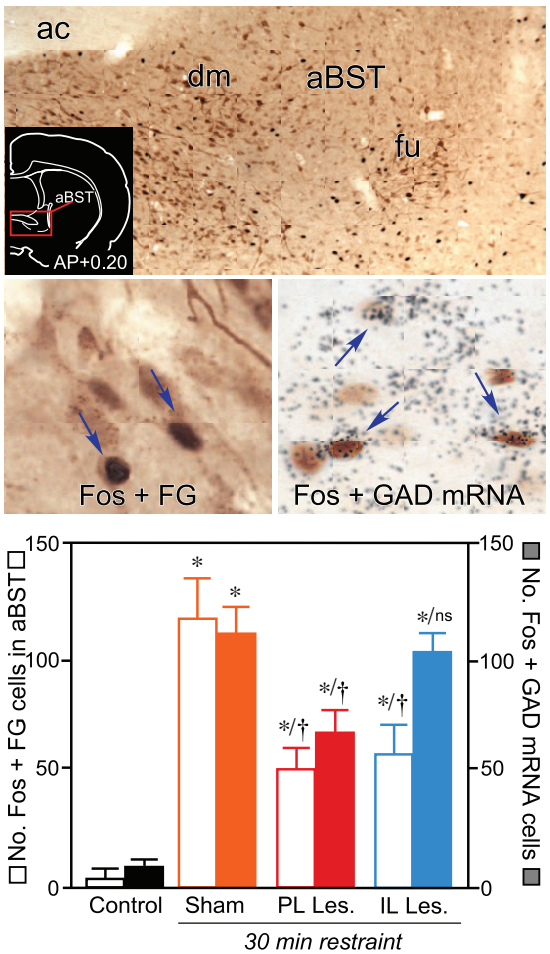

FIGURE 2 | Top: Brightfield photomicrograph showing stress-induced Fos immunoreactivity (black nuclei) and Fluoro-Gold (FG; brown cytoplasm) in anterior bed nuclei of the stria terminalis (aBST). Retrogradely-labeled cells are concentrated in fusiform (fu) and dorsomedial (dm) subnuclei of aBST following tracer injections centered in the PVH. **Inset:** Coronal section showing the approximate location of aBST corresponding to the region comprising the relevant subdivisions (red box). **Middle left:** Following restraint stress, cells doubly-labeled for Fos and Fluoro-Gold (arrows) are abundant in sham-lesioned animals. **Middle right:** Concurrent labeling for Fos (brown) with glutamic acid decarboxylase (GAD67) mRNA (black grains) showing comparable increases in doubly-labeled cells (arrows) in the sham-lesioned group following restraint stress. **Bottom:** Mean + SEM number of neurons co-labeled for Fos and Fluoro-Gold, and for Fos and GAD67 mRNA, in aBST of treatment groups. Whereas both PL and IL lesions reliably diminished stress-induced activation of PVH-projecting neurons in aBST, only PL lesions resulted in a decrease in the activation of GABAergic neurons in this subregion, implicating different relays for prefrontal modulation of the stress axis. *Differs significantly from sham-lesioned control animals, $p < 0.05$. †Differs significantly from sham-lesioned stressed animals, $p < 0.05$. Portions of these data have been derived from Radley et al. (2009), and Radley and Sawchenko (2011). Data on IL lesion effects on stress-induced aBST activation are previously unpublished.

an immunotoxin in aBST that preferentially ablates GABAergic, while sparing non-GABAergic, neurons (Radley et al., 2009). Ablation of GABAergic cell groups in aBST enhanced activation of PVH and hormonal indices of HPA axis output in response to acute restraint. Previous reports that indiscriminate lesions to aBST attenuate stress-induced HPA output (Choi et al., 2007), whereas stimulation of aBST may either facilitate or inhibit HPA activity (Dunn, 1987), are consistent with the idea that distinct HPA-regulatory influences arise from neurochemically heterogeneous subpopulations. Thus, opposing influences of the dorsal and ventral mPFC may commingle within the same region of aBST onto separate populations of PVH-projecting GABAergic and non-GABAergic neurons, respectively, to modulate emotional stress-induced HPA output (**Figure 3**).

Subsequent anatomical pathway tracing studies have that PL is the cortical subfield that provides the source of HPA-inhibitory influences that emanate from the mPFC (Radley et al., 2006b, 2008b, 2009). Whereas the subcortical projections of dorsal and ventral mPFC are considered to be highly divergent (e.g., Vertes, 2004), their projections to aBST distribute in a topographically

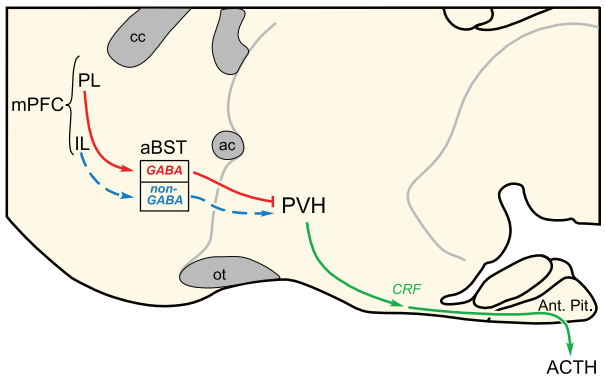

FIGURE 3 | Two disynaptic pathways from medial prefrontal cortex (mPFC) are proposed to account for the differential modulation of emotional stress-induced HPA output. Whereas evidence highlighted in the text [see also, Radley et al. (2009)] supports GABAergic neurons in the anterior bed nuclei of the stria terminalis (aBST) as interceding for the HPA-inhibitory influences of prelimbic cortex (PL; red lines), the pathway from infralimbic cortex (IL) is suggested, and remains to be verified with functional studies. ac, anterior commissure; ACTH, adrenocorticotropic hormone; Ant. Pit., anterior pituitary gland; cc, corpus callosum; CRF, corticotropin-releasing factor; ot, optic tract; PVH, paraventricular nucleus of the hypothalamus.

graded, increasing dorsal-to-ventral manner (Radley et al., 2009). Anterograde tracer injections centered in the most dorsal aspect of mPFC (ACd) fail to label any projections to aBST, more ventrally placed injections label progressively more inputs, with PL providing a moderate innervation of aBST, and IL providing the densest input. Acute restraint stress increases activation of aBST-projecting neurons throughout PL and IL, and most prominently in the medial-to-rostral aspect of PL (Radley and Sawchenko, unpublished observations). Finally, dual tracing experiments show that PL projections overlap extensively, and make appositions with, PVH-projecting cell groups in aBST (Radley and Sawchenko, 2011).

Insight into the broader organization of HPA axis control has been gleaned from examination of a second limbic forebrain region implicated in the inhibitory regulation of the neuroendocrine stress response. The hippocampal formation (HF) is similar to mPFC from the standpoint that its extrinsic projections are excitatory, it avoids direct innervation of PVH proper, and is capable of inhibiting emotional-stress induced HPA output (Swanson and Cowan, 1977; Walaas and Fonnum, 1980; Canteras and Swanson, 1992; Cullinan et al., 1993). Previous studies have shown that HPA-inhibitory influences of HF are localized to vSUB (Herman et al., 1995; Herman and Mueller, 2006), implicating a disynaptic GABAergic relay to PVH. vSUB contains the bulk of the extrinsic projections of HF that innervate candidate PVH-projecting GABAergic cell groups, such as various subregions of the BST and hypothalamus, and preoptic area (Cullinan et al., 1993). Nonetheless, attempts to define the precise relays subserving vSUB influences on the stress axis, or its relation to other relays involved in HPA axis inhibition, had not been previously clarified.

We have found that vSUB influences on the HPA axis are also interceded for by aBST, similar in nature to PL (Radley and Sawchenko, 2011). First, GABAergic PVH-afferent cell groups in aBST showed a diminished functional activation in animals bearing excitotoxin lesions of vSUB. In these experiments, vSUB lesions were also noted to increase multiple indices of acute restraint-induced HPA activation, as previously reported (Herman et al., 1995). Although HF is not typically regarded as one of the more stress-responsive regions in the limbic forebrain (Li and Sawchenko, 1998), vSUB, particularly its aBST-projecting neurons, does in fact display a moderate degree of engagement following acute restraint stress (Radley and Sawchenko, 2011). Finally, animals bearing dual tracer deposits show that vSUB projections overlap extensively, and make appositions with, stress-sensitive PVH-projecting cell groups in aBST. Collectively, these studies highlight a neural circuitry from vSUB → aBST (GABA) → PVH, with each node in the pathway showing functional activation in response to acute restraint stress, and lesions of vSUB resulting in corresponding alterations in output (i.e., decreased aBST, increased PVH/HPA activation).

A key feature of aBST, in addition to its role as a site of convergence, is that it appears to integrate limbic cortical influences (Radley and Sawchenko, 2011). For instance, animals bearing excitotoxin lesions of both PL and vSUB were found to exhibit more exaggerated central indices of stress-induced HPA responses as compared to lesions of either alone. Furthermore, ablation of GABAergic cell groups in aBST produced a greater enhancement of hormonal indices of HPA activation in response to acute restraint, as compared to animals with vSUB lesions alone. Given that dual lesions of PL and vSUB, or separately, by disruption of their interceding inhibitory relay, result in a greater overall effect on stress-induced HPA output than lesions of either, implicates aBST as a key integrator of stress-inhibitory influences emanating from the limbic cortex. Indeed, our examination of the projections of PL and vSUB reveal extensive overlap in their terminal innervation of PVH-projecting neurons within aBST, with evidence of some convergence onto single neurons (**Figure 4**).

IMPLICATIONS OF AN HPA-INHIBITORY NETWORK

The elucidation of this network should help to address some of the lingering questions concerning the central organization of HPA control. First is the generality of aBST as a site of convergence and integration of additional forebrain limbic influences on emotional stress-induced HPA output (**Figure 5**). None of the other forebrain cell groups implicated in the inhibition of emotional stress-induced HPA activity (i.e., septum, posterior paraventricular thalamic nucleus) provides any substantial direct innervation of PVH, although each projects to aBST (Shin et al., 2008). Thus, aBST GABAergic neurons are poised to receive and integrate these along with prefrontal and hippocampal influences. The amygdala is generally considered to exert an excitatory influence on HPA axis activation (Prewitt and Herman, 1997; Sullivan et al., 2004), however, the circuits and mechanisms accounting for this are poorly understood. Both CeA and MeA issue a massive GABAergic input into GABAergic regions of aBST (Sun and Cassell, 1993; Tsubouchi et al., 2007), particularly the aforementioned PVH-projecting population. This suggests that excitatory effects on HPA output may be mediated via disinhibition of this modulatory pathway. The basolateral amygdala (BLA), which

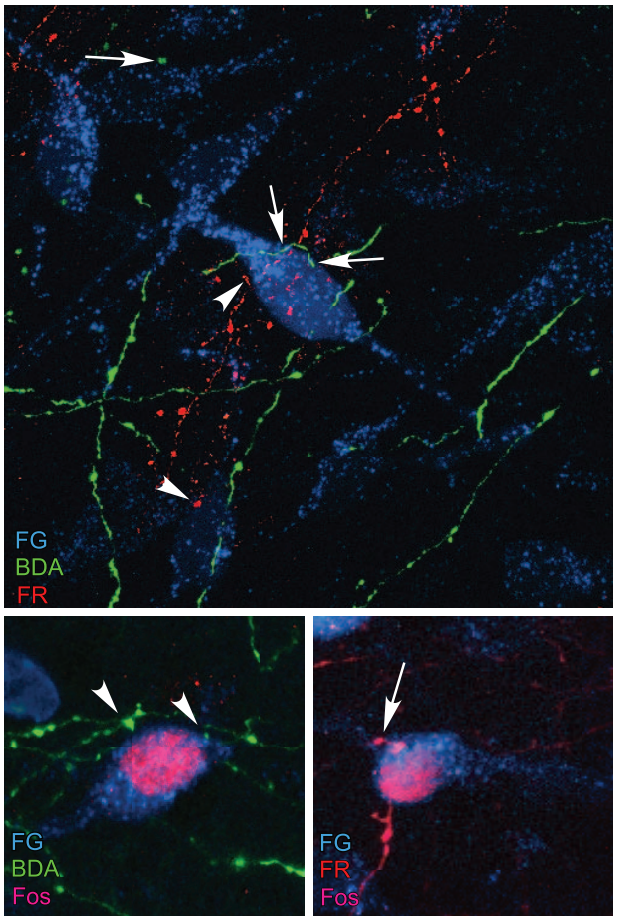

FIGURE 4 | Top: Overlap of retrograde tracer injections in PVH (Fluoro-gold, FG; cyan), and anterograde tracers in PL (BDA; green) and vSUB (FlouroRuby, FR; red) was evaluated in fluorescence preparations using confocal microscopy. Instances of BDA- (arrows) and FR-labeled (arrowhead) terminals were found to make appositions onto single PVH-projecting neurons in aBST, by analysis of single optical planes containing fluorescence labeling for all three markers. **Bottom row:** After a single stress exposure, numerous instances of Fos-labeled nuclei are evident in PVH-projecting neurons containing appositions from BDA- (left) and FR-labeled (right) terminals. Data are based upon Radley and Sawchenko (2011).

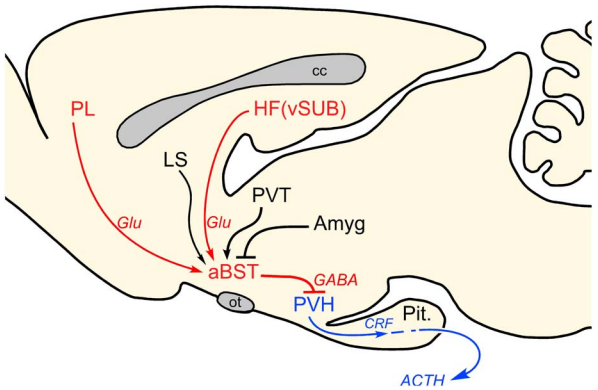

FIGURE 5 | Proposed role of anterior bed nuclei of the stria terminalis (aBST) as an integrator of limbic forebrain influences on emotional stress-induced HPA output. Previous work of ours supports the pathways highlighted in red, with aBST providing an important source of GABAergic innervation of PVH, and relaying limbic cortical influences. Other forebrain cell groups known to influence HPA output (highlighted in black), notably via GC receptor-mediated negative feedback, also project to aBST, whose integrated output targets PVH directly. Like ventral subiculum (vSUB) and prelimbic cortex (PL), these regions do not provide any appreciable innervation of PVH, but do issue projections to the aBST. ACTH, adrenocorticotropic hormone; Amyg, amygdala; cc, corpus callosum; CRF, corticotropin-releasing factor; Glu, glutamate; HF, hippocampal formation; LS, lateral septum; ot, optic tract; Pit., pituitary gland; PVH, paraventricular nucleus of the hypothalamus; PVT, paraventricular thalamic nucleus.

consists predominantly of pyramidal-like glutamatergic neurons, is also implicated in stimulating emotional stress-induced HPA activation (Bhatnagar et al., 2004). One likely scenario is for BLA to access the PVH via the BLA-to-CeA pathway widely implicated as the direction of information flow for the genesis of fear-related autonomic and behavioral responses (Pitkanen et al., 1997; LeDoux, 2000), then proceeding via a CeA (GABA) → aBST (GABA) → PVH pathway.

The model as proposed may also help to advance our understanding of the circuits and mechanisms accounting for GC receptor-mediated negative feedback. A number of cell groups implicated in inhibiting emotional stress-induced HPA activation (i.e., PL, HF, PVTp, lateral septum) are also capable of imparting GC receptor-mediated negative feedback on the axis (Feldman and Conforti, 1980a; Jacobson and Sapolsky, 1991; Diorio et al., 1993; Jaferi and Bhatnagar, 2006). This raises the possibility that aBST may integrate steroid-dependent feedback information from the limbic forebrain for conveyance to the PVH. Evidence increasingly suggests that GC negative feedback in the limbic forebrain may be mediated via an endocannabinoid signaling mechanism. For example, Hill and colleagues (2011) recently reported that GC receptor activation in mPFC neurons mobilizes the release of endocannabinoids and increases excitatory outflow from principal neurons via the presynaptic inhibition of GABA release from local interneurons (Hill et al., 2011). Understanding of a broader circuitry for imparting inhibitory influences over the stress axis should allow for assessment of whether GC negative feedback is restricted to upstream mediators, or whether aBST can intercede for these influences as a proximate source of steroid-mediated feedback, and, the generality of endocannabinoid signaling in relaying GC-dependent feedback in other components of the network.

Finally, if the proposed framework is inhibitory in nature, this helps to clarify a fundamentally important question of what drives the initial activation of PVH and HPA output during stress. As previously noted, IL appears to exert an excitatory influence on HPA output via a distinct realy in aBST, and may comprise one of the upstream components for an activating network. The idea that a non-GABAergic subpopulation of aBST neurons relays excitatory influences from IL to the PVH is consistent with evidence that indiscriminate excitotoxin lesions in aBST reliably attenuate acute emotional stress-induced HPA output (Choi et al., 2007). One proposal from Choi and colleagues (2007) is that the non-neuroendocrine CRF-expressing subpopulation within aBST (corresponding to the fusiform subdivision of Dong et al., 2001)

may provide a source of excitatory input into PVH, and future studies will help to clarify this relationship further. At least with regard to physiological stress, HPA output appears to be mediated predominantly via medullary aminergic inputs to PVH, as ablation of this pathway completely blocks central and peripheral indices of HPA activation under exposure to these challenges (Ritter et al., 2003; Schiltz and Sawchenko, 2007). By contrast, this pathway does not mediate HPA activation during acute emotional stress (Ritter et al., 2003; Schiltz and Sawchenko, 2007), and evidence for an equivalent activating system under this category of challenges remains elusive.

CHRONIC STRESS-INDUCED NETWORK REORGANIZATION

Chronic stress induces profound structural and synaptic changes in a variety of limbic forebrain regions. mPFC (ACd, PL, and IL) and CA3 hippocampal neurons show regressive alterations in apical dendritic and synapse morphology (Watanabe et al., 1992; Magarinos and McEwen, 1995a; Cook and Wellman, 2004; Radley et al., 2004, 2006b; Stewart et al., 2005; Hajszan et al., 2009), whereas amygdala (BLA) neurons show increases in these indices (Vyas et al., 2002, 2006). These changes are paralleled by reductions in gray matter volume and functional impairments in mPFC and HF of depressed individuals (Sheline et al., 1996, 2003; Drevets et al., 1997). Generally speaking, stress-induced structural plasticity is dependent on elevated GCs and excitatory glutamatergic signaling (Liu et al., 2008; Magarinos and McEwen, 1995b). Such structural alterations have been linked with disruptions in learning and memory (Luine et al., 1994; Stewart et al., 2005; Liston et al., 2006; Dias-Ferreira et al., 2009; Holmes and Wellman, 2009; cf. Conrad, 2010), and increases in anxiety-like behaviors (e.g., elevated plus maze performance; Mitra et al., 2005). HPA axis hyperactivity (i.e., sensitization, facilitation) is also widely documented to result from chronic stress (Ottenweller et al., 1989; Dallman et al., 1992; Willner, 1997; Bhatnagar and Dallman, 1998; Figueiredo et al., 2003a; Weinberg et al., 2010), although its relation to structural plasticity in the limbic forebrain remains to be thoroughly examined.

Much of the previous literature relevant to studying the effects chronic stress on structural plasticity entailed examination of dendritic branching patterns. This is likely due to the fact that stress and GCs produce robust changes on neurons that are readily manifest at the morphological level, and that changes in dendritic branching patterns (i.e., complexity, length, branch numbers) generally were thought to correlate with changes in synaptic connectivity. Nonetheless, increasing attention has been given to more detailed analyses of synaptic alterations in neural circuits following chronic stress, given their critical role as junctional points of connectivity that mediate information flow between neurons. Dendritic spines represent sites of postsynaptic contact for the majority of excitatory synaptic input in cortical structures. Spines are highly motile and dynamic structures that exhibit a wide degree of morphological diversity, with alterations in shape and number providing a cellular correlate for learning capacity, learning, and memory (Bailey and Kandel, 1993; Sorra and Harris, 2000; Kasai et al., 2003; Wilbrecht et al., 2010). Despite the heterogeneity of spine morphology, their classification into broad categories has proven useful. For instance, long and thin spines tend to be regarded as immature, and are more abundant during development, whereas mushroom-shaped spines (large diameter head, small diameter neck) represent stronger, more well-established excitatory synapses.

A number of studies suggest that chronic stress leads to a net loss of excitatory synapses in PL neurons (Radley et al., 2006b; Michelsen et al., 2007; Liu and Aghajanian, 2008; Arnsten, 2009). Notably, dendritic spines in the distal portions of the apical dendritic tree appear to be most profoundly impacted by chronic stress, inclusive of retraction of distal processes and decreases in spine density (**Figure 6**) (Radley et al., 2006b; Liu and Aghajanian, 2008). We conducted a high-throughput analysis of over 17,000 dendritic spine morphologies in PL and ACd pyramidal neurons (Radley et al., 2008a), and found that chronic stress resulted in an overall decrease in apical dendritic spine density, manifested by a loss of mushroom-shaped spines, and an increased frequency of long and thin spines. Another recent report employing two-photon microscopic *in vivo* imaging of spines in has provided the most compelling evidence to date for the capacity of GCs to mediate stress-induced spine alterations in the cortex (i.e., primary motor, secondary motor, somatosensory; Liston and Gan, 2011). Whereas acute GC exposure increased the rate of spine turnover (elimination and formation), prolonged GC exposure selectively increased the elimination of spines, particularly ones that were older and more stable. These, and other studies (Michelsen et al., 2007; Liu and Aghajanian, 2008), support the idea that chronic stress, via increases in GC levels, may selectively target the mature, stable population of excitatory synapses throughout cortical structures.

Chronic stress has also been shown to decrease synapse and spine density in hippocampal neurons (Sousa et al., 2000; Sandi et al., 2003; Stewart et al., 2005; Hajszan et al., 2009). In one of the more rigorous demonstrations of this phenomenon, one study employed electron microscopy (EM) and stereological 3-D reconstructions in, finding that chronic stress induced significant decreases in dendritic spine density and synapse number, and was reversible following a stress-free recovery period (Sandi et al., 2003). In another study employing EM, Magarinos and colleagues (1997) reported ultrastructural differences in presynaptic terminals of synapses in the mossy fiber pathway in CA3 neurons following chronic stress, indicative of an up-regulation of presynaptic activity and release of glutamate. Taken together, decreases in density and in overall numbers of postsynaptic excitatory contacts may help to limit the extent of excitotoxic damage that would otherwise result from prolonged activation of glutamatergic synapses under chronic stress.

A number of studies have begun to identify the cellular mechanisms underlying chronic stress-induced spine synapse loss (for reviews, see Arnsten, 2009; Duman and Voleti, 2012). For example, reduced expression of certain neurotrophic/growth factors (notably, brain-derived neurotrophic factor) in the hippocampus, and more recently in mPFC, may contribute to dendritic spine synaptic compromise in these regions (Liu et al., 2012; Nibuya et al., 1995; Kuipers et al., 2003). Alterations in protein kinase C signaling have also been shown to underlie dendritic spine loss in mPFC (Hains et al., 2009). Finally, the mammalian target of rapamycin (mTOR) signaling pathway has recently been

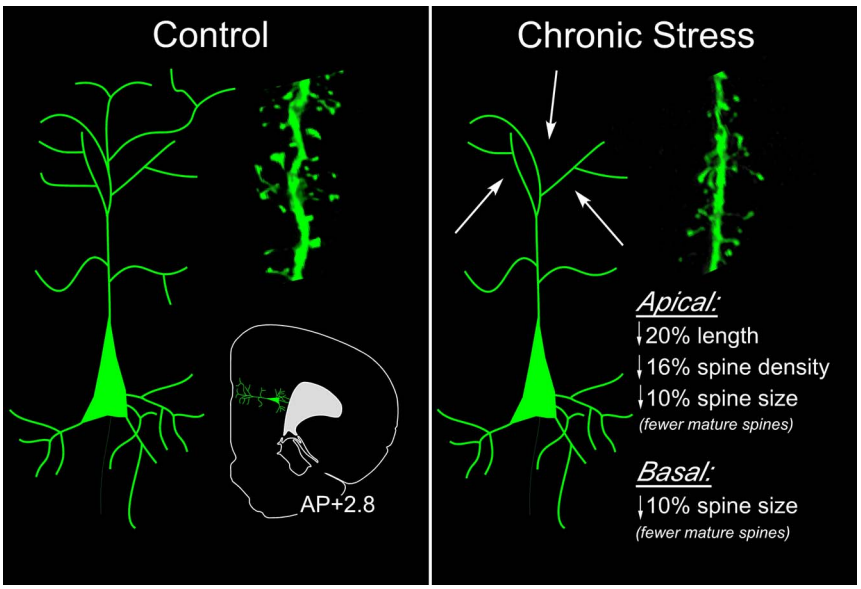

FIGURE 6 | Summary of effects of chronic stress on structural plasticity in mPFC pyramidal neurons. In these studies (Radley et al., 2004, 2006b, 2008a), high resolution analyses were performed in digitally reconstructed dendritic segments from fluorescent dye-injected pyramidal neurons in dorsal anterior cingulate (ACd) and prelimbic (PL) areas. An atlas plate (lower left) depicts the approximate region within mPFC that neurons were filled for morphologic analyses. Distance in millimeters relative to bregma is indicated. Arrows highlight the fact that dendritic atrophy and spine/excitatory synapse loss is most prominent on distal apical dendrites (right). Spine morphologic analyses reveal fewer spines with mature (stubby, mushroom-shaped), and a greater number with immature (long and thin) phenotype.

implicated in synaptic deficits that result from excessive glutamatergic stimulation, such as that which ensues under chronic stress (Magarinos and McEwen, 1995b; Li et al., 2011). These studies highlight potentially important cellular mechanisms for investigating their role in the circuit alterations underlying neuroendocrine adjustments following chronic stress.

From the network perspective, large-scale decreases and destabilization of the excitatory synapse population in mPFC and HF could uncouple excitatory afferent input from excitatory outflow in PL and/or HF, resulting in their diminished influence over PVH-projecting GABAergic neurons in aBST. Concurrent increases in BLA neuronal dendritic branching and spine densities could also drive disinhibition of PVH-projecting GABAergic neurons, via increasing activation in the extrinsic GABAergic projections from CeA. One challenge concerns whether changes throughout the entire network are necessary for HPA axis hyperactivity following chronic stress, or whether this phenotype is regulated by a distinct pathway or mechanism. As many of the stress-related changes in HF have been demonstrated more dorsally in CA3, and to some extent in DG and CA1 (e.g., Sousa et al., 2000; Snyder et al., 2011) it is unclear whether vSUB serves as a way station, or whether stress effects within vSUB proper (or ventral hippocampus) account for alterations in excitatory outflow to aBST. Another issue concerns the fact that little is known about how chronic stress impacts other cell groups implicated in the stress-inhibitory network, such as PVTp and lateral septum. The fact that GCs appear capable to exert widespread effects throughout the cortex, inclusive of sensorimotor regions (Liston and Gan, 2011), poses additional challenges in teasing apart neural circuits that underlie stress-related behavioral and physiological alterations.

SUMMARY

It has been previously established that the HPA axis response to emotional stress involves a network of limbic forebrain afferents that exert their effects on the PVH via multisynaptic and parallel pathways. Recent evidence lends support for at least two limbic cortical regions, mPFC and HF, that impart their inhibitory influences over the stress axis by converging on a discrete target, the aBST, that in turn inhibits the PVH and HPA activity. Importantly, GABAergic neurons in the aBST exhibit the capacity to integrate the inhibitory prefrontal and hippocampal influences that they impart on the stress axis. There are a number of hypotheses that derive from this model that should help to inform future work. One idea is that aBST serves as a neural hub for receiving and integrating stress-modulatory influences from other limbic forebrain regions (i.e., PVTp, septum, amygdala). Another implication is that this network, notably via GABAergic relays in aBST, may serve to integrate GC receptor-mediated negative feedback signals from some, if not all of, these limbic forebrain regions via a presynaptic endocannabinoid signaling mechanism. As this network is inhibitory, this may help to inform the search for HPA-activating networks; i.e., something akin to the medullary aminergic inputs to PVH that are known to drive HPA output in response to physiological stressors.

Chronic stress-induced neuroplasticity throughout the limbic cortex, or within key regions, may lead to an attrition of their excitatory influence on the PVH-projecting GABAergic cell population in aBST, producing HPA axis hyperactivity. A key feature of this hypothesis is that regressive changes are evident in regions that normally serve to inhibit HPA axis activation during emotionally stressful experiences, and hypertrophic

changes (i.e., increased branching and synapse number) are evident in regions that contribute an excitatory influence on HPA output. Whereas considerable gains have been made in understanding the cellular mechanisms underlying dendritic spine dynamics, much of this work has not been applied to enhance our knowledge of how chronic stress leads to long-term changes in neuroendocrine function. Moreover, examination of why stress and GCs have bidirectional effects on excitatory synapse plasticity in BLA, relative to HF and mPFC, may shed light on what are likely to be categorically distinct effects on the regulation of gene expression in these cell types that are imparted by GCs. Finally, susceptibility to stress-related psychiatric illnesses depends on a number of factors (e.g., genetics, early-life experiences, previous stress exposure) that may help to explain why some individuals go on to develop stress-related disorders while others do not. The extent to which structural plasticity in limbic cortical regions is predictive of adaptation or failure of stress/HPA control systems is of fundamental importance for informing the issue of how these more complex hereditary and environmental factors may tip the balance between stress resilience and pathology.

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Regulation of excitatory synapses and fearful memories by stress hormones

Harm J. Krugers[1]*, Ming Zhou[1], Marian Joëls[1,2] and Merel Kindt[3]

[1] *Center for Neuroscience, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, Netherlands*
[2] *Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, Netherlands*
[3] *Department of Clinical Psychology, University of Amsterdam, Amsterdam, Netherlands*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA
Sarina Rodrigues, Oregon State University, USA
Bruce McEwen, The Rockefeller University, USA

****Correspondence:***
Harm J. Krugers, Center for Neuroscience, Swammerdam Institute for Life Sciences, University of Amsterdam, Science Park 904, 1098 XH, Amsterdam, Netherlands.
e-mail: h.krugers@uva.nl

Memories for emotionally arousing and fearful events are generally well retained. From the evolutionary point of view this is a highly adaptive behavioral response aimed to remember relevant information. However, fearful memories can also be inappropriately and vividly (re)expressed, such as in posttraumatic stress disorder. The memory formation of emotionally arousing events is largely modulated by hormones, peptides, and neurotransmitters which are released during and after exposure to these conditions. One of the core reactions in response to a stressful situation is the rapid activation of the autonomic nervous system, which results in the release of norepinephrine in the brain. In addition, stressful events stimulate the hypothalamus–pituitary–adrenal axis which slowly increases the release of glucocorticoid hormones from the adrenal glands. Here we will review how glucocorticoids and norepinephrine regulate the formation of fearful memories in rodents and humans and how these hormones can facilitate the storage of information by regulating excitatory synapses.

Keywords: glucocorticoids, norepinephrine, fear conditioning, AMPA

INTRODUCTION

In our daily life we face many emotionally arousing and stressful experiences, ranging from small displeasures to major life events such as accidents or loss of relatives. The perception of these events results in behavioral and physiological responses which enable adaptation to these potentially threatening situations (Chrousos, 1998; Kim and Diamond, 2002; de Kloet et al., 2005). Enhanced memory for stressful experiences is a highly adaptive behavioral response, which helps to remember relevant information (McGaugh, 2000) and prepares individuals to cope appropriately with similar events in the future (de Kloet et al., 1999).

One of the core neuro-endocrine reactions in response to a stressful situation is the rapid activation of the autonomic nervous system (ANS), which results in the release of norepinephrine in the brain, in part by neurons located in the locus coeruleus. These noradrenergic projections regulate neuronal function via β-adrenergic receptors in areas that are critically involved in learning and memory such as the hippocampus, prefrontal cortex, and amygdala (Foote et al., 1983; Gibbs and Summers, 2002; Roozendaal et al., 2009). Stressful events also stimulate activation of the hypothalamus–pituitary–adrenal (HPA) axis, which leads to a slow increase in the release of glucocorticoid hormones from the adrenal cortex (corticosterone in most rodents; cortisol in humans). These hormones enter the brain and bind to two subtypes of discretely localized receptors, i.e., the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), which (like adrenergic receptors) are expressed in regions that are critical for memory formation such as hippocampus, amygdala, and prefrontal cortex (de Kloet et al., 2005). MRs are occupied when hormone levels are low; these receptors exert their effects classically via the genome. GRs have a 10-fold lower affinity for corticosterone, become substantially activated when hormone levels rise after stress and exert slow genomic actions in cells carrying the receptor. Recent evidence has revealed that corticosteroid hormones can also regulate synaptic function via non-genomic effects, both via activation of MRs and GRs (Orchinik et al., 1991; Venero and Borrell, 1999; Di et al., 2003; Karst et al., 2005, 2010; Groc et al., 2008).

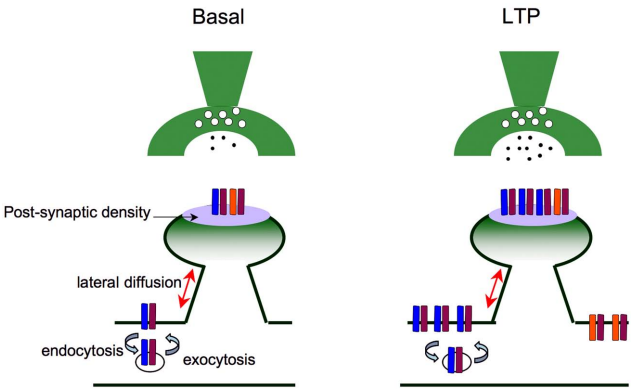
In this review we will highlight behavioral studies emphasizing how norepinephrine and glucocorticoids, via their receptors, regulate fearful memories, both in rodents and humans. Second, we will address the cellular mechanism by which norepinephrine and glucocorticoids promote learning and memory processes by focusing on regulation of excitatory synapses. Recent studies have revealed that these hormones modulate these synapses by regulating the function of AMPA type glutamate receptors (Karst et al., 2005; Hu et al., 2007; Groc et al., 2008; Martin et al., 2009; Yuen et al., 2009, 2011; Krugers et al., 2010; Liu et al., 2010; Tenorio et al., 2010), which are critically involved in synaptic transmission and activity-dependent changes in synaptic transmission – a major cellular model for learning and memory (Malinow and Malenka, 2002; Malenka, 2003; Neves et al., 2008; Kessels and Malinow, 2009; **Box 1**).

FEAR CONDITIONING AND INHIBITORY AVOIDANCE

Various tasks are being used to examine hormonal regulation of emotional memories. Here we will briefly address two of the most used behavioral tasks, Pavlovian fear conditioning and

Box 1 | Excitatory synapses, plasticity, and memory.

Changes in synaptic connectivity are generally believed to underlie learning and memory processes (Doyere and Laroche, 1992; Bliss and Collingridge, 1993; Neves et al., 2008). Plasticity at synapses can be regulated at the presynaptic site (by changing the release of neurotransmitters) and/or the postsynaptic site (by changing the function and number of their receptors; Malinow and Malenka, 2002). The most explored forms of plasticity at excitatory synapses are *N*-methyl-D-aspartic acid receptor (NMDAR)-dependent long-term potentiation (LTP) and long-term depression (LTD), which have been associated with changes in postsynaptic signaling (Bliss and Collingridge, 1993; Neves et al., 2008).



Long-term potentiation (LTP) reflects a long-lasting increase in synaptic connectivity (Neves et al., 2008) that can be experimentally elicited by high-frequency stimulation or by afferent stimulation in combination with postsynaptic depolarization (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). NMDA receptors play a critical role in the induction of LTP. This receptor is a unique ligand-gated ion channels since activation requires binding of glutamate as well as membrane depolarization which is needed to release the magnesium block of the channel and to open the channel with high probability (Nowak et al., 1984). Therefore, the NMDA receptor functions as a coincidence detector that determines specificity and associativity of synaptic potentiation. Activation of NMDA receptors allows Ca^{2+} influx into dendritic spines of postsynaptic neurons which activates calcium-dependent enzymes, such as calcium/calmodulin-dependent calcium kinase II (CaMKII; Barria et al., 1997), protein kinase A (PKA; Man et al., 2007), and protein kinase C (Boehm et al., 2006). These kinases impact synaptic transmission, including regulation of the function of AMPA receptors (Lledo et al., 1995; Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997; Lee et al., 2000; Boehm et al., 2006; Derkach et al., 2007). Moreover, these enzymes may help to organize structural process that leads to the incorporation of AMPA receptor-binding proteins into the postsynaptic density (PSD), followed by subsequent anchoring or additional AMPA receptors (Lisman and Zhabotinsky, 2001).

AMPA receptors are highly mobile and the link between AMPA receptor surface diffusion and cycling is evident in synaptic plasticity paradigms. Recent studies have shown that AMPA receptor trafficking is regulated by both exocytotic and endocytotic processes and by their surface lateral diffusion in the plasma membrane (Kennedy and Ehlers, 2006; Shepherd and Huganir, 2007; Newpher and Ehlers, 2008). Endocytosis of AMPA receptors is important for the number of AMPA receptors at the membrane surface and recycling endosomes supply AMPA receptors for LTP (Park et al., 2004). Receptor recycling from postsynaptic endocytic zones appears to be crucial for maintaining a mobile population of surface AMPA receptors that can be synaptically inserted to increase synaptic strength (Blanpied et al., 2002; Lu et al., 2007; Petrini et al., 2009). Together, the regulation of synaptic AMPA receptor number relies on a dynamic equilibrium between intracellular, extrasynaptic, and synaptic pools, and is regulated by the activity status of the neuronal network (Makino and Malinow, 2009; Petrini et al., 2009).

The trafficking of AMPA receptors governs rules that appear to be dependent on the subunit composition: the GluA1 carboxyl terminus mediates regulated delivery of AMPARs onto synapses upon synaptic activation while the GluA2 carboxyl terminus determines the continuous delivery of AMPARs onto synapses independent from synaptic stimulation (Shi et al., 2001). Upon LTP induction, GluA1-containing calcium-permeable AMPA receptors are incorporated into synaptic membrane, rapidly, and transiently from intracellular reserve pool (Shi et al., 2001), and are replaced by GluA1-lacking calcium-impermeable AMPA receptors shortly after LTP induction (Plant et al., 2006). Functionally, these GluA1-lacking AMPA receptors (such as GluR2/3) are calcium-impermeable (Burnashev et al., 1992; Kauer and Malenka, 2006; Plant et al., 2006) and may play a role in maintaining synaptic strength (Malinow and Malenka, 2002; Malenka, 2003; Kauer and Malenka, 2006; Plant et al., 2006).

inhibitory avoidance (IA) learning. Pavlovian fear conditioning is a behavioral paradigm that can be used to study the memory formation of emotionally arousing events, both in rodent animals and humans (e.g., Nader et al., 2000; Kindt et al., 2009). In fear conditioning, an emotionally neutral conditioned stimulus (CS) such as a tone or light is paired with an aversive CS such as a foot shock unconditioned stimulus (US). After pairing, the CS elicits defensive behavior, of which freezing behavior is most frequently studied (Rodrigues et al., 2009). The amygdala is critically involved in fear conditioning (LeDoux, 2000): the lateral amygdala (LA) receives auditory, visual, olfactory, and somatosensory information from the thalamus and cortex, and plasticity in the LA is believed to underlie the association between the CS (cue) and US (Rogan et al., 1997). The hippocampus also plays a role in fear conditioning in that it provides information about the context of a fearful event (LeDoux, 2000). Finally, the medial prefrontal cortex regulates the expression and control of fear responses (LeDoux, 2000). A second task that is widely used to examine the memory formation of emotionally arousing events is IA training. In IA training, rodents are placed in a light chamber and can subsequently enter a dark chamber. Upon entry of this chamber, animals receive a footshock, which is well remembered. Inhibitory avoidance memory formation is believed to be hippocampal dependent (e.g., Whitlock et al., 2006) with the amygdala playing a modulatory role (McGaugh, 2000). In addition, regulating prefrontal cortex function by the amygdala regulates memory consolidation in this task (e.g., Barsegyan et al., 2009).

NOREPINEPHRINE, GLUCOCORTICOIDS, AND FEARFUL MEMORIES IN RODENTS

Norepinephrine and corticosteroid hormones, via their receptors, mediate (at least in part) the memory enhancing effects of stress

and emotion (Joëls et al., 2006, 2011; Roozendaal et al., 2009). Norepinephrine enhances memory formation of emotional events via brain β-adrenergic receptors: application of norepinephrine or β-adrenergic receptor agonists promotes memory consolidation in various aversive memory tasks such as IA task, fear conditioning, and in Morris water-maze learning (Hu et al., 2007; Roozendaal et al., 2009; but see also Hatfield and McGaugh, 1999; Lee et al., 2001; Bush et al., 2010), and blocking β-adrenergic receptors reduces contextual fear memories (Ji et al., 2003). Activation of α-adrenergic receptors also enhances memory, but presumably act by enhancing β-adrenergic actions (Ferry et al., 1999a,b). Finally, noradrenaline has been reported to enhance reconsolidation of information (e.g., Debiec and LeDoux, 2006).

Corticosteroid hormones, via MRs have been implicated in the appraisal, and response selection during the learning process (Oitzl and de Kloet, 1992; Sandi and Rose, 1994). Recent studies provide evidence that MRs are also involved in encoding of information, possibly linked to effects on appraisal, and/or response selection: application of the MR antagonist spironolactone prior to training lastingly suppress the expression of fear (Zhou et al., 2010). Moreover, genetic deletion of MRs in the forebrain led to various cognitive impairments, including impaired learning in a Morris water-maze task (Berger et al., 2006) and reduced fear learning (Zhou et al., 2010). Via GRs, corticosteroid hormones have been reported to promote long-term consolidation of information (de Kloet et al., 1999; Joëls et al., 2006; Roozendaal et al., 2009). For instance, a point mutation in the mouse GR was found to impair spatial memory formation (Oitzl et al., 2001), and blocking GRs impairs fear conditioning (Pugh et al., 1997a; Donley et al., 2005). In agreement, in several fearful learning paradigms, including fear conditioning and IA learning, post-training application of corticosterone, or GR agonists promoted the consolidation of information (Corodimas et al., 1994; Sandi and Rose, 1994; Pugh et al., 1997b; Hui et al., 2004; Roozendaal et al., 2009). These studies imply that GRs are involved in consolidation of fearful information and that genomic actions are involved. This does not exclude the possibility that other GR-dependent pathways are also involved. For instance, a recent study suggested that membrane-associated GRs also promote long-term memory in an object recognition task via chromatin modification (Roozendaal et al., 2010). Thus, it is possible that both non-genomic as well as genomic actions of corticosteroid hormones, via GRs, promote the storage of relevant information.

In addition to these well-documented effects of stress and glucocorticoids on consolidation processes, these hormones also affect memory retrieval mechanisms (de Quervain et al., 1998) and extinction processes (Brinks et al., 2009). Exposure to stress and elevated corticosteroid levels hampers the retrieval of already stored information (de Quervain et al., 1998) and glucocorticoids promote the extinction of information (de Kloet et al., 1999). Finally, blocking GRs has been reported to hamper reconsolidation of cue-conditioned fear (Pitman et al., 2011). Taken together, there is ample evidence that corticosteroid hormones, via activation of MRs and GRs, exert a repertoire of behavioral effects that promote the consolidation of relevant (fearful) information, facilitate the extinction of information that is no longer relevant, and ultimately favor behavioral adaptation (de Kloet et al., 1999).

Corticosteroids act in concert with other hormones such as norepinephrine (Roozendaal et al., 2009), endocannabinoids (Campolongo et al., 2009), corticotropin releasing hormone (CRH; Roozendaal et al., 2008) for optimal memory performance both in humans and rodents (de Quervain et al., 2009; Roozendaal et al., 2009). It is generally thought that noradrenergic activation is essential for the memory enhancing effects and that glucocorticoids play a permissive role in noradrenergic actions, thereby promoting memory formation (Hui et al., 2006; Roozendaal et al., 2006, 2009). These studies emphasize that concerted action of various stress-related mediators is required for optimal memory performance in rodents (Joëls and Baram, 2009).

NOREPINEPHRINE, GLUCOCORTICOIDS, AND FEARFUL MEMORIES IN HUMANS

The involvement of noradrenergic receptor activation in human emotional memory has been investigated by either stimulating or decreasing the release of norepinephrine (**Table 1**). Blocking the β-adrenergic receptors with propranolol selectively impairs memory performance for emotional arousing information (Cahill et al., 1994; Van Stegeren et al., 1998; Hurlemann et al., 2005; Van Stegeren, 2008). Conversely, adrenergic receptor agonist epinephrine (Cahill and Alkire, 2003) or the α_2-adrenergic receptor-antagonist yohimbine – which stimulates central noradrenergic activity by blocking the α_2-adrenergic autoreceptor (Charney et al., 1987; Peskind et al., 1995) – enhances memory consolidation of emotionally arousing information (Southwick et al., 2002). These findings support that noradrenergic receptors are critically involved in the formation of human emotional memory (McGaugh, 2004).

The effect of glucocorticoids on memory formation, is typically studied by either a stress manipulation such as the Trier Social Stress Test (TSST; Kirschbaum et al., 1993), the cold pressor test (CPT), or by administering cortisol directly. Although stress or cortisol treatment generally impairs memory retrieval (de Quervain et al., 2000), the same hormone has been reported to enhance memory consolidation (Het et al., 2005; Wolf, 2009). These memory effects of the corticosteroids are often stronger for emotional arousing material (Wolf, 2009).

Even though the memory enhancing effects of emotional arousal are extremely functional from an evolutionary perspective, the impact of emotion on memory can also have long-term detrimental consequences. Research into the effects of stress on emotional memory is highly relevant for a better understanding

Table 1 | Role noradrenergic hormones in emotional memories.

	Enhanced noradrenergic tone	
Emotional memory formation	↑	Cahill et al. (1994); Van Stegeren et al. (1998); Van Stegeren (2008); Peskind et al. (1995); Southwick et al. (2002); Soeter and Kindt (2011a)
Extinction fear conditioning	↓	Soeter and Kindt (2011a)
Reconsolidation fear	↑	Kindt et al. (2009); Soeter and Kindt (2010)
Fear generalization	↑	Soeter and Kindt (2011a)

of the etiology and maintenance of emotional disorders, such as anxiety disorders. In humans the effects of stress on memory are traditionally investigated for non-associative and distinct emotional stimuli such as emotional stories and pictures (McGaugh, 2004; Wolf, 2009). Given that patients with anxiety disorders either fear for stimuli that are intrinsically non-threatening or they persist in fear responding whilst the acute threat already disappeared (e.g., after traumatic experiences), the emotional memory literature seems to be inconclusive for the understanding of these disorders. Indeed, an important aspect of the pathogenesis of anxiety disorders is that they originate from a learned association between a previously neutral event (CS; such as a stranger) and an anticipated disaster (US; such as physical assault). This can be experimentally modeled in a differential human fear conditioning paradigm. In contrast to animal research, the effect of stress hormones such as noradrenaline on associative fear memory is not extensively studied in humans.

Another notable aspect of research into human emotional memory is that most studies did not assess the emotional response but the declarative memory for the emotional stimuli. However, not the factual recollection but the concomitant excessive emotional expression is the main problem in emotional disorders (Ehlers et al., 2004). In particular, hyper-noradrenergic activity in the wake of a life-threatening event may contribute to the "overconsolidation" of memory for trauma, generating disturbing intrusive memories that are characteristic of posttraumatic stress disorder (PTSD; Pitman and Delahanty, 2005; Glannon, 2006; Henry et al., 2007). In patients with PTSD, these involuntary traumatic memories may be experienced as reenactments of the original trauma ("flashbacks") and are associated with significant emotion and distress (DSM-IV-R; American Psychiatric Association, 2000).

In two human fear conditioning studies, we recently demonstrated that the systemic administration of the α_2-adrenergic receptor-antagonist yohimbine (20 mg) during memory formation strengthened the later expression of human associative fear memory (fear potentiated startle reflex; Soeter and Kindt, 2011a,c). More specifically, stimulation of the noradrenergic system by the administration of yohimbine during memory formation did not directly augment the differential startle fear response. Yet, the retention tests presented 48 h later uncovered that the earlier administration of yohimbine extensively delayed the process of extinction learning and generated a superior recovery of fear (reinstatement and reacquisition). The competition between the original excitatory fear association and the newly formed inhibitory memory trace determines the behavioral outcome of extinction learning (Bouton, 1993). Given that yohimbine was administered during fear conditioning (48 h prior to fear extinction), the noradrenergic manipulation apparently delayed the process of extinction by strengthening the original excitatory fear association. In addition, the yohimbine administration promoted fear generalization, a core feature of anxiety disorders (Soeter and Kindt, 2011c). In rodents, the generalization of fear seems to be dependent on the strength of the memory as operationalized by training intensity (both US intensity and the number of CS+ and US applied; Laxmi et al., 2003). Allegedly, the strengthening of a specific fear memory trace by α_2-adrenergic receptor-manipulation may produce fear generalization similar to training intensity.

The effect of β-adrenergic interference has not yet been demonstrated for the consolidation of associative fear memory. For reconsolidation, however, a series of studies showed a robust memory impairing effect of the β-adrenergic receptor blocker propranolol (Kindt et al., 2009; Soeter and Kindt, 2010, 2011b,c). Disrupting reconsolidation by propranolol (40 mg) – administered before or after memory retrieval – "deleted" the emotional expression of a fear memory in humans (Kindt et al., 2009; Soeter and Kindt, 2010, 2011b,c). The anxiolytic properties of propranolol could not explain the fear erasure, as omission of memory reactivation after propranolol intake yielded intact fear responding. Together, these recent studies illustrate the involvement of noradrenergic modulation in the (re)consolidation and generalization of human associative fear memory. Given that fear generalization is a main characteristic of anxiety disorders, these findings suggest that norepinephrine may play an important role in the etiology and maintenance of anxiety disorders.

In contrast to the noradrenergic modulation of associative fear memory, the modulatory role of cortisol seems to be more complex. A mixture of fear conditioning paradigms reveals ambiguous findings regarding the effect of cortisol on the emotional expression of associative fear memory in humans. Cue or context fear conditioning and eyeblink conditioning studies – using either a trace or delay reinforcement scheme – have shown impairing as well as enhancing effects of cortisol on associative fear memory. First, a relatively low dose of hydrocortisone (30 mg) affected cue fear conditioning, decreasing it in men and increasing it in women (Stark et al., 2006; Merz et al., 2010; Tabbert et al., 2010). In contrast to this gender effect, exposure to a stress manipulation (elevating both the sympathetic and the glucocorticoid stress response) facilitated cue fear conditioning in men but not in women (Zorawski et al., 2005, 2006; Jackson et al., 2006). Furthermore, a high dose of hydrocortisone (60 mg) exclusively enhanced context fear conditioning in both sexes, while leaving cue fear conditioning unaffected (Grillon et al., 2011). Finally, delay eyeblink conditioning is impaired in men and women after a stress manipulation (TSST; Wolf et al., 2009), whereas trace eyeblink conditioning is improved by a stress manipulation (CPT; Duncko et al., 2007) as well as by cortisol (2 mg, administered intravenously; Kuehl et al., 2010), but also by a cortisol inhibitor (1500 mg metyrapone; Nees et al., 2008). In summary, future research is required to clarify the modulatory role of cortisol on associative fear memory in humans and the possible interaction with the noradrenergic system.

EXCITATORY SYNAPSES AND LEARNING AND MEMORY

An import question that remains to be addressed is which mechanisms are involved in the effects of norepinephrine and glucocorticoids on fear learning. The current view of how memories are formed is that neurons are activated during the learning process thereby changing synaptic communication (Neves et al., 2008). AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) type glutamate receptors mediate most of the fast excitatory synaptic transmission in the brain and controlling the number of synaptic AMPA receptors on the postsynaptic membrane is an

essential mechanism to regulate synaptic transmission and plasticity (Malinow and Malenka, 2002; Plant et al., 2006; Kessels and Malinow, 2009). The best-studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD) of excitatory synaptic transmission (Malinow and Malenka, 2002; Bredt and Nicoll, 2003). LTP involves the activity-dependent recruitment of AMPA receptors to the postsynaptic membrane and a concurrent increase in AMPA-mediated transmission whereas LTD reflects a decrease in synaptic AMPA receptor function.

AMPA receptors are heteromeric tetramer complexes formed of different combinations of GluA1, GluA2, GluA3, and GluA4 subunits (Keinanen et al., 1990; Tanabe et al., 1992; Wisden and Seeburg, 1993; Hollmann and Heinemann, 1994; Wenthold et al., 1996). In adult hippocampal pyramidal neurons, two main populations of AMPA receptor complexes are found: GluA1/GluA2 and GluA2/GluA3 containing AMPA receptors. The trafficking of AMPA receptors to and from the synapse is regulated by (1) exocytotic/endocytotic recycling between intracellular and membrane receptor pools (Passafaro et al., 2001; Gerges et al., 2006); and (2) surface diffusion between extrasynaptic and synaptic receptor pools (Adesnik et al., 2005; Ashby et al., 2006; Ehlers et al., 2007; Makino and Malinow, 2009; Petrini et al., 2009; **Box 1**). The leading model for constitutive and activity-dependent AMPA receptor trafficking is that activity-dependent processes (such as induction of LTP) promote synaptic delivery of GluA1-containing AMPA receptors which are believed to be gradually replaced by the cycling GluA2/GluA3 heteromers after LTP induction (Shi et al., 2001; Plant et al., 2006).

AMPA receptors have been shown to underlie memory formation. Inhibitory avoidance training rapidly (and reversibly) increases hippocampal synaptic insertion of GluA1 and GluA2 AMPA receptor subunits (Whitlock et al., 2006). Studies using mutant mice reveal that GluA1 mutant mice are hampered in short-term memory processes (Reisel et al., 2002; Schmitt et al., 2005; Sanderson et al., 2007, 2009, 2011), while the mutation leaves Morris water-maze spatial navigation unaffected (Zamanillo et al., 1999). Moreover, GluA2 mutant mice are impaired in a spatial working memory task and elevated Y-maze (Shimshek et al., 2006). These studies indicate that GluA1 and GluA2 subunits are at least relevant for short-term memory processes. Finally, the observation that preventing synaptic insertion of GluA1-containing AMPA receptors in the amygdala hampers tone-cue fear conditioning implies that trafficking of GluA1-containing AMPA receptors is critical for fear learning (Rumpel et al., 2005).

STRESS HORMONES AND HIPPOCAMPAL EXCITATORY SYNAPSES

The cellular mechanisms via which norepinephrine and corticosterone facilitate learning and memory processes are starting to be unraveled. Here we summarize studies – mainly in the rodent hippocampus – that have examined how these hormones regulate synaptic transmission and synaptic plasticity. Recent studies have revealed that AMPA receptors are regulated by norepinephrine and glucocorticoid hormones. Via activation of β-adrenergic receptors, norepinephrine can rapidly – but reversibly – activate PKA and CaMKII (Wang et al., 2004; Hu et al., 2007) and increase the phosphorylation of GluA1 at Ser845 and Ser831. Likewise, stress, via activation of β-ARs increases phosphorylation of Ser831 and Ser845 (Hu et al., 2007). In agreement with the observations that phosphorylation of AMPA receptors at these sites is critical for LTP, activation of β-adrenergic receptors facilitates the induction of hippocampal (LTP; Thomas et al., 1996; Winder et al., 1999; Hu et al., 2007; Tenorio et al., 2010) and enhances activity-dependent synaptic insertion of AMPA receptors (Hu et al., 2007). Interestingly, activation of β-adrenergic receptors facilitates LTP in a time-dependent manner; these receptors only facilitate LTP when these receptors are activated during and shortly after induction of LTP, i.e., when the adrenergic receptors enhance phosphorylation of GluA1 (Hu et al., 2007).

Also corticosteroid hormones can rapidly and reversibly promote hippocampal synaptic transmission. Within minutes after application, glucocorticoids increase synaptic transmission in the hippocampus (Karst et al., 2005), via activation of low affinity MRs which are located in the cellular membrane. This rapid and reversible increase in synaptic transmission after glucocorticoid exposure most likely results from an increase in the presynaptic release of glutamate (Karst et al., 2005) in which the Erk pathway is critically involved (Olijslagers et al., 2008). At the same time scale, glucocorticoid exposure, via membrane MRs rapidly increases the lateral diffusion of GluA1 and GluA2 subunits, without altering the number of postsynaptic AMPA receptors (Groc et al., 2008; Martin et al., 2009). At this time, glucocorticoids, via MRs, promote the activity-dependent synaptic insertion of GluA2-containing AMPA receptors (Groc et al., 2008). Finally, glucocorticoids also facilitate LTP in at time-dependent manner; LTP is only facilitated when elevated corticosteroid levels are present at the moment of high-frequency stimulation (Wiegert et al., 2006). These studies show that both norepinephrine and glucocorticoids can rapidly facilitate synaptic plasticity and thereby increase the ability to encode information at the cellular level (**Figure 1**). While glucocorticoids and norepinephrine act in concert for optimal memory performance, they also affect synaptic function in a synergistic fashion (Joëls et al., 2011). Application of a β-adrenergic receptor agonist together with corticosterone facilitates the induction of LTP in the hippocampus (Pu et al., 2007). Moreover, activation of β-adrenergic receptors together with corticosterone enhances AMPA receptor function (Zhou et al., 2011).

After exposure to a stressful event, plasma corticosteroid levels slowly return to their pre-stress level in about 2 hours (de Kloet et al., 2005). Nevertheless, these hormones exert – via a slow, genomic mode of action – long-lasting effects on excitatory synapses (**Figure 1**). Elevated glucocorticoid levels increase the membrane expression and synaptic insertion of GluA2-containing AMPA receptors in the hippocampal neurons (Groc et al., 2008; Martin et al., 2009). These effects are mediated via GRs, require time as well as the synthesis of new proteins, and most likely result from increased lateral diffusion and/or altered ratio of endocytosis/exocytosis of GluA2-containing AMPA receptors (Groc et al., 2008; Martin et al., 2009). Functionally, glucocorticoids also slowly increase the amplitude of evoked as well as spontaneous AMPA receptor-mediated synaptic currents in hippocampal primary cultures and hippocampal slices (Karst and Joëls, 2005; Martin et al., 2009), thereby enhancing AMPA receptor-mediated

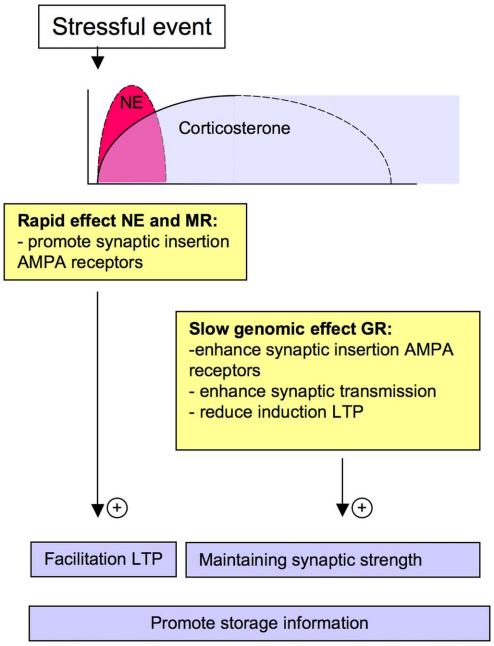


FIGURE 1 | Norepinephrine and glucocorticoids rapidly increase activity-dependent synaptic insertion of AMPA receptors. Slowly, corticosteroid hormones enhance AMPA receptor mediated synaptic transmission and reduce the ability to encode novel information. This might preserve and promote the retention of the original (fearful and relevant) memory trace (see text for details).

synaptic transmission. Furthermore, glucocorticoids – via a slow mode of action – suppress the induction of LTP (Alfarez et al., 2002; Wiegert et al., 2005), facilitate LTD (Coussens et al., 1997; Xu et al., 1997) and increase endocytosis of synaptic AMPARs upon stimuli that weaken synaptic transmission (Martin et al., 2009).

STRESS HORMONES: FROM EXCITATORY SYNAPSES TO FEARFUL MEMORIES

The release of norepinephrine and glucocorticoids promotes the consolidation of fearful memories in rodents and humans (Roozendaal et al., 2009). Recent findings indicate that stress hormones like norepinephrine and corticosterone both rapidly and slowly increase AMPA receptor mediated synaptic transmission. These differential effects on AMPA receptor trafficking may provide a cellular mechanism that underlies the memory enhancing effects of these hormones. Initially glucocorticoids and norepinephrine promote the AMPA receptor mediated synaptic transmission and synaptic insertion of AMPA receptors (Karst et al., 2005; Hu et al., 2007; Groc et al., 2008; Olijslagers et al., 2008). These effects are accompanied by an increased ability to elicit LTP (Thomas et al., 1996; Winder et al., 1999; Wiegert et al., 2006; Hu et al., 2007) and may therefore contribute to an enhanced capacity to acquire and store information (**Figure 1**).

Next, glucocorticoids via a slow genomic action enhance synaptic insertion of AMPA receptors. At the same time, glucocorticoids suppress activity-dependent increase in synaptic AMPA receptors (Groc et al., 2008), activity-dependent increase in AMPA receptor-mediated synaptic transmission (Hui Xiong, unpublished observations), and synaptic plasticity (e.g., Wiegert et al., 2005). Thus, these hormones slowly reduce the ability to encode novel information. The consequence could be that these hormones also prevent the ability to overwrite information that is present in the network, in a meta-plastic manner (Joëls et al., 2006; Krugers et al., 2010), thereby preserving the original memory trace. Furthermore, glucocorticoids promote the loss of synaptic AMPA receptors which is enhanced upon stimuli that reduce synaptic transmission (Martin et al., 2009), thereby accentuating synaptic efficacy. This provides a picture where glucocorticoids, via MRs, and β-adrenergic receptor activation rapidly enhance the ability to store information, which is consolidated and accentuated via activation of GRs (Krugers et al., 2010; **Figure 1**).

FUTURE PERSPECTIVES

There are a number of relevant issues which need to be addressed:

(1) First, it is unknown how activation of MRs and GRs enhance (activity-dependent) synaptic insertion of AMPA receptors. Potential candidates are enzymes that regulate the phosphorylation of AMPA receptors, regulators of endocytosis/exocytosis (Liu et al., 2010), and/or proteins that promote transport and synaptic retention of AMPA receptors (Nicoll et al., 2006).

(2) A behaviorally very relevant question is whether AMPA receptors mediate the memory enhancing effects of stress hormones. Studies using mice carrying mutations in the GluR1 phosphorylation sites indicate that norepinephrine-regulated phosphorylation of GluR1 facilitates emotional memory (Hu et al., 2007). Moreover, application of pep2m, which blocks trafficking of GluA2-containing AMPA receptors also prevents the memory enhancing effects of stress (Conboy and Sandi, 2010), and fearful memories (Migues et al., 2010). Also, stress-induced regulation of Rab4/SGK may underlie stress-effects on AMPA receptor function and stress-effects on working memory (Yuen et al., 2011). Studies using temporal erasure of functional AMPA receptors will be required to reveal whether regulation of AMPA receptor function is critical for stress-induced facilitation of the different learning phases (such as acquisition and/or consolidation of information).

(3) The studies carried out so far mainly focused on the hippocampal formation. However, region-specific effects of stress hormones on excitatory synapses – even in the hippocampus – need to be considered. For example, in an elegant series of studies it was shown that corticosteroid hormones may have different effects on synaptic plasticity within the hippocampal formation; corticosteroid hormones suppress synaptic plasticity in de dorsal hippocampus but enhance synaptic plasticity in the ventral hippocampus (Maggio and Segal, 2007, 2009; Segal et al., 2010).

Moreover, other brain areas such as prefrontal cortex and amygdala are also critically involved in the regulation of fearful memories. It will therefore also be necessary to carefully investigate the effects of stress hormones on excitatory synapses in

these brain areas. Indeed corticosteroid hormones have been reported to affect AMPA receptor mediated synaptic transmission in the amygdala (Karst et al., 2010) differently from the hippocampus (see Karst et al., 2005), and stress and corticosteroid hormones regulate AMPA receptors (Yuen et al., 2011) and function of the prefrontal cortex (Arnsten, 2009).

(4) Behaviorally, several neurotransmitters (e.g., norepinephrine, enodocannabinoids, dopamine), neuropeptides, and steroid hormones (e.g., corticosteroid hormones; Joëls and Baram, 2009) may act together for optimal memory performance (de Quervain et al., 2009; Roozendaal et al., 2009) and cellular plasticity (Pu et al., 2007). It will therefore be relevant to examine whether and how these stress-mediators interact to regulate AMPA receptor function as well as learning and memory.

(5) In susceptible individuals, memories for aversive events may remain inappropriately present and lead to anxiety disorders such as in (PTSD; de Kloet et al., 2005). This underscores the importance of understanding how individual differences in cognitive development, and the ability to cope with threatening events later in life, are determined. These differences are largely regulated by environmental factors, in particular during the early postnatal period – in conjunction with genetic factors – (Hackman et al., 2010). When comparing rodent offspring of mothers that exhibited low levels of maternal care with the adult offspring of mothers that exhibited high levels of maternal care, enhanced memories for fearful events and increased anxiety was observed (Weaver et al., 2006; Champagne et al., 2008). Also, maternal deprivation results in enhanced fear learning (Oomen et al., 2010). It will therefore be important to examine how stress hormones promote the retention of stressful memories and regulate molecular mechanisms that are fundamental for learning and memory (such as AMPA receptors) in individuals who suffered from negative early life experiences.

(6) Finally, studies over the past decade have shown that stored memories are rendered labile after being retrieved, and require *de novo* protein synthesis for reconsolidation (Nader et al., 2000). Reconsolidation has been demonstrated in various tasks and species (Nader et al., 2000; Eisenberg et al., 2003; Sangha et al., 2003), including humans (Kindt et al., 2009; Schiller et al., 2010). The notion that stored memories can be turned into a labile state has opened new avenues to reduce the expression of fear more permanently than the traditional extinction procedure (Pitman and Delahanty, 2005), e.g., by targeting noradrenergic receptors (Pitman et al., 2002; Orr et al., 2006; Brunet et al., 2008; Kindt et al., 2009; Soeter and Kindt, 2010, 2011b,c) and corticosteroid receptors (Barrett and Gonzalez-Lima, 2004; Cai et al., 2006; Abrari et al., 2008). Future studies will be needed to test whether targeting stress hormones and their receptors can be used to effectively reduce fear and whether these fear-reducing effects are mediated via AMPA receptors (Clem and Huganir, 2010).

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The roles of the actin cytoskeleton in fear memory formation

Raphael Lamprecht*[1,2,3]

[1] Faculty of Natural Sciences, Department of Neurobiology and Ethology, University of Haifa, Haifa, Israel
[2] Center for Gene Manipulation in the Brain, University of Haifa, Haifa, Israel
[3] Center for Brain and Behavior, University of Haifa, Haifa, Israel

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Farah Lubin, University of Alabama at Birmingham, USA
Michael J. Schell, Uniformed Services University, USA
Brian Morris, University of Glasgow, UK

***Correspondence:**
Raphael Lamprecht, Department of Neurobiology and Ethology, University of Haifa, Haifa 31905, Israel.
e-mail: rlamp@research.haifa.ac.il

The formation and storage of fear memory is needed to adapt behavior and avoid danger during subsequent fearful events. However, fear memory may also play a significant role in stress and anxiety disorders. When fear becomes disproportionate to that necessary to cope with a given stimulus, or begins to occur in inappropriate situations, a fear or anxiety disorder exists. Thus, the study of cellular and molecular mechanisms underpinning fear memory may shed light on the formation of memory and on anxiety and stress related disorders. Evidence indicates that fear learning leads to changes in neuronal synaptic transmission and morphology in brain areas underlying fear memory formation including the amygdala and hippocampus. The actin cytoskeleton has been shown to participate in these key neuronal processes. Recent findings show that the actin cytoskeleton is needed for fear memory formation and extinction. Moreover, the actin cytoskeleton is involved in synaptic plasticity and in neuronal morphogenesis in brain areas that mediate fear memory. The actin cytoskeleton may therefore mediate between synaptic transmission during fear learning and long-term cellular alterations mandatory for fear memory formation.

Keywords: actin cytoskeleton, fear memory, synaptic plasticity, amygdala, hippocampus

Long-term memory (LTM) formation is believed to involve alterations of synaptic efficacy produced by modifications in neural transmission caused by physiochemical and/or structural modifications of synaptic communication within neuronal networks (Konorski, 1948; Hebb, 1949; Dudai, 1989; Bliss and Collingridge, 1993; Martin et al., 2000; Tsien, 2000; Kandel, 2001; Lamprecht and LeDoux, 2004). A prime challenge is to identify molecules involved in sustaining synaptic alterations and memory formation. Actin is a most attractive candidate to play a key role in memory formation as it is responsive to synaptic signaling, such as triggered during learning, and consequently may mediate cellular events that underlie changes in synaptic efficacy, such as synaptic transmission and morphology.

Actin cytoskeleton is involved in many key cellular processes including cellular morphogenesis, motility, division, and intracellular transport. Actin exists in two states in cells, either as a globular monomer (G-actin) or following head-to-tail interaction as a polymer to form filamentous F-actin. Actin remodeling and the structure of F-actin network are tightly regulated by actin-binding proteins (Luo, 2000; Dillon and Goda, 2005). These actin cytoskeleton-regulatory proteins mediate between intrinsic and extrinsic cellular signals and actin-dependent cellular functions. Thus, by forming such intricate network of filaments responsive to regulatory signals, actin mediates a large variety of cellular functions from supporting cellular morphology to providing contractile forces needed for cellular activities including cell division and transport of vesicles. Actin monomers and filaments are abundant in presynapses and postsynapses and act to regulate key neuronal processes such as alterations in synaptic transmission and morphology (Luo, 2002; Dillon and Goda, 2005; Cingolani and Goda, 2008). Changes in synaptic transmission and neuronal morphology are involved in the process of memory formation (Lamprecht and LeDoux, 2004).

This review is focused on the roles of the actin cytoskeleton in fear memory formation, in particular in the lateral amygdala (LA) and hippocampus brain regions shown to be involved in fear conditioning. Fear conditioning is a useful behavioral paradigm used to study brain mechanisms underlying fear memory formation. In fear conditioning an association is formed between a neutral conditioned stimulus (CS), such as a tone, and an aversive unconditioned stimulus (US), typically a mild footshock (LeDoux, 2000; Davis and Whalen, 2001; Schafe et al., 2001; Sah et al., 2003; Rodrigues et al., 2004; Maren, 2005). Fear conditioning leads to LTM of the CS that acquires affective properties and will subsequently elicit responses that typically occur in the presence of danger. The lateral nucleus of the amygdala receives information about the CS and US from thalamus and cortex and cells in LA are responsive to CS or US and some LA cells respond to both stimuli (e.g., LeDoux et al., 1984; LeDoux et al., 1990a; Turner and Herkenham, 1991; Mascagni et al., 1993; Romanski and LeDoux, 1993; Romanski et al., 1993; Shi and Cassell, 1997; McDonald, 1998; Shi and Davis, 1998; Doron and LeDoux, 2000; LeDoux, 2000; Linke et al., 2000). Damage or functional inactivation of the LA during acquisition prevents the learning from taking place (e.g., LeDoux et al., 1990b; Helmstetter and Bellgowan, 1994; Muller et al., 1997; Fanselow and LeDoux, 1999; Wilensky et al., 1999; Nader et al., 2001), and neural activity changes in LA by learning (e.g., Quirk et al., 1995; Quirk et al., 1997; Collins and Pare, 2000; Repa et al., 2001). LA is connected directly or indirectly to other amygdala nuclei including the central nucleus of the amygdala (CE) shown to participate in fear

memory formation and also to serve as output nucleus to brain areas involved in fear responses (e.g., LeDoux, 2000; Wilensky et al., 2006; Ciocchi et al., 2010; Haubensak et al., 2010; Duvarci et al., 2011). The hippocampus is involved in contextual fear conditioning where the environmental contex is associated with an aversive event (e.g., Kim and Fanselow, 1992; Phillips and LeDoux, 1992).

As noted above, actin is involved in neuronal transmission and morphogenesis and in synaptic plasticity (Luo, 2002; Dillon and Goda, 2005; Cingolani and Goda, 2008) neuronal processes that have been shown to be implicated in fear memory formation in LA and hippocampus (see below). These findings beg the questions: is the actin cytoskeleton an essential component of the molecular events needed for long-term fear memory formation in these brain regions? If so, which cellular mechanisms are modulated by actin cytoskeleton and how they mediate fear memory formation?

ACTIN AND FEAR MEMORY FORMATION

Several studies have shown that the actin cytoskeleton is needed for both cued fear conditioning (tone–footshock pairing) and contextual fear conditioning memory formation in amygdala and hippocampus. It was shown that intra-hippocampal infusion of actin cytoskeleton assembly inhibitors (latrunculin A or cytochalasin D) impaired the consolidation of contextual fear memory (Fischer et al., 2004). Moreover, microinjection of these compounds into the hippocampus impaired the extinction of contextual fear memory, a form of learning whereby the animal re-learns that the context is not fearful (Fischer et al., 2004). Microinfusion of cytochalasin D, an actin polymerization inhibitor, into rat LA immediately before fear conditioning training interfered with the formation of long-term fear memory (LTM) but not short-term fear memory (STM; Mantzur et al., 2009). Furthermore, microinfusion of cytochalasin D into rat LA immediately after fear conditioning dampened LTM. Cytochalasin D had no effect on fear conditioning memory retrieval when injected immediately before LTM test. Rehberg et al. (2010) showed that auditory cued but not contextual fear memory is disrupted, when the actin depolymerization inhibitor phalloidin was injected into basolateral complex of the amygdala (BLA) 6 h after conditioning. Re-consolidation of memory is also dependent on regulation of actin polymerization (Rehberg et al., 2010). Microinjection of cytochalasin D into the BLA or CA1 was shown to impair the return of fear after reconditioning at the last extinction session indicating that actin polymerization is also needed for reconditioning (Motanis and Maroun, 2011). Actin cytoskeleton was shown to be involved in other types of memory formation (e.g., conditioned taste aversion: Bi et al., 2010; aversive memories of drug withdrawal: Hou et al., 2009). In summary, convincing evidence is available indicating that actin cytoskeleton is involved in fear memory formation.

THE ROLES OF ACTIN REGULATORY PROTEINS IN FEAR MEMORY

How does neuronal activation in amygdala or hippocampus during fear conditioning lead to changes in actin cytoskeleton needed for fear memory formation? Actin cytoskeleton polymerization and depolymerization are tightly controlled by regulatory proteins (Luo, 2000). Other actin-mediated function such as intracellular transport and contractility are also mediated by actin-binding proteins (Kamm and Stull, 2001; Somlyo and Somlyo, 2003). These regulatory proteins (**Figure 1**) could mediate actin involvement in fear memory formation as they are functionally linked with synaptic receptors that participate in fear conditioning such as the glutamate receptors, Eph receptors, and adhesion molecules such as cadherin (Gerlai et al., 1999; Rodrigues et al., 2004; Schrick et al., 2007; Maguschak and Ressler, 2008; Savelieva et al., 2008). For example, actin dynamics in spines are inhibited by activation of either α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) glutamate receptors (Fischer et al., 2000). Moreover, activation of either receptor inhibited actin-based protrusive activity from dendritic spine head. In addition, several actin regulatory proteins, such as members of the Rho GTPase family, are activated by glutamate receptor to regulate neuronal morphogenesis. Studies are available suggesting that RhoA mediates the promotion of normal dendritic arbor development by NMDA receptor activation (Li et al., 2000), and recruitment and activation of RhoA underlies spines morphology in a glutamate receptor-dependent manner (Schubert et al., 2006). Two-photon glutamate uncaging leads to long-term volume increase of single spine and to rapid activation of RhoA and Cdc42 in stimulated spine (Murakoshi et al., 2011). Moreover, NMDA or its downstream signaling pathways stimulation may lead to regulation of Rho or Rac GTPases activity (e.g., Tejada-Simon et al., 2006; Nakazawa et al., 2008; Saneyoshi et al., 2008). Eph receptors are also regulators of the Rho/Rac/CDC42 GTPases proteins and affect actin dynamics and neuronal morphology (Shamah et al., 2001; Irie and Yamaguchi, 2002; Penzes et al., 2003; Klein, 2009). Adhesion molecules may regulate Rho/Rac/CDC42 GTPases proteins to affect actin cytoskeleton (e.g., Brusés, 2006).

Indeed, several actin regulatory proteins have been shown to be involved in fear memory formation. Following fear conditioning, the tyrosine phosphorylated p190 RhoGAP becomes associated with a molecular complex in LA (Lamprecht et al., 2002). Importantly, evidence is available that p190 RhoGAP is involved in mediating actin reorganization. Specifically, in p190 RhoGAP mutant mice, polymerized actin accumulates extensively in cells of the neural tube floor, suggesting that p190 RhoGAP plays a role in regulating actin assembly (Brouns et al., 2000). P190 RhoGAP regulates Rho GTPase protein, a molecular switch that controls many key cellular processes including actin dynamics. Inhibition of the Rho GTPase effector, the Rho-associated kinase (ROCK), a kinase that affects actin cytoskeleton (Amano et al., 2010), in LA impaired the formation of long- but not short-term fear memory formation (Lamprecht et al., 2002). Interestingly, the activation of Rho and Rac GTPases led to rearrangement of cerebral actin cytoskeleton, enhanced neurotransmission and synaptic plasticity, and facilitation of fear conditioning (Diana et al., 2007). In addition, RhoB, a member of the Rho GTPase family, is involved in short-term plasticity in hippocampus, in the regulation of cofilin and dendritic and spine morphology (McNair et al., 2010). Intracerebroventricular injection of ROCK inhibitor leads to increase in anxiety-related behaviors (Saitoh et al., 2006). ROCK regulates actin cytoskeleton via other signaling molecules such as the LIM kinase (LIMK) that regulates actin dynamics. LIMK exerts its effect on actin polymerization by phosphorylating and thus inactivating the actin depolymerization factor (ADF)/cofilin

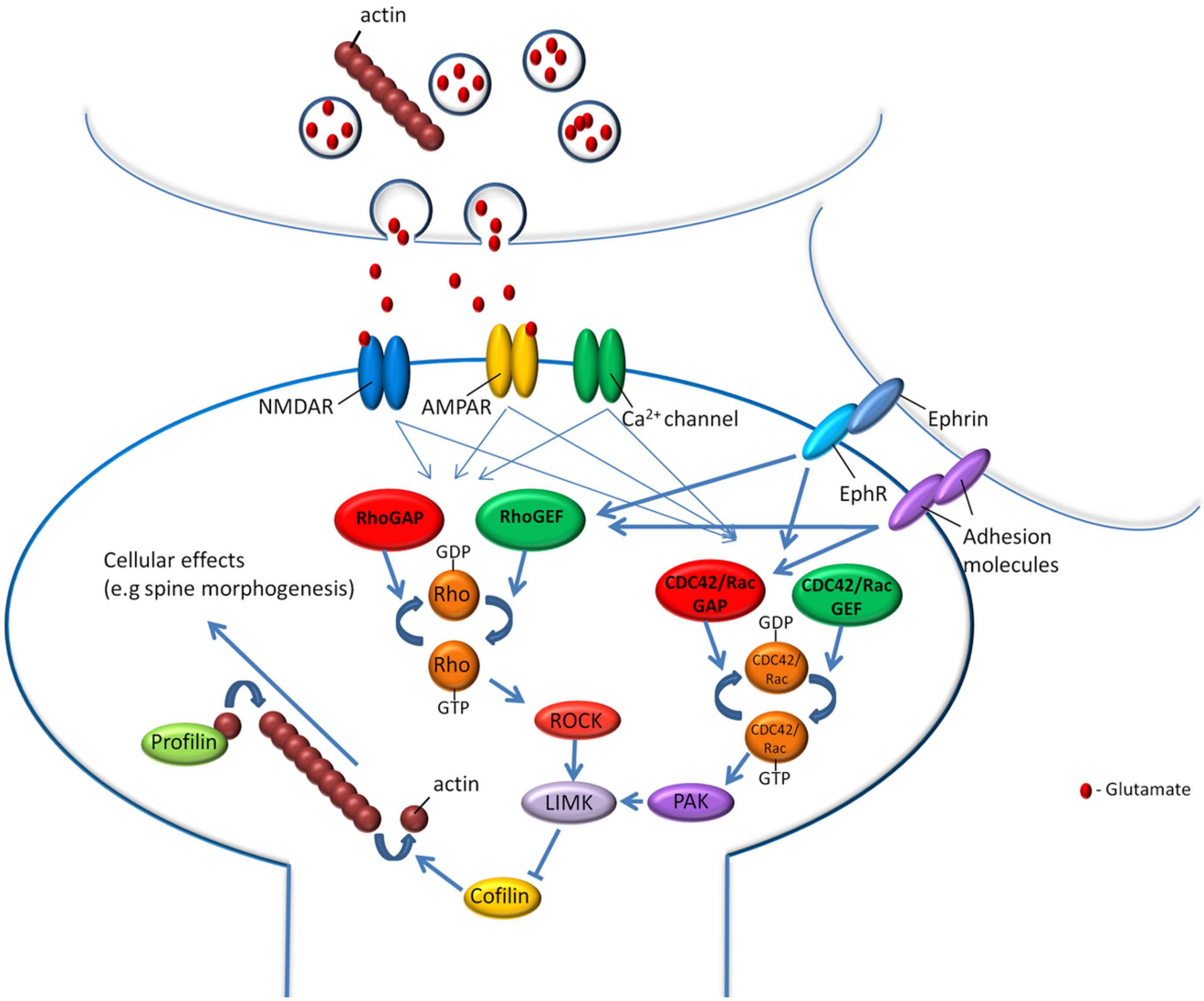

FIGURE 1 | Actin cytoskeleton and its regulatory proteins are involved in fear memory formation. Fear conditioning memory formation depends on the activation of glutamate receptors, calcium channels, receptors tyrosine kinases such as Eph receptors and adhesion molecules. Activation of these receptors and channels during or after fear learning may lead to regulation of intracellular signaling cascades that affect actin dynamics and cellular processes such as neuronal morphogenesis. Among these regulated molecules are the Rho, Rac, and CDC42 GTPases and their effectors and actin-binding proteins such as profilin shown to be involved in fear memory formation.

(Arber et al., 1998; Yang et al., 1998; Sumi et al., 1999). Indeed, in LIMK-1 knockout mice, spine-dendrite F-actin levels were reduced compared to wild type mice (Meng et al., 2002). Furthermore, the knockout mice exhibited significant abnormalities in spine and axonal morphology. In addition, hippocampal long-term potentiation (LTP) is enhanced indicating that synaptic function was altered. The LIMK-1 knockout mice also showed enhanced cued fear conditioning LTM. These results indicate that the regulation of actin polymerization by the LIMK pathway is essential for normal fear memory formation. The LIMK effector cofilin is also involved in fear conditioning. Mice in which n-cofilin was removed from principal neurons of the postnatal forebrain are impaired in long- and short-term fear memory (Rust et al., 2010).

Profilin is another actin cytoskeleton-regulatory protein that regulates actin polymerization by funneling ATP-actin to the growing actin filaments (Witke, 2004). Profilin was shown to be translocated into dendritic spines in cultured hippocampal neurons after neuronal stimulation, LTP and long-term depression (LTD; Ackermann and Matus, 2003; Neuhoff et al., 2005). The translocation of profilin is associated with the suppression of actin dynamics in the spine head and the stabilization of spine morphology. Fear conditioning in rats leads to the movement of profilin into dendritic spines in the LA (Lamprecht et al., 2006a). Profilin-containing spines were shown to be larger compared to spines devoid of profilin. A greater proportion of profilin-containing spines with enlarged PSDs could contribute to the enhancement

of associatively induced synaptic responses in LA following fear learning. Mice with knockdown of one of the profilin isoforms, profilin2, are hyperactive and show increased novelty-seeking behavior (Pilo Boyl et al., 2007). Freezing after fear conditioning is similar in control and knockout mice when number of freezings, but not time of freezing, is measured during LTM test (Pilo Boyl et al., 2007).

Myosin light chain kinase (MLCK) is a calcium/calmodulin-dependent protein kinase that phosphorylates the myosin regulatory light chain (RLC), leading to contraction of the actomyosin filaments (Kamm and Stull, 2001; Somlyo and Somlyo, 2003). MLCK is involved in regulating cellular events related to synaptic transmission, such as neurotransmitter release (Mochida et al., 1994; Ryan, 1999; Polo-Parada et al., 2001), *N*-methyl-D-aspartate receptor activity (Lei et al., 2001) and potassium channel function (Akasu et al., 1993). In addition, MLCK participates in neural morphogenesis, including the regulation of growth cone motility (Gallo et al., 2002; Zhou et al., 2002) and dendritic branching (Ramakers et al., 2001). MLCK is present in cells throughout the LA and is localized to dendritic shafts and spines that are postsynaptic to the projections from the auditory thalamus to lateral nucleus of the amygdala, a pathway specifically implicated in fear learning (Lamprecht et al., 2006b). Inhibition of MLCK in LA leads to the enhancement of fear memory formation but has no effect on retrieval of fear memory (Lamprecht et al., 2006b). In addition, inhibition of myosin light chain kinase enhances LTP in the auditory thalamic pathway to the LA (Lamprecht et al., 2006b). MLCK inhibition immediately after fear conditioning training has no effect on fear memory formation. The short time window of involvement of MLCK in fear conditioning is consistent with its ability to rapidly regulate synaptic transmission (Ryan, 1999; Lei et al., 2001). In addition, anatomical findings showing that MLCK is located in LA presynaptic terminals and in postsynaptic densities suggest that MLCK might be involved in regulating events in these sites such as vesicle release (Ryan, 1999) or receptor activity (Lei et al., 2001). Moreover, the observation that MLCK inhibition does not affect fear memory retrieval implies that MLCK does not regulate transmission during memory activation, but only during acquisition. Consistent with this view is the observation that application of ML-7 (an MLCK inhibitor) to amygdala slices has no effect on basal transmission but rather specifically on the induction of associative LTP. These findings showing that the inhibition of MLCK enhances conditioning and the synaptic plasticity underlying conditioning indicate that MLCK normally inhibits fear learning.

Other proteins that are involved in actin polymerization and some in spine morphology have been implicated in fear memory formation such as beta-adducin shown to be essential for contextual and cued fear conditioning (Rabenstein et al., 2005), drebrin A needed for context-dependent freezing after fear conditioning (Kojima et al., 2010), Ndr which expression is increased in amygdala 6 h after Pavlovian fear conditioning training (Stork et al., 2004), neurabin needed for contextual fear memory and hippocampal LTP but not auditory fear memory and LTD (Wu et al., 2008) and p21-activated kinase which is not needed for normal short-term contextual fear conditioning but is needed for normal consolidation/retention of fear memory (Hayashi et al., 2004).

Cumulatively, the aforementioned studies show that actin regulatory proteins are involved in fear memory formation. Modulation of the actin cytoskeleton by these proteins may serve as a signaling connection between synaptic activation induced by learning and cellular changes underlying fear memory formation.

To further elucidate possible roles of actin cytoskeleton in fear memory formation its roles in synaptic morphology, transmission and plasticity in amygdala and hippocampus are discussed.

ACTIN CYTOSKELETON IN SYNAPTIC TRANSMISSION

Alteration of synaptic efficacy either by affecting synaptic release of neurotransmitters and/or the level of synaptic receptors for neurotransmitters is associated with memory formation and synaptic plasticity. Changes in synaptic efficacy are induced by fear learning. For example, it was shown that fear-conditioned animals exhibit a presynaptic facilitation of AMPA receptor-mediated transmission in LA neurons (McKernan and Shinnick-Gallagher, 1997) and conditioned fear is accompanied by the enhancement in transmitter release at cortico-amygdala synapses (Tsvetkov et al., 2002). At the postsynapse fear conditioning drives AMPA receptors into the synapses of neurons in the LA, incorporation process that is needed for fear conditioning memory formation (Rumpel et al., 2005; Yeh et al., 2006; Nedelescu et al., 2010).

Actin cytoskeleton is found in pre- and post-synapse and is involved in the regulation of synaptic transmission in these sites and may mediate changes in synaptic efficacy following fear conditioning. In the presynapse actin cytoskeleton contacts synaptic vesicle through short strands of synapsin, a phosphoprotein associated with synaptic vesicle membrane (e.g., Landis et al., 1988; Hirokawa et al., 1989; Doussau and Augustine, 2000). It is possible that actin regulates the availability of the vesicle in the reserve pool (RP) by forming a barrier (e.g., Wang et al., 1996) or may serve as a scaffold protein to retain synapsin in presynapse, thereby indirectly influencing neurotransmission (Sankaranarayanan et al., 2003). Neuronal stimulation may redistribute synapsin enabling access to the RP of vesicles (Greengard et al., 1994; Chi et al., 2001, 2003). Actin may also promote vesicle delivery to the readily releasable pool (RRP) by providing cytoskeletal routes of vesicle to the RRP (Prekeris and Terrian, 1997; Evans et al., 1998; Watanabe et al., 2005). In addition, actin may be involved in the endocytosis of vesicle at the presynapse, possibly by forming a link with dynamin or by promoting the transport of endocytosed vesicles to the internal RP cluster (Shupliakov et al., 2002; Bloom et al., 2003; Engqvist-Goldstein and Drubin, 2003). Synaptic vesicles endocytosed at one bouton can be recruited into the functional pool of nearby boutons where they undergo exocytosis (Darcy et al., 2006). Such distribution of vesicles between nearby boutons requires actin turnover (Darcy et al., 2006).

The postsynaptic actin cytoskeleton may also contribute to synaptic transmission as it is involved in the regulation of glutamate and GABA receptors clustering and trafficking and thereby in the postsynaptic response to neurotransmitters. F-actin depolymerization reduces the number of AMPA and NMDA receptors clusters at excitatory synapses (Allison et al., 1998). Actin also mediates glutamate receptor trafficking via myosins, the main actin-dependent motor proteins. Myosin Va mediates translocation of GluR1-containing AMPA receptor (AMPAR) from the dendritic

shaft into spines and is required for LTP (Correia et al., 2008). Myosin Vb is also involved in AMPAR trafficking (Lisé et al., 2006). Actin regulatory and associated proteins also mediate receptor trafficking. For example, ADF/cofilin-mediated actin dynamics regulates AMPAR receptor trafficking during synaptic potentiation (Gu et al., 2010). The reversion induced LIM protein (RIL) is involved in actin-dependent trafficking of GluR1 (Schulz et al., 2004) and the actin adaptor protein 4.1N stabilizes the surface expression of GluR1 (Shen et al., 2000). Actin also mediates AMPAR internalization. AMPAR internalization can be induced by the actin assembly inhibitor latrunculin A, and this process is blocked by jasplakinolide, a drug which stabilizes actin filaments (Zhou et al., 2001) and myosin VI plays a role in the clathrin-mediated endocytosis of AMPARs (Osterweil et al., 2005). Actin cytoskeleton can also affect inhibitory transmission by mediating GABA receptor trafficking to the synapse (e.g., Graziane et al., 2009).

Taken together, the aforementioned studies show that actin cytoskeleton is involved in regulating synaptic transmission by affecting pre- and post-synapse molecular and cellular events that are also involved in synaptic plasticity and fear memory formation. Additional research is warranted to elucidate whether actin cytoskeleton is needed for presynaptic or postsynaptic changes during and following fear conditioning training.

ACTIN CYTOSKELETON IN SYNAPTIC MORPHOGENESIS

It has been shown that alteration in neuronal morphology is associated with memory formation (Bailey and Kandel, 1993; Lamprecht and LeDoux, 2004) and may serve to modulate neuronal connectivity needed to form or alter memory. Most excitatory synapses in the brain terminate on dendritic spines, which have been the focus of recent work in the mammalian brain. Dendritic spines receive the majority of excitatory synaptic inputs in the brain, compartmentalize local synaptic signaling pathways, and restrict the diffusion of postsynaptic molecules (Nimchinsky et al., 2002; Lamprecht and LeDoux, 2004; Newpher and Ehlers, 2009). Modulation of the number of dendritic spines and/or their morphology has been proposed to contribute to alterations in excitatory synaptic transmission during learning (Lamprecht and LeDoux, 2004). Changes in number and shape of dendritic spines where observed following fear conditioning. For example, contextual fear conditioning leads to a time-dependent increase in dendritic spine density in the CA1 hippocampal region and the anterior cingulate cortex (Restivo et al., 2009; Vetere et al., 2011) and auditory fear conditioning leads to an increase in spinophilin-immunoreactive dendritic spines in the LA (Radley et al., 2006). Postsynaptic density (PSD) area on a smooth endoplasmic reticulum (sER)-free spines increases with fear conditioning while the spines head volume of these spines decreases (Ostroff et al., 2010).

Actin cytoskeleton is involved in neuronal morphogenesis in postsynaptic dendritic spines. The base, neck, and head of mature spine consist of a mixture of branched and linear actin filaments. The neck contains both linear and branched filaments, whereas branched actin filament network is a dominant feature of spine head (Korobova and Svitkina, 2010). The actin cytoskeleton is intimately involved in the formation and elimination, stability, motility, and morphology of dendritic spines (e.g., Halpain et al., 1998; Matus, 2000; Korkotian and Segal, 2001; Luo, 2002; Ethell and Pasquale, 2005; Tada and Sheng, 2006; Schubert and Dotti, 2007; Honkura et al., 2008; Hotulainen and Hoogenraad, 2010). In addition, actin plays a role in stabilizing postsynaptic proteins (Allison et al., 1998; Kuriu et al., 2006; Renner et al., 2009) and in modulating spine head structure in response to synaptic signaling (Fischer et al., 2000; Star et al., 2002; Okamoto et al., 2004).

Alteration in axonal morphology is also implicated in memory formation and synaptic plasticity (Bailey and Kandel, 1993; Lamprecht and LeDoux, 2004). Actin polymerization mediates morphological changes involved in axonal growth, guidance, shape, collateral branching, branch retraction, and regeneration (Luo, 2002; Letourneau, 2009).

Additional research is warranted to elucidate whether actin is involved in neuronal morphogenesis seen in amygdala and hippocampus following fear memory formation and whether such changes are essential for memory formation. Some supporting evidence comes from studies showing that interference with actin regulatory proteins activity impairs fear memory formation and spine and axonal morphology (e.g., LIMK-1, Meng et al., 2002).

THE ROLES OF ACTIN CYTOSKELETON IN SYNAPTIC PLASTICITY

As mentioned above actin cytoskeleton plays key roles in modulating synaptic transmission and neuronal morphogenesis, cellular processes believed to underlie synaptic plasticity (e.g., Bailey and Kandel, 1993; Lamprecht and LeDoux, 2004). The role of actin cytoskeleton in synaptic plasticity was studied mainly by elucidating its involvement in LTP or LTD, physiological models of memory (e.g., Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000). Findings suggest that LTP occur in the LA and hippocampus during fear conditioning. LTP induction at thalamic auditory inputs to the LA enhances auditory-induced responses in the LA in a manner similar to the increase of CS-evoked responses observed during auditory fear conditioning (Rogan and LeDoux, 1995). Fear conditioning-altered auditory CS-evoked responses in LA changes in conjunction with conditioned fear responses (Rogan et al., 1997). Thalamic inputs or cortical inputs to the LA were enhanced in slices from trained animals compared to naive or unpaired animal groups (McKernan and Shinnick-Gallagher, 1997). Moreover, fear conditioning inhibits the induction of LTP at cortical inputs suggesting that LA synapses that have already undergone LTP by training are less capable of showing additional LTP (Tsvetkov et al., 2002; Schroeder and Shinnick-Gallagher, 2004; and Schroeder and Shinnick-Gallagher, 2005). It was shown that contextual fear conditioning increased synaptic response in hippocampal CA1 (e.g., Sacchetti et al., 2001) and that contextual fear conditioning modified the ability to induce LTP in hippocampus (Sacchetti et al., 2002).

To study the roles of actin in LTP Okamoto et al. (2004) used the fluorescence resonance energy transfer (FRET) technique to show that in rat hippocampal dendritic spines LTP induction led to persistent shift of F-actin/G-actin equilibrium toward F-actin within seconds of a tetanic stimulus. In the dentate gyrus, LTP increased F-actin content in dendritic spines lasting up to 5 weeks (Fukazawa et al., 2003). The increase in F-actin correlates with a stable increase in the size of the spine head and inhibition of actin polymerization impaired LTP-induced spine head enlargement (Matsuzaki et al.,

2004; Okamoto et al., 2004; Fortin et al., 2010). LTP also induces changes in axonal morphology and actin cytoskeleton leading to formation of new axonal varicosities and new axonal actin puncta (Colicos et al., 2001; De Paola et al., 2003). The new presynaptic actin puncta become associated with recycling synaptic vesicle pool (Colicos et al., 2001). Long-term facilitation induced the growth of new synapses and presynaptic actin remodeling in *Aplysia* mechanosensory neurons (Hatada et al., 2000). In addition, cytochalasin D, an actin polymerization inhibitor, selectively blocks long-term but not short-term facilitation (Udo et al., 2005).

Actin cytoskeleton is needed for synaptic plasticity in brain areas mediating fear memory formation such as amygdala and hippocampus (LeDoux, 2000; Davis and Whalen, 2001; Schafe et al., 2001; Sah et al., 2003; Rodrigues et al., 2004; Maren, 2005). In LA, 5-HT-induced L-LTP is blocked by the actin inhibitor cytochalasin D (Huang and Kandel, 2007). Furthermore, LTP in interneurons in LA is maintained by trafficking of GluR2-lacking AMPA receptors that require an interaction with SAP97 and the actin cytoskeleton (Polepalli et al., 2010). Inhibition of actin polymerization in hippocampus or disruption of F-actin lead to impairment of LTP formation and facilitation (e.g., Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003; Kramár et al., 2009). In addition, inhibition of actin polymerization affects protein synthesis-independent early LTP, prevents late-LTP, and interferes with synaptic tagging in apical dendrites of hippocampal CA1 (Ramachandran and Frey, 2009). Furthermore, chemical forms of LTP in dissociated hippocampal cultures forms GluR1 and synaptophysin puncta and these cellular and molecular events require actin polymerization (Antonova et al., 2001).

Actin cytoskeleton is also involved in LTD which in many instances induces opposite synaptic, morphological, and molecular events compared to LTP (e.g., Zhou et al., 2004). LTD induces shifts the F-actin/G-actin equilibrium toward G-actin and decreases spine head volume with the disappearance of some spines (Okamoto et al., 2004). Furthermore, LTD-inducing paradigm has stabilizing effects on actin (Star et al., 2002).

Cumulatively, the aforementioned studies show that actin cytoskeleton serves as regulator of synaptic plasticity possibly by affecting synaptic morphology and transmission and thereby tuning synaptic strength. Furthermore, actin cytoskeleton is intimately involved in synaptic plasticity in amygdala and hippocampus areas that mediate fear memory formation. Further studies are needed to elucidate whether actin cytoskeleton is needed for LTP of synapses in the amygdala following fear conditioning and how it can affect plasticity.

FUTURE RESEARCH

Much evidence indicates that the actin cytoskeleton and its regulatory proteins are involved in fear memory formation. However, key questions remain unresolved. For example, are the morphological changes shown to be mediated by actin cytoskeleton needed for fear memory formation? Such changes may include alteration of spines and axonal morphology. Does actin cytoskeleton regulate changes in synaptic transmission needed for fear conditioning memory formation? If so, are they related to presynaptic (changes in vesicle release) or postsynaptic (changes in receptor trafficking) alterations or to both? Studies aimed to elucidate such questions will undoubtedly provide key insights into the roles of actin cytoskeleton in fear memory and also on the cellular processes essential for fear memory formation and greatly contribute to a better understanding of the intricate molecular and cellular processes governing fear memory formation.

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Interaction between diazepam and hippocampal corticosterone after acute stress: impact on memory in middle-aged mice

Daniel Béracochéa[1]*†, Christophe Tronche[1,2†], Mathieu Coutan[2], Rodolphe Dorey[1,2], Frédéric Chauveau[2] and Christophe Piérard[2]

[1] *UMR-CNRS 5287, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, Universités de Bordeaux, Talence, France*
[2] *Département Environnements Opérationnels, Institut de Recherche Biomédicale des Armées, Brétigny sur Orge, France*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Gernot Riedel, University of Aberdeen, UK
Mouna Maroun, University of Haifa, Israel

****Correspondence:***
Daniel Béracochéa, UMR-CNRS 5287, Institut de Neurosciences Cognitives et Intégratives d'Aquitaine, Universités de Bordeaux, Bat B2, Avenue des Facultés, 33405 Talence cédex, France. e-mail: d.beracochea@cnic.u-bordeaux1.fr

†Daniel Béracochéa and Christophe Tronche share the first place in the list.

Benzodiazepines (BDZ) are widely prescribed in the treatment of anxiety disorders associated to aging. Interestingly, whereas a reciprocal interaction between the GABAergic system and HPA axis has been evidenced, there is to our knowledge no direct evaluation of the impact of BDZ on both hippocampus (HPC) corticosterone concentrations and HPC-dependent memory in stressed middle-aged subjects. We showed previously that an acute stress induced in middle-aged mice severe memory impairments in a hippocampus-dependent task, and increased in parallel hippocampus corticosterone concentrations, as compared to non-stressed middle-aged controls (Tronche et al., 2010). Based on these findings, the aims of the present study were to evidence the impact of diazepam (a positive allosteric modulator of the GABA-A receptor) on HPC glucocorticoids concentrations and in parallel on HPC-dependent memory in acutely stressed middle-aged mice. Microdialysis experiments showed an interaction between diazepam doses and corticosterone concentrations into the HPC. From 0.25 to 0.5 mg/kg, diazepam dose-dependently reduces intra-HPC corticosterone concentrations and in parallel, dose-dependently increased hippocampal-dependent memory performance. In contrast, the highest (1.0 mg/kg) diazepam dose induces a reduction in HPC corticosterone concentration, which was of greater magnitude as compared to the two other diazepam doses, but however decreased the hippocampal-dependent memory performance. In summary, our study provides first evidence that diazepam restores in stressed middle-aged animals the hippocampus-dependent response, in relation with HPC corticosterone concentrations. Overall, our data illustrate how stress and benzodiazepines could modulate cognitive functions depending on hippocampus activity.

Keywords: glucocorticoids, benzodiazepines, hippocampus, microdialysis, aging, stress

INTRODUCTION

It is well known that both stress-induced and age-induced cognitive dysfunctions are major public health issues nowadays. More particularly, it has been shown that stress and aging impair in humans declarative memory, and more specifically memory processes involving either the hippocampus (HPC) and/or prefrontal cortex activity (de Quervain et al., 2003; Cappell et al., 2010). Moreover, both stress-induced and aged-induced memory impairments would involved dysfunction of the HPA axis activity (Sapolsky et al., 1986; de Kloet et al., 1991; Lupien et al., 1999; Pardon, 2007; Pardon and Ratray, 2008; Comijs et al., 2010).

In line with these observations, we recently showed that stressed middle-aged mice exhibited contextual memory impairments associated with a dramatic increase in intra-HPC corticosterone concentration (Tronche et al., 2010a). We already evidenced the causal role of HPC corticosterone on memory dysfunction in stressed middle-aged mice insofar as the administration of metyrapone (an inhibitor of corticosterone synthesis) totally alleviated both the stress-induced corticosterone rise and memory impairments (Tronche et al., 2010a). In addition, we also showed that the direct infusion of corticosterone into the HPC of young adult mice induced memory impairments similar to those observed in stressed middle-aged mice (Chauveau et al., 2009, 2010).

Because of the impact of benzodiazepines (BDZ) on GABAergic neurons, these compounds are widely prescribed in the treatment of anxiety disorders associated to aging. Indeed, it has been shown that aging causes organisms to become vulnerable to stress, which might be mediated by dysfunction of the brain system regulating emotional and stress responses (Pardon and Ratray, 2008; Shoji and Mizoguchi, 2010). However, it is also known that compounds modulating GABA-A receptors such as BDZ, also affect HPC-dependent memory functions (for review, see Beracochea, 2006). Furthermore, it has already been demonstrated that GABAergic neurons also act on hypothalamic nuclei of the HPA axis (Jones et al., 1984; Hillhouse and Milton, 1989; Stotz-Potter et al., 1996; Cullinan et al., 2008) and that peripheral injection of GABA-A receptor positive modulators such as BDZ reduced HPA axis activity (Imaki et al., 1995; Grottoli et al., 2002).

Given the reciprocal interaction between the GABAergic system and HPA axis, it is of importance to determine the impact of BDZ administration on HPA axis activity and its consequence on memory

function, in particular in middle-aged subjects that are vulnerable to stress. So far, the aim of the present study was to evidence more specifically the impact of BDZ administration on HPC glucocorticoids concentration and its consequence on memory in a HPC-dependent contextual memory task in stressed middle-aged mice.

For that purpose, we studied in a first experiment the effects of a diazepam administration on contextual memory, using the contextual serial discrimination task (CSD). This task allowed to evidence a hippocampal-dependent memory impairment in stressed (Celerier et al., 2004; Piérard et al., 2009) as well as in middle-aged (Tronche et al., 2010a) and aged subjects (Tronche et al., 2010b). More precisely, the CSD task involves two serial discriminations (D1 and D2) learned on two different contexts. We found from *in situ* brain lesions and pharmacological experiments, that the memory retrieval of D1 but not of D2 is HPC-dependent (Chauveau et al., 2008, 2009, 2010). In a second experiment, we evaluated the emotional status of diazepam-treated stressed middle-aged mice in an elevated plus-maze task. Finally, in a third experiment, we measured by *in vivo* microdialysis the intra-HPC corticosterone concentration following diazepam administration in freely moving mice. Microdialysis allows a direct and dynamic measurement of the interaction between the HPA axis and the HPC, as a function of the administered dose of diazepam. Whereas peripheral measurements of circulating glucocorticoids have already been performed (Comijs et al., 2010), there is to our knowledge no direct evidence for such a dynamic interaction at the hippocampal level, which is surprising given the known importance of the hippocampus both in memory processes and in the negative feedback exerted by this brain area on HPA axis activity.

MATERIALS AND METHODS

ANIMALS

Upon arrival in the laboratory, all animals were 3-month-old male mice of the BALB/c inbred strain obtained from Charles River (L'Arbresle, France). Animals were housed in collective cage in the colony room (12 h light–dark cycle in a temperature controlled and ventilated room) until they were either 16 months. Two weeks before the experiments, they were housed individually. The animal's weights were ranged between 28 and 35 g at the time of experiments. All procedures were carried out during the light phase of the cycle, between 08:00 and 12:00 a.m. During the food deprivation phase, mice were handled daily so as to become familiar with the experimenter. During that phase, all subjects were maintained at 85–90% of their *ad libitum* body weight throughout the behavioral study. All animal experimentation reported in the present paper has been conducted in accordance with the guidelines laid down by the European Communities Council.

MEMORY TEST

Apparatus

The CSD has been extensively described in earlier studies (Chauveau et al., 2009, 2010; Pierard et al., 2010). All tests were performed in a four-hole board apparatus (45 cm × 45 cm × 30 cm high) enclosed by gray Plexiglas. The four-hole board apparatus was placed on the floor of the room (3.0 m × 3.0 m × 2.40 m high). The floor of the board was interchangeable (white and rough; black and smooth). On the floor, four holes opening on a food cup (3 cm in diameter × 2.5 cm in depth) were located 6 cm away from the sidewalls. The apparatus was placed in a room exposed to a 60-dB background noise and a light centered over the apparatus provided 20 lx intensity at the position of the apparatus. The apparatus was cleaned with 95% ethanol, then with water before each mouse behavioral testing. Data were automatically monitored by photoelectric cells and video recording.

Acquisition phase

In the CSD, the acquisition phase took place in room A where animals learned two consecutive spatial discriminations (D1 and D2; see **Figure 1**). Both discriminations differed in terms of the color and texture of the floor (internal context of the four-hole board)

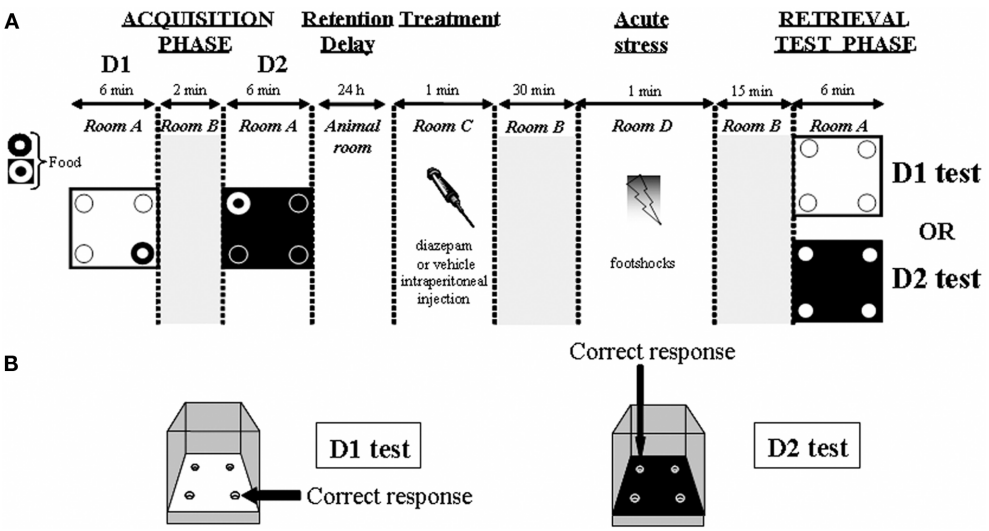

FIGURE 1 | Description of the contextual serial discrimination (CSD) behavioral task. (A) Behavioral procedures. **(B)** Type of responses for the retrieval test phase. D1, discrimination 1; D2, Discrimination 2; CSD, contextual serial discrimination. During retrieval test phase, each mice is randomly evaluated either on D1 floor or D2 floor without food reward.

and were separated by a 2-min delay interval. During this delay, the mouse was returned to its home cage in room B. At the beginning of acquisition and retrieval phases, mice were placed in the center of the four-hole board in an opaque PVC tube for 5 s to provide the animal with a random start in the apparatus. For D1, ten 20-mg food pellets were available only in one of the four holes on the board for 6 min. Location of the baited hole for D1 was randomized for each subject. For D2, ten 20-mg food pellets were consistently located in the opposite symmetrical hole, for 6 min likewise. The environmental spatial cues were made of colored and striped paper sheets positioned at 1.00 m above the four-hole board. These allocentric cues remained at the same place for both D1 and D2 discriminations and for the retrieval phase. Thus, both discriminations D1 and D2 differed only by the internal (floor) contextual cues. Both floors were positioned in a mixed random order during the acquisition of the first and second discrimination tasks. At the end of the acquisition phase, mice returned to their home cage in the animal room for 24 h. Only mice having eaten all the pellets during both acquisition sessions were used for the retrieval test phase.

Acute stress

Twenty-four hours after acquisition, mice were placed in the stress delivery cage for 1 min, in room D. Stress was induced 15 min before the test session. Stressed mice received three consecutive inescapable electric foot-shocks (0.9 mA; 10 ms). All animals were then returned to their home cage during the delay preceding the test session.

Test phase

Fifteen minutes after acute stress, mice were replaced in the four-hole board (room A) without any pellet in the apparatus. Mice were placed either on the D1 floor *or* on the D2 floor and were allowed to freely explore the apparatus for 6 min. For all mice, the retrieval test phase occurred 24 h after the acquisition phase and was performed on independent groups for either D1 or D2. Performance was assessed by measuring the number of head dips in each hole during 6 min.

Measurements

Memory retrieval performance was evaluated through the exploration rates into the different holes. *Correct responses* were defined as head dips into the hole previously baited on the same floor-context during the acquisition phase, and were calculated as follows: (head dips into the baited hole/total number of head dips in the four holes) × 100.

EVALUATION OF EMOTIONAL STATUS

Elevated plus maze

In order to verify the anxiolytic action of diazepam and the absence of sedative effect for the selected doses, stressed animals were submitted to the elevated plus-maze task 15 min after electric shock delivery. Stress was similar as the one previously described in the CSD experiments. The elevated plus maze, which was constructed of gray Plexiglas, consisted of four arms arranged in the shape of a plus sign. Each arm was 30 cm long, 7 cm wide, and was elevated 40 cm above the ground. The four arms were joined at the center by a 7-cm square platform. Two opposite arms of the plus maze were enclosed by sidewalls 17 cm high, but open on the top. The remaining arms did not have sidewalls. These walls did not extend from the center of the maze. The experiment was performed between 08:30 and 12:00 a.m. At the beginning of the session, mice were placed at the center of the plus maze in a cylinder (8 cm diameter, 17 cm high) for 30 s. Then, the cylinder was removed and mice were allowed to freely explore all arms of the maze for 6 min. An entry was counted only when a mouse entered an arm with all four paws. "Anxiety-like" behavior was measured by the ratio of entries into the open arms divided by the total number of entries in all arms (entry ratio). Results were expressed in percentages (ratio × 100). The elevated plus-maze test has been performed in four independent groups of mice (i.e., not submitted to the CSD task and microdialysis experiment) distributed as follows: vehicle: $n = 7$; diazepam 0.25 mg/kg: $n = 7$; diazepam 0.5 mg/kg: $n = 7$; diazepam 1.0 mg/kg: $n = 7$).

SURGERY AND HISTOLOGY

Mice were anesthetized with a ketamine (100 mg/kg body weigh)–xylazine (10 mg/kg body weigh) mixture and placed into a stereotaxic frame. A single guide cannula microdialysis (CMA/7 Microdialysis probe, CMA Microdialysis, Sweden) was implanted in the bottom of the parietal cortex at the following coordinates from the bregma (Paxinos and Franklin, 2001): Antero-posterior = −2000 μm, Lateral = +1400 μm, and Vertical = −800 μm. The guide cannula was fixed with dental cement and three micro screws attached to the skull. All operated mice were allowed to recover in their home cages in the animal room for at least 7 days before the microdialysis experiment. On the day of the experiment, the microdialysis probe was lowered 1 mm below through the guide cannula so that the microdialysis membrane is located into the dorsal HPC. At the end of the microdialysis experiment, mice were anesthetized and then transcardially perfused in the left ventricle with saline solution (NaCl 0.9%) followed by formaldehyde (4%). Brains were then postfixed in a 4% formaldehyde solution for 10 days, then in a saccharose–formaldehyde solution (30 and 4% v/v) for 2 days. All the brains were sectioned coronally (50 μm thickness). A cresyl violet stain was used to locate the microdialysis probe with utmost accuracy.

IN VIVO MICRODIALYSIS

Seven days after surgery, a dialysis probe (CMA/7; CMA Microdialysis AB, Sweden; length: 1 mm; molecular cut-off 6 kDa and membrane outer diameter: 0.24 mm) was carefully implanted into the right dorsal HPC under light anesthesia induced by a ketamine (50 mg/kg body weigh)–xylazine (5 mg/kg body weigh) mixture. Mice were then individually housed in a system allowing animals to move freely (CMA/120; CMA Microdialysis AB, Sweden) overnight. After the overnight perfusion at 1 μl/min to equilibrate extracellular metabolites concentrations, freely moving animals were continuously perfused with a sterile-filtered saline solution (Dulbecco's phosphate buffered saline; SIGMA; in g/l: $CaCl_2$, 0.133; $MgCl_2$, 0.1; KCl, 0.2; KH_2PO_4, 0.2; NaCl 8.0; Na_2HPO_4, 1.15; pH between 7.1 and 7.5) at a 1-μl/min flow rate through a micro-infusion pump. The foot-shock delivery system was inside the dialysis cage in order to deliver acute stress. Microdialyzates were sampled every 15 min using tubes with a dead volume of 1.2 μl/100 mm length (CMA Microdialysis AB). Samples were stored at −80°C.

Baseline dialyzates were collected for 1 h before *ip* injection of diazepam ($n = 8$ for each dose) or vehicle ($n = 8$) and 30 min before acute stress delivery. Free corticosterone levels measured in the dialyzate (in nanomolar) were expressed as the percentage of the averaged baseline values collected before the injection.

DRUG ADMINISTRATION

Five days before experiments, mice were daily prepared for intraperitoneal (*ip*) administration by exerting light pressure on the body with the syringe. On the day of the experiment, 30 min before acute stress administration, mice received an *ip* injection of a diazepam solution. Diazepam (Valium®, Roche, 1, 0.5, 0.25 mg/kg body weigh dissolved in saline solution) and vehicle (saline solution) solutions were injected in a room (room C, **Figure 1**) different from the behavioral room (room A).

INTRA-HIPPOCAMPAL CORTICOSTERONE ASSAY

A commercially prepared Enzyme Immunoassay kit was used to measure HPC corticosterone concentrations in the microdialyzates (Correlate-EIA™, Assay Designs, Ann Arbor, USA). The sensitivity of the assay was 0.08 nmol/l. Therefore, baseline sample concentration was more than 10-fold superior than sensitivity threshold.

STATISTICAL ANALYSES

Statistical analyses were performed using the Sigma Plot 11.0 software. Behavioral data were analyzed using 1 way or 2 way factorial analyses of variance (ANOVAs) with either "Treatments" and "Discriminations" as factors followed, – when adequate, with *post hoc* comparisons (Bonferroni's *t*-test). In the CSD task, comparisons of retrieval performances with chance level were calculated with paired-samples *t*-test (with hypothesized mean = chance level = 25% for correct responses).

For microdialysis, basal free extracellular corticosterone levels were compared with one sample Student's *t*-test. Stress effects on intra-HPC corticosterone levels are expressed in percentage of baseline variation. They were compared using two-way repeated-measures ANOVA (RM-ANOVA) with both "Treatments" and "Time" factors. When appropriate, *post hoc* analyses were performed with Bonferroni's *t*-test.

All the data were expressed as mean ± SEM and "NS" means that "*p*" values exceed 0.05 and are considered as non-statistically significant.

ETHICAL STATEMENT

The present study was carried out in compliance with the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes, under the agreement #2010/11 delivered by the French Ministry of Defence after the protocol was examined by the local ethical committee. Guidelines for proper laboratory animal care were fully implemented.

RESULTS

EXPERIMENT 1: CSD TASK

Acquisition phase

The acquisition phase has been analyzed according to the further random attribution of mice to D1 (Vehicle: $n = 10$; 0.25 mg/kg: $n = 9$; 0.5 mg/kg: $n = 9$; 1.0 mg/kg: $n = 9$) or D2 (Vehicle: $n = 10$; 0.25 mg/kg: $n = 9$; 0.5 mg/kg: $n = 9$; 1.0 mg/kg: $n = 9$) as regards the retrieval test phase and the post-stress delay. Total numbers and percentage of head dips are reported in **Table 1**. The Student's *t*-test is used for comparisons between D1 and D2.

Test phase

A two-way ANOVA performed on all groups (vehicle; Diazepam 0.25 mg/kg, Diazepam 0.5 mg/kg, and Diazepam 1.0 mg/kg) showed a significant interaction between Discriminations × Treatments [$F_{(3,66)} = 33.049$; $p \leq 0.001$; **Figure 2**].

(i) Vehicle. Vehicle-treated mice exhibited performance for D1 and D2 significantly different from chance level (25%; 17.8 ± 2.5%; $t = 3.552$; $p \leq 0.01$ and 46.3 ± 2.5%; $t = 8.663$; $p \leq 0.001$ respectively as compared to chance level). Bonferroni's *t*-test reveals significant memory performances differences between D1 and D2 (17.8 ± 2.5 and 46.3 ± 2.5% respectively; $t = 8.218$; $p \leq 0.001$).

(ii) Diazepam 0.25 mg/kg. Within-group analyses showed that D1 performance in 0.25 mg/kg treated mice was at chance (28.7 ± 5.7%; $t = 1.943$; NS) but was significantly above

Table 1 | Total number of head dips and % number of head dips in the rewarded hole of acquisition 1 and acquisition 2 in vehicle and diazepam-treated groups.

	Total number of head dips		Percent of head dips in baited hole		Student's *t*-test on the percentage of head dips in baited hole	
Groups	**D1**	**D2**	**%D1**	**%D2**	**D1 vs D2**	**vehicle vs doses**
Vehicle $n = 20$	56.5 ± 1.7	64.9 ± 1.4	60.7 ± 2.8	56.5 ± 4.9	$t = 0.739$, NS	
1 mg/kg $n = 18$	63.7 ± 4.2	62.8 ± 6.2	65.6 ± 6.4	67.8 ± 3.8	$t = 0.631$, NS	for D1, $t = 0.738$, NS for D2, $t = 1.793$, NS
0.5 mg/kg $n = 18$	58.1 ± 3.4	61.1 ± 6.9	56.3 ± 2.8	63.6 ± 5.4	$t = 1.131$, NS	for D1, $t = 1.107$, NS for D2, $t = 0.917$, NS
0.25 mg/kg $n = 18$	60.1 ± 3.8	65.4 ± 4.4	58.4 ± 8.1	62.4 ± 5.5	$t = 0.413$, NS	for D1, $t = 0.283$, NS for D2, $t = 0.802$, NS

There is no significant between groups difference.

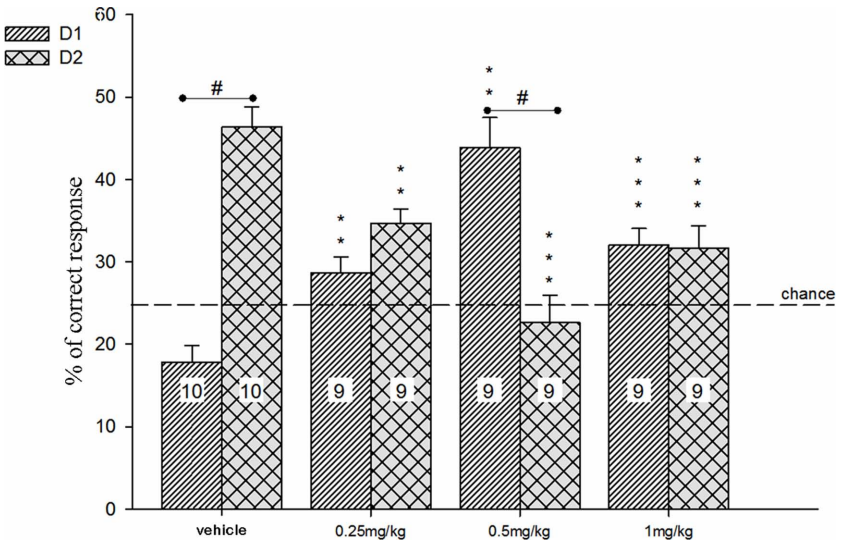

FIGURE 2 | Effect of stress on contextual memory in vehicle and diazepam (0.25, 0.5, and 1 mg/kg) treated mice. Memory performance was evaluated by the percentage of correct responses for D1 and D2 in the CSD task. Each animal were evaluated either on the D1 either on D2. All groups received *ip* injection (vehicle or diazepam) 30 min before the stress delivery. All animals were evaluated 15 min after the stress delivery. Numbers of animals used for each group are mentioned in histograms. Comparison to vehicle groups: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. Dotted lines represent chance level.

chance level for D2 (34.6 ± 6.3%; $t = 5.434$; $p \leq 0.001$). Bonferroni's *t*-test did not reveal any differences between D1 and D2 (28.7 ± 5.7 and 34.6 ± 6.3% respectively; $t = 1.631$; NS). In addition, cross-analyses were performed as compared to vehicle-treated group on independent groups of mice. Inter-group comparisons evidenced a significant difference for D1 performance between the vehicle-treated and the 0.25-mg/kg groups (17.8 ± 2.5 and 28.7 ± 5.7% respectively; $t = 3.042$; $p \leq 0.01$). Furthermore, a significant difference for D2 performance was already observed between the vehicle-treated and the 0.25-mg/kg groups (46.3 ± 2.5 and 34.6 ± 6.3% respectively; $t = 3.284$; $p \leq 0.01$).

(iii) Diazepam 0.5 mg/kg. Within-group analyses showed that D1 performance in 0.5 mg/kg treated mice was significantly above chance level (43.8 ± 3.7%; $t = 5.090$; $p \leq 0.001$) but was at chance for D2 (22.6 ± 3.3%; $t = 0.719$; NS). Bonferroni's *t*-test revealed a significant differences between D1 and D2 performance in 0.5 mg/kg treated mice (43.8 ± 3.7 and 22.6 ± 3.3% respectively; $t = 5.799$; $p \leq 0.001$). In addition, cross-analyses were performed as compared to vehicle-treated group on independent groups of mice. Inter-group comparisons evidenced a significant difference for D1 performance between the vehicle-treated and the 0.5-mg/kg groups (17.8 ± 2.5 and 43.8 ± 3.7% respectively; $t = 7.286$; $p \leq 0.001$). Furthermore, a significant difference for D2 performance was already observed between the vehicle-treated and the 0.5-mg/kg groups (46.3 ± 2.5 and 22.6 ± 3.3% respectively; $t = 6.662$; $p \leq 0.001$).

(iv) Diazepam 1 mg/kg. The 1-mg/kg treated-mice exhibited significant memory performance for D1 and D2 as compared to chance level (25%; 32.1 ± 1.9%; $t = 3.576$; $p \leq 0.01$ and 31.7 ± 2.7%; $t = 2.513$; $p \leq 0.05$ respectively as compared to chance level). Bonferroni's *t*-test did not reveal any difference between D1 and D2 (32.1 ± 1.9 and 31.7 ± 2.7% respectively; $t = 0.103$; NS). In addition, inter-group comparisons evidenced a significant difference for D1 performance between the vehicle-treated and the 1-mg/kg groups (17.8 ± 2.5 and 32.1 ± 1.9% respectively; $t = 3.990$; $p \leq 0.001$). Furthermore, a significant difference for D2 performance was already observed between the vehicle-treated and the 1-mg/kg groups (46.3 ± 2.5 and 31.7 ± 2.7% respectively; $t = 4.114$; $p \leq 0.001$).

Elevated plus-maze task

Results are represented in **Figure 3**. Diazepam administration in stressed middle-aged mice induced a significant decrease of entry ratio as compared to placebo (39.9 ± 5.0%) for the dose of 0.5 mg/kg (58.9 ± 2.3%; $p < 0.01$) and 1.0 mg/kg (54.7 ± 3.1%; $p < 0.05$). In contrast, diazepam at 0.25 mg/kg did not significantly modified performance as compared to vehicle (46.9 ± 2.3%; NS).

EXPERIMENT 2: HIPPOCAMPAL CORTICOSTERONE LEVELS

Basal levels

The basal corticosterone levels in the dialyzate have been analyzed according to the further random attribution of mice to vehicle or diazepam-treated groups.

Basal corticosterone levels in the dialyzate obtained from vehicle-treated mice were 2.71 ± 0.48 nmol/l ($n = 8$), 2.29 ± 0.56 nmol/l ($n = 8$) for 0.25 mg/kg, 1.51 ± 0.49 nmol/l ($n = 8$) for 0.5 mg/kg, 1.77 ± 0.15 nmol/l ($n = 8$) for 1 mg/kg. There were no significant differences in basal extracellular corticosterone levels between: (i) vehicle and 0.25 mg/kg treated mice ($t = 0.567$; NS), (ii) vehicle and 0.5 mg/kg treated mice ($t = 1.888$; NS), (iii) vehicle and 1 mg/kg treated mice ($t = 1.857$; NS).

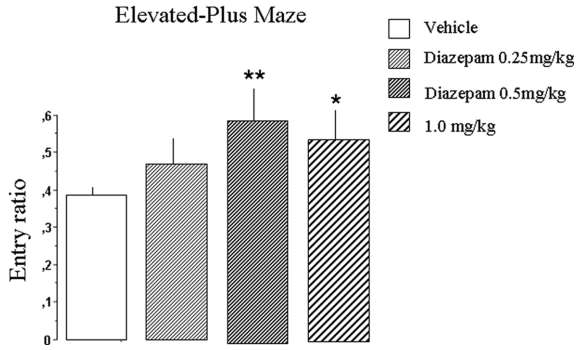

FIGURE 3 | Entry ratio in the elevated plus-maze task in vehicle and diazepam (0.25, 0.5, and 1 mg/kg) treated mice. As can be observed, diazepam at 0.5 and 1.0 mg/kg increased the entry ratio as compared to vehicles, $*p < 0.01$; $**p < 0.05$. The lower diazepam dose (0.25 mg/kg) induced no significant effect as compared to vehicles.

Effect of stress

Figure 4 represents corticosterone levels in the dorsal HPC. Results are expressed in percentage of variation of baseline. Two-way repeated-measures ANOVAs performed on corticosterone kinetic evidenced a significant interaction between Treatments × Time [$F_{(13,364)} = 2.801$; $p \leq 0.001$]. Bonferroni's *t*-test did not reveal any difference between the groups (vehicle, 1, 0.5, and 0.25 mg/kg) in the pre-stress period for each factor.

- Vehicle. As compared to the last pre-stress sample (120.42 ± 25.01%; "time = 0"), stress induced a fast and important increase in corticosterone levels from 15 min after stress (223.77 ± 34.23%; $t = 4.009$; $p \leq 0.001$) to 120 min (215.27 ± 28.04; $t = 3.623$; $p \leq 0.01$).
- 0.25 mg/kg. During the post-stress period, stress induced a fast and rapid increase in corticosterone levels from 15 min after stress (203.36 ± 25.52%; $t = 3.895$; $p \leq 0.01$) to 105 min (190.56 ± 47.28%; $t = 3.254$; $p \leq 0.01$) as compared to the last pre-stress sample (113.52 ± 18.10%; "time = 0"). Only the 120 point was no significant (184.03 ± 35.09%; $t = 1.258$; NS) with the last pre-stress sample (113.52 ± 18.10%; "time = 0"). Furthermore, after the stress delivery, the increase in corticosterone levels observed in 0.25 mg/kg diazepam-treated mice and vehicle-treated mice showed non-significant differences during all the post-stress period (NS in all comparisons).
- 0.5 mg/kg. As compared to the last pre-stress sample (91.23 ± 14.09%; "time = 0"), stress induced a progressive, and significant increase in corticosterone levels from 15 min after stress (169.89 ± 25.47%; $t = 3.004$; $p \leq 0.05$) to 75 min (191.21 ± 18.71%; $t = 3.819$; $p \leq 0.01$). Furthermore, the highest difference was observed 60 min after stress administration (243.51 ± 23.93; $t = 5.817$; $p \leq 0.001$).
  In addition, a faster increase in corticosterone levels in vehicle-treated mice was observed 15 min after stress delivery as compared 0.5 mg/kg diazepam-treated mice (223.77 ± 34.23 and 169.89 ± 25.47%, respectively; $t = 2.414$; $p \leq 0.05$) as well as 30 min after stress delivery (238.22 ± 24.60 and 166.62 ± 15.50%, respectively; $t = 2.295$; $p \leq 0.05$). In contrast, no significant difference was observed between vehicle and 0.5 mg/kg diazepam-treated mice 45, 60, 75, and 90 min after stress delivery (NS in all comparisons). Finally, a faster decrease of corticosterone levels in 0.5 mg/kg diazepam-treated mice was observed as regards to vehicle-treated mice for the 105-min point (172.45 ± 37.95 and 232.86 ± 26.74%, respectively; $t = 2.690$; $p \leq 0.05$).
- 1 mg/kg. As compared to the last pre-stress sample (107.78 ± 8.58%; "time = 0"), the stress-induced rise in corticosterone levels was not observed in 1 mg/kg diazepam-treated mice.
  In consequence, the significant fast and important increase in corticosterone levels in vehicle-treated mice was observed 15 min to the end of the post-stress delay, as compared to 1 mg/kg diazepam-treated mice (223.77 ± 34.23 and 89.63 ± 10.61%, respectively; $t = 4.575$; $p \leq 0.001$ for 15 post-stress delay; 215.27 ± 28.04 and 104.35 ± 23.27%, respectively; $t = 3.738$; $p \leq 0.001$ for 120 post-stress delay).

DISCUSSION

The main findings of our study are as follows:

(i) Diazepam significantly decreases anxiety-like reactivity in the elevated plus maze, at the doses of 0.5 and 1.0 mg/kg (but not at the dose of 0.25 mg/kg), as compared to stressed vehicle-injected animals.

(ii) Stressed middle-aged mice exhibit a highly significant memory of the second discrimination (D2), while responding at chance (25%) for the first one (D1). Diazepam administration at 0.5 mg/kg totally reverses the memory retrieval pattern in acutely stressed middle-aged animals (D1 > D2). In contrast, animals receiving the lowest (0.25 mg/kg) and highest (1.0 mg/kg) diazepam doses exhibit similar memory performance for both discriminations even though just above chance level.

(iii) Microdialysis experiment shows a significant interaction between diazepam doses and corticosterone concentrations into the HPC. From 0.25 to 0.5 mg/kg, diazepam dose-dependently reduces intra-HPC corticosterone concentrations and in parallel, dose-dependently increased hippocampal memory performance in the CSD task. In contrast, the highest diazepam dose (1.0 mg/kg) induces a reduction in hippocampal corticosterone concentration, which was of greater magnitude as compared to the two other diazepam doses, but however decreased the hippocampal-dependent memory performance (D1) in the CSD task.

Our previous data (Chauveau et al., 2008, 2010; Tronche et al., 2010a,b) showed that both stress and aging, as well as hippocampal chemical lesions, selectively reduced the retrieval of D1 to chance level (25%) while sparing the retrieval of D2. In the present study, we focused on stressed condition only insofar as stressed middle-aged mice exhibit memory retrieval pattern comparable to non-stressed middle-aged animals, except that stressed subjects exhibit an increase of D2 response of greater magnitude as compared to non-stressed ones. Thus, since our goal was only to determine if diazepam is able to restore a

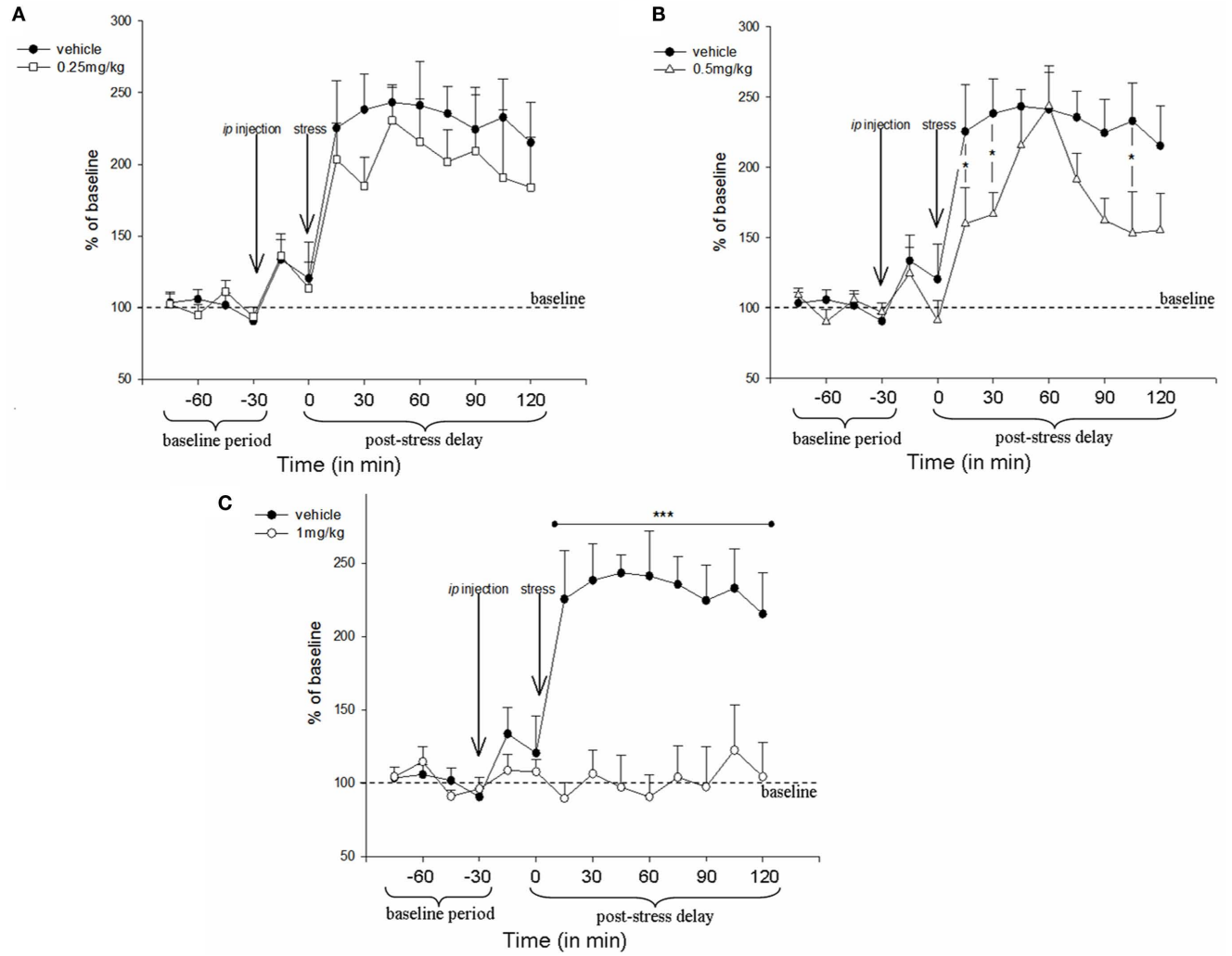

FIGURE 4 | Effect of stress on corticosterone concentration into the dorsal HPC. (A) Effect of 0.25 mg/kg diazepam on corticosterone concentration into the dorsal HPC after stress delivery. **(B)** Effect of 0.5 mg/kg diazepam on corticosterone concentration into the dorsal HPC after stress delivery. **(C)** Effect of 1 mg/kg diazepam on corticosterone concentration into the dorsal HPC after stress delivery. Numbers of animals used for each group are mentioned in graphs legends. Dotted lines represent baseline level. Comparisons to vehicle-treated animals: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

memory retrieval pattern comparable to the level of the one observed in young non-stressed mice (that is to say is able to restore the hippocampal-dependent D1 response), we decided therefore to analyze the effects of diazepam only in the more deleterious condition (middle-aged stressed mice) as compared to non-stress condition.

Our present study confirms the therapeutic anxiolytic action of diazepam. However, in our experimental conditions, this effect is more important for the dose of 0.50 mg/kg ($p < 0.01$), as compared to 1.0 mg/kg ($p < 0.05$). Data from the elevated plus-maze test also shows the absence of any sedative effect of diazepam on locomotion for the range of the doses used in our study.

We already showed that the memory retrieval of the first discrimination (D1) but not of the second one (D2) is dependent on the HPC activity, and that both stress and aging affected the memory retrieval of D1 but not of D2 (Celerier et al., 2004; Chauveau et al., 2008, 2009, 2010; Tronche et al., 2010a,b). Moreover, we also showed previously that in non-stress condition, aging increases HPC corticosterone concentration and abolished the HPC-dependent memory retrieval pattern, as compared to non-stressed young adult mice (Tronche et al., 2010a,b). Thus, acute stress amplified such endocrinal and cognitive effects of aging, as compared to the non-stress condition.

Our present data evidences a significant memory-enhancing effect of diazepam on HPC-dependent memory performance at the dose of 0.5 mg/kg in stressed middle-aged mice. This finding is at first sight surprising, because of the well-known anterograde and retrograde amnesic properties of BDZ in healthy young adult subjects (for review, Beracochea, 2006). However, the memory-enhancing effect observed in the present study as compared to both control animals and chance level for the dose of 0.5 mg/kg may rely on the specificity of the studied population, that is to say stressed middle-aged subjects. The microdialysis experiment shows that diazepam dose-dependently reduces HPC corticosterone

concentrations but the memory-enhancing effect is observed only at the 0.5-mg/kg dose. In contrast, whereas the 1.0-mg/kg dose continues to decrease HPC corticosterone concentration, there is a decrease of HPC-dependent memory performance as compared to the 0.5-mg/kg diazepam dose (see **Figure 5**). Thus, the result found with the highest diazepam dose confirms a pejorative effect of diazepam on HPC-dependent memory performance, as compared to the 0.5-mg/kg dose.

In addition to the effects of diazepam on hippocampal corticosterone, other actors could also be involved in the dose-dependent effect of BDZ on memory in stress condition, mainly catecholamine, GABA, glutamate, and aspartate. Moreover, changes in GABA/BDZ receptor number and affinity (desensitization) could also be involved.

As shown in **Figure 5**, a key finding is the continuous decrease in HPC corticosterone concentration measured 15 min after stress, as a function of the administered dose of diazepam. From a dynamic point of view (**Figure 4**), the highest diazepam dose totally inhibits the stress-induced HPC corticosterone rise. However, for the 0.50-mg/kg dose, diazepam attenuates the stress-induced corticosterone rise as compared to vehicle-treated animals and accelerates the return to baseline.

From a cognitive point of view, **Figure 5** also shows a bimodal effect of diazepam as a function of the administered dose. Indeed, from 0.0 to 0.5 mg/kg, we observed an inverse relationship between HPC-dependent memory performance and HPC corticosterone concentration. In contrast, from 0.50 to 1.0 mg/kg, the memory performance varies in the same way as the HPC corticosterone level.

The action of diazepam on HPC corticosterone concentrations and memory performance could be explained via the interaction between the GABAergic system and HPC corticosterone as a result of the modulation of HPA axis activity by diazepam.

Indeed, numerous data have clearly demonstrated that HPA axis activity is regulated by non-glucocorticoid inhibitors. There is evidence that HPC-mediated mechanisms of glucocorticoid feedback could involve hypothalamic CRH secretion and GABAergic pathways (Calogero et al., 1988; Arvat et al., 2002; Cullinan et al., 2008).

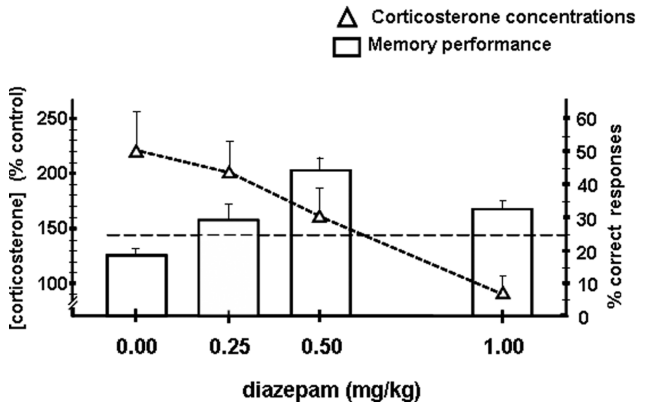

FIGURE 5 | Synoptic view of the effects of diazepam on both hippocampus corticosterone concentrations (left) and memory performance (D1 test; right). Comments are mentioned in the text.

Benzodiazepines (BDZ) activate central GABA receptors, which are importantly distributed in the HPC (Laviv et al., 2010; Lehner et al., 2010). It has been hypothesized that the effects of GABA/BDZ on HPA activity are mediated by CRH and/or AVP (see Cullinan et al., 2008).

The neurotransmitter GABA is a well-known inhibitor of ACTH release (Makara and Stark, 1974), probably through a central action on hypothalamic CRH. *In vivo* injection of the GABA-A receptor antagonist bicuculline into the dorsomedial hypothalamus resulted in increased plasma ACTH and corticosterone (Keim and Shekhar, 1996). Thus, the HPA axis appears to be under tonic GABA inhibition at the hypothalamic level, mediated through GABA receptors (Häusler et al., 1993). Moreover, neuroanatomical and pharmacological studies have established GABA-mediated inhibition of the HPA axis at the level of the PVN (Cullinan et al., 2008). Our study is however the first to evidence an *in vivo* direct dynamic interaction between BDZ and corticosterone level in the hippocampus in stress condition.

Glucocorticoids can impair HPC long term potentiation (LTP) *in vitro* (Dubrovsky et al., 1987; Pavlides et al., 1993) as well as increasing after hyperpolarization mediated by small conductance calcium-activated potassium channels (Joëls and de Kloet, 1989) that have been implicated in arousal. Because of the "inverted-U" response to these hormones (Diamond et al., 1992), low concentrations maintain, moderate concentrations promote, and high concentrations impair neuronal function. LTP is dependent on adrenal output *in vivo*, and adrenalectomy results in a significant decrease in the extent of LTP (Shors et al., 1990). Conversely, stress and excess glucocorticoids impair neuronal function and HPC-dependent memory (reviewed by Sapolsky et al., 1986; de Kloet et al., 1991; Filipini et al., 1991; McEwen and Sapolsky, 1995; Lupien and McEwen, 1997).

So far, the bimodal modulation of HPC-dependent memory performance according to corticosterone concentrations is in agreement with the study of Diamond et al. (1992), showing that corticosterone exerts a concentration-dependent biphasic influence on the expression of hippocampal plasticity.

CONCLUSION

Our data evidences a direct interaction between diazepam, HPC corticosterone concentrations, and HPC-dependent memory performance in stressed middle-aged mice. To our knowledge, it is shown here for the first time that diazepam restores memory performance sustained by the hippocampus as previously evidenced (Chauveau et al., 2010) so that stressed middle-aged animals receiving the 0.5-mg/kg diazepam dose exhibit a memory pattern similar to the one of young adult non-stressed mice. This effect is related to the level of HPC corticosterone. Overall, our data illustrate how stress and benzodiazepines could modulate cognitive functions depending on hippocampus activity.

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Analysis of kinase gene expression in the frontal cortex of suicide victims: implications of fear and stress†

Kwang Choi*, Thien Le, Guoqiang Xing, Luke R. Johnson and Robert J. Ursano

Department of Psychiatry, Center for the Study of Traumatic Stress, Uniformed Services University of Health Sciences, Bethesda, MD, USA

Edited by:
Regina M. Sullivan, Nathan Kline Institute and NYU School of Medicine, USA

Reviewed by:
Fabrício A. Pamplona, Universidade Federal de Santa Catarina, Brazil
Michael R. Bruchas, Washington University in St. Louis, USA

****Correspondence:***
Kwang Choi, Department of Psychiatry, Uniformed Services University of Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA.
e-mail: kwang.choi@usuhs.mil

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Suicide is a serious public health issue that results from an interaction between multiple risk factors including individual vulnerabilities to complex feelings of hopelessness, fear, and stress. Although kinase genes have been implicated in fear and stress, including the consolidation and extinction of fearful memories, expression profiles of those genes in the brain of suicide victims are less clear. Using gene expression microarray data from the Online Stanley Genomics Database[1] and a quantitative PCR, we investigated the expression profiles of multiple kinase genes including the calcium calmodulin-dependent kinase (*CAMK*), the cyclin-dependent kinase, the mitogen-activated protein kinase (*MAPK*), and the protein kinase C (*PKC*) in the prefrontal cortex (PFC) of mood disorder patients died with suicide ($N = 45$) and without suicide ($N = 38$). We also investigated the expression pattern of the same genes in the PFC of developing humans ranging in age from birth to 49 year ($N = 46$). The expression levels of *CAMK2B, CDK5, MAPK9*, and *PRKCI* were increased in the PFC of suicide victims as compared to non-suicide controls (false discovery rate, FDR-adjusted $p < 0.05$, fold change >1.1). Those genes also showed changes in expression pattern during the postnatal development (FDR-adjusted $p < 0.05$). These results suggest that multiple kinase genes undergo age-dependent changes in normal brains as well as pathological changes in suicide brains. These findings may provide an important link to protein kinases known to be important for the development of fear memory, stress associated neural plasticity, and up-regulation in the PFC of suicide victims. More research is needed to better understand the functional role of these kinase genes that may be associated with the pathophysiology of suicide.

Keywords: brain development, frontal cortex, posttraumatic stress disorder, anxiety disorder, psychiatric genomics, bioinformatics

INTRODUCTION

Physical and mental threat can induce fear responses, and fear can be associated with objects and places through a process of Pavlovian fear conditioning (Ledoux, 2000). The process of fear learning and, importantly, its "overriding" or fear extinction is dependent on the amygdala and the prefrontal cortex (PFC). Stress is a multi-dimensional challenge to physical and mental homeostasis that can be triggered by fear (Kim and Diamond, 2002; McEwen, 2007), and both acute and chronic stress can alter the properties of fear (Conrad et al., 1999; Rodrigues et al., 2009). A growing body of evidence suggests that fear and traumatic stress may contribute to the pathophysiology of suicide. For instance, suicidal ideation was significantly associated with traumatic life events and the effects of traumatic stress on suicidal behavior may be mediated by feelings of hopelessness (Tarrier and Picken, 2010; Guerra and Calhoun, 2011). A study using a large scale of civilian population ($N = 34{,}653$) found that over 70% of the individuals who reported a lifetime history of a suicide attempt had anxiety disorders (Nepon et al., 2010). Interestingly, individuals with comorbidity of personality disorders such as neuroticism and posttraumatic stress disorder (PTSD) showed a much stronger association with suicide attempts than those who had PTSD alone. In a military population, more U.S. service members have been deployed since September 2001 than in the previous 40 years. A greater number of these deployed service members are surviving, which has increased the incidence of combat-related PTSD among those veterans (Callahan, 2010). For example, Iraq and Afghanistan War veterans who showed PTSD symptoms were four times more likely to endorse suicidal ideation than their non-PTSD counterparts (Jakupcak et al., 2009). These evidences suggest that fearful memories and PTSD symptoms may contribute to suicidal ideation and attempts, and individual vulnerability to traumatic events is one of the risk factors for suicide (Ursano et al., 2010). However, precise molecular mechanisms underlying how fear and stress trigger suicidal behavior in humans are not clearly understood.

Multiple brain regions have been implicated in emotional learning and memory. Among those, the PFC is one of the key brain regions that integrates stress signals and subsequent decision-making process in humans. Protein phosphorylation in the brain plays a critical role in triggering synaptic changes that are associated with emotional learning and memory (Fischer et al., 2003). Intracellular phosphorylation is orchestrated by a complex network of many different kinases including protein kinase C (*PKC*), calcium/calmodulin-dependent protein kinase (*CAMK*), mitogen-activated protein kinase (*MAPK*), and cyclin-dependent kinase (*CDK*). *PKC* is a critical phosphorylating enzyme in the phosphoinositide signaling pathway. Previous

[1]www.stanleygenomics.org

studies have suggested that these kinases such as *PKC, CAMK, MAPK,* and *CDK* also regulate fear conditioning and extinction in animals (Schafe et al., 2000; Li et al., 2002; Frankland et al., 2004; Lepicard et al., 2006; Sananbenesi et al., 2007; Bergstrom et al., 2011). For example, chronic administration of a *PKC* inhibitor reduced the acquisition of conditioned fear memory suggesting the involvement of *PKC* in the synaptic plasticity and memory (Li et al., 2002). On the contrary, another study reported that inhibition of *PKC* signaling protected dendritic spines in the PFC and rescued working memory impairment caused by chronic stress (Hains et al., 2009). The authors suggested that *PKC* inhibitors may act as neuroprotective agents in fear and stress-related disorders. *CAMK* is also involved in hippocampal-dependent contextual learning in rodents (Kouzu et al., 2000). Among the individuals with psychiatric disorders, the expression levels of *CaMKII* beta (*CAMK2B*) were increased in the PFC of individuals with schizophrenia (27%) and individuals with depression (36%) as compared to the unaffected controls (Novak et al., 2006). Because *CAMK2B* regulates important functions in the brain such as neurotransmitter signaling, neural outgrowth, and pruning, its increased expression in the PFC of the individuals with psychiatric disorders may have important implications. Moreover, inhibition of the *MAPK* pathway in the hippocampus abolished the increased contextual fear conditioning induced by glucocorticoids in mice (Revest et al., 2005). This suggests that the *MAPK* pathway interacts with the glucocorticoid system in fear learning and memory. Another kinase, cyclin-dependent kinase 5 (CDK5), has been implicated in learning and memory (Fischer et al., 2003). *CDK5* plays a role during neurodevelopmental processes, such as interactions with distinct cytoplasmic and synaptic target molecules, and synaptic plasticity underlying memory consolidation in the adult brain. It has been shown that extinction of fear memory requires down-regulation of *CDK5* activity in the mouse hippocampus (Sananbenesi et al., 2007). Taken together, these studies indicate that multiple kinase systems contribute to regulation of emotional learning and memory, and subsequent behavioral responses in animals and in humans.

Recent advances in genomic technologies utilizing postmortem brain tissue have made significant progress toward more analytical and informative research in psychiatry (Harrison, 2011; Mccullumsmith and Meador-Woodruff, 2011). However, identifying potential susceptibility genes associated with suicide has been challenging (Mann et al., 2009; Tsai et al., 2011). We have shown that gene expression changes in postmortem brain tissue are subtle possibly due to a relatively small sample size, known and unknown confounding factors, and diagnostic heterogeneity among psychiatric patients (Choi et al., 2008). Given that multiple kinase genes are implicated in the mechanisms of fear and stress, and fear and stress are the major risk factors for suicidal behavior, we hypothesized that the kinases genes may be involved in the pathophysiology of suicidal behavior. Thus, we investigated the expression profiles of four major kinase genes including protein kinase C (*PKC*), calcium/calmodulin-dependent protein kinase (*CAMK*), mitogen-activated protein kinase (*MAPK*), and *CDK* in the PFC of mood disorder patients died with and without suicide. In order to study the developmental expression pattern of the same genes, we measured mRNA levels in the PFC of normal individuals ranging in age from birth to 49 years. Using gene expression microarrays from the postmortem brain tissue, we aimed to identify potential kinase genes that are associated with both chronological age and suicide.

MATERIALS AND METHODS

POSTMORTEM BRAIN TISSUE

Gene expression microarray datasets from the individuals with mood disorders with suicide ($N = 45$) and without suicide ($N = 38$) were obtained from the Stanley Online Genomics database (see text footnote 1). The details of the brain sample collection have been described previously (Torrey et al., 2000). Developmental brain tissue from the PFC of subjects ($N = 46$) ranging in age from 1 month to 49 years was obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders (UMBB; NICHHD contract# NO1-HD8-3283). The collection protocol was reviewed and approved by the Institutional Review Board of the University of Maryland, Baltimore. For the developmental brains, all subjects were free of neurological and psychiatric symptoms at the time of death as described previously (Choi et al., 2009). Developmental brain microarray raw data are available from the gene expression omnibus (GEO) with an Accession number GSE11512.

MICROARRAY EXPERIMENT

Total RNA was extracted from gray matter of the PFC (BA 46) and using the Trizol method (Invitrogen, Carlsbad, CA, USA). Samples were included only if the RNA was of good quality (RNA integrity number, RIN > 7) as determined by the Bioanalyzer 2100 electrophoresis system (Agilent Technologies, Foster City, CA, USA). Purified RNA was carried through the Affymetrix preparation protocol[2], and each sample was hybridized to the different Affymetrix platform such as HGU95av2, HGU 133a, HGU 133b, or HGU133 plus 2.0 GeneChip to assess genome-wide expression profiles. RNA processing and microarray data generation was performed by the individual investigators at their own facilities as described previously (Choi et al., 2008).

QUALITY CONTROL OF MICROARRAYS

Raw microarray data were processed and analyzed using the R statistical language[3] and the Bioconductor packages (Gentleman et al., 2004). A robust multi-array average (RMA) algorithm was used for normalization of expression values (log base 2) for each transcript (Irizarry et al., 2003). Microarray data quality was assessed using a pair-wise sample correlation coefficient with hierarchical clustering. Transcripts were filtered out if 20% or more of the subjects had expression values of less than a 1.1-fold change in either direction from the transcript's median value and if the percent of subjects with an absent gene call exceeded 33% using the Affymetrix calls.

MICROARRAY ANALYSIS OF THE DEVELOPMENTAL BRAINS

First, we analyzed individual demographic factors including brain pH, postmortem interval (PMI), RIN, race, and sex to identify potential confounds affecting the expression of a significant number of genes ($p < 0.001$). Following the demographic variable analyses,

[2]www.Affymetrix.com

[3]http://www.r-project.org

gene expression across chronological age was analyzed in a series of multiple regression models, one model for each gene, including age (log base 2) and brain pH as independent variables and gene expression (log base 2) as a dependent variable (Choi et al., 2009). To correct for multiple testing of the genes, the calculated *p*-values corresponding to the age covariate for each gene were adjusted to give an overall false discovery rate (FDR) of 5% using the *q*-value (*qv*) package[4]. The criteria of significance were set at $qv < 0.05$.

MICROARRAY ANALYSIS OF MOOD DISORDER WITH AND WITHOUT SUICIDE

Mood disorder subjects were divided into two groups: those with suicide ($N = 45$) and those without suicide ($N = 38$). Each pre- and post-mortem variable was compared between the suicide and the non-suicide group. We identified the variables including age, mood disorder, and duration of illness that were different between the two groups ($p < 0.05$). Thus, we adjusted for these variables in the following suicide analysis using multiple regression models. For an individual study analysis, we performed a linear regression analysis to calculate an adjusted fold change, SE, and *p*-value for each gene in each study. We then performed a cross-study comparison based on scaled representations of individual study-level analysis across nine microarray studies. Consensus fold change was calculated for each gene based on a weighted combination of the individual fold changes and the SEs for the microarray probe sets that map to each gene across the studies as described previously (Choi et al., 2008). Weights were determined in a probe set specific manner to account for the different levels of precision associated with each probe set that map to a given gene across the studies. The weights were equal to 1/SE*i*, where SE*i* is the SE of the *i*th probe set for the gene across all the studies. To adjust for multiple testing of the genes, the calculated *p*-values corresponding to the suicide group were adjusted to give an overall FDR of 5% using the *qv* package. The criteria of significance were set at $qv < 0.05$ and fold change >1.1.

BIOINFORMATICS MAPPINGS

The NCBI's Database for Annotation, Visualization, and Integrated Discovery was used as the standard source for gene annotation information [22]. The primary fields extracted from the DAVID include: Entrez ID, gene symbol, gene name, and gene summary. For the microarrays, queries were based on the Affymetrix probe set ID (AFFYID).

RESULTS

A summary of the subject characteristics included in the microarray studies is shown in **Table 1**. There were no significant differences in sex, race, brain pH, and PMI between the suicide and the non-suicide group; this is important since these pre- and post-mortem variables appear to influence gene expression in the postmortem brain tissue. The number of bipolar disorder subjects was slightly higher in the non-suicide group (66 vs. 51%) while the number of major depression subjects was higher in the suicide group (49 vs. 34%). Other variables such as age (47.2 vs. 41.7) and duration of illness (19.4 vs. 15) were different between the suicide and the non-suicide group ($p < 0.05$). Thus, we adjusted for age, mood disorder (bipolar disorder and major depression) and duration of illness in the following suicide analysis using a multiple regression model. Among the 45 suicide cases, a majority of the subjects died of drug overdose (36%), hanging (29%), jumped (11%), and gun shot wound (9%).

Figure 1 shows the expression profiles of *CAMK2B* in the PFC of normal individuals ranging in age from birth to 49 years (A) and suicide victims as compared to the non-suicide subjects (B). The expression levels of *CAMK2B* in the PFC were gradually decreased during the postnatal development ($r^2 = 0.69$, $qv = 1.1E\text{-}11$). Each subject was color-coded based on the arbitrary age group such as neonate (red), infant (green), toddler (blue), school age (magenta), teenage (pink), young adult (yellow), and adult (gray). A combined analysis of the microarray studies shows that *CAMK2B* expression levels are significantly increased in the PFC of suicide victims as compared to the non-suicide subjects (FC: 1.13, $qv = 0.009$). There are multiple microarray probes ($N = 2$–5) for *CAMK2B* in each study and those probes show consistent changes in expression levels. Although most of the individual studies did not show significant changes, the combined analysis (shown on the bottom) showed consensus increases in expression in the suicide group as compared to the non-suicide group.

The expression levels of *CDK5* in the PFC during postnatal development show inverted U-shape (**Figure 2A**). Specifically, the expression levels of *CDK5* in the PFC were increased until 10 years of age ($r^2 = 0.18$, $qv = 0.035$) and then gradually decreased until 49 years ($r^2 = 0.23$, $qv = 0.029$). A combined analysis of the microarray studies shows that *CDK5* expression levels are increased in the PFC of suicide victims as compared to the non-suicide subjects (FC: 1.19, $qv = 0.04$) as shown in **Figure 2B**. Individual studies did not show significant changes but a combined analysis (shown on the bottom) showed a significant up-regulation of *CDK5* in the PFC of suicide victims.

Figure 3A shows that the expression levels of mitogen-activated protein kinase 9 (*MAPK9*) in the PFC are gradually increased in the PFC of normal individuals ranging in age from birth to 49 years ($r^2 = 0.64$, $qv = 1.2E\text{-}05$). The expression levels consistently increased

Table 1 | A summary of subject characteristics included in suicide microarray analysis.

	Non-suicide	Suicide
Number of subjects	38	45
Age	47.2 (1.6)	41.7 (1.6)
Sex (male)	55.3%	55.6%
Race (caucasian)	94.7%	95.6%
Bipolar disorder	65.8%	51.1%
Major depression	34.2%	48.9%
PMI	32.9 (2.5)	33.0 (2.3)
Brain pH	6.4 (0.05)	6.5 (0.04)
Duration of illness	19.4 (1.7)	15.0 (1.4)
Lifetime antipsychotics	11287 (4021)	4981 (1707)

For each variable, mean ± SE or percentage value is reported. PMI, postmortem interval.

[4]www.bioconductor.org

[5]http://david.abcc.ncifcrf.gov/

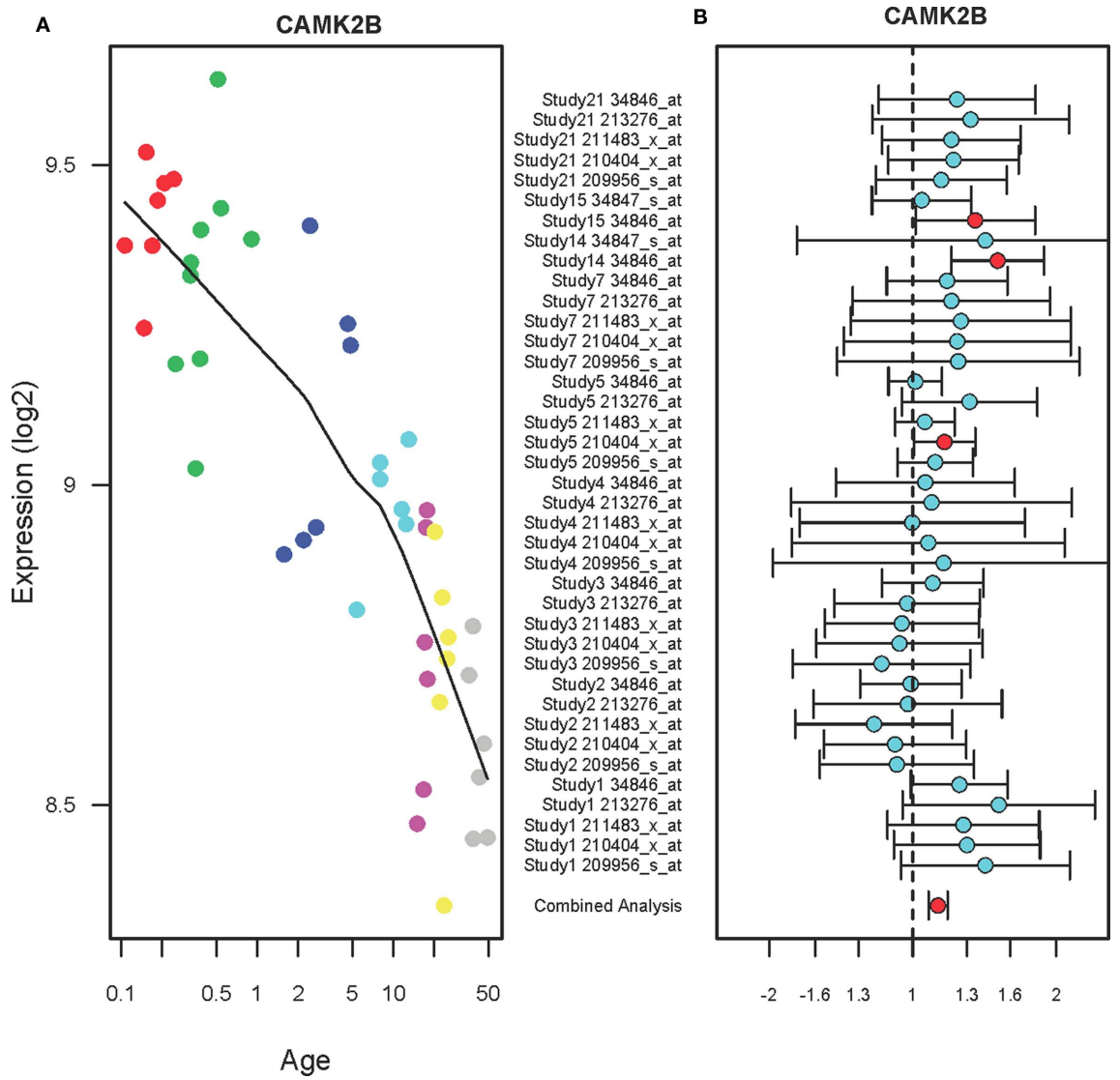

FIGURE 1 | The expression profiles of *CAMK2B* in the PFC of normal developing individuals and suicide victims. (A) The scatter plot with a locally weighted scatter plot smoothing (LOWESS) line demonstrates gradual decreases in expression levels of *CAMK2B* in the PFC of normal humans ranging in age from birth to 49 years. Points are colored by the predefined age groups: red = neonate, green = infant, blue = toddler, light blue = school age, pink = teenage, yellow = young adult, gray: adult. **(B)** The plot with fold changes and 95% confidence intervals shows consistent up-regulation of *CAMK2B* in the PFC of suicide victims as compared to the non-suicide controls across nine microarray studies. A combined analysis shown on the bottom represents the weighted fold change and 95% confidence intervals. Individual microarray probes for *CAMK2B* across multiple studies are shown on the left (Study ID and Affymetrix probe ID). Red circle: adjusted $p < 0.05$, Cyan circle: adjusted $p > 0.05$.

until 20 years of age then stabilized until 49 years. In the PFC of suicide victims, *MAPK9* expression levels were increased as compared to the non-suicide subjects (FC: 1.21, $qv = 0.002$) as shown in **Figure 3B**. Each study has two microarray probes for *MAPK9* and most of the probes showed a tendency toward increase in expression. A combined analysis of microarrays revealed increased expression levels of *MAPK9* in the suicide victims as compared to the non-suicide controls.

A developmental expression pattern of the protein kinase C iota (*PRKCI*) gene showed a gradual decrease in the PFC of normal individuals ranging in age from birth to 49 years ($r^2 = 0.66$, $qv = 4.2\text{E-}06$) as shown in **Figure 4A**. A combined analysis of the microarrays showed that *PRKCI* expression levels were increased in the PFC of suicide victims as compared to the non-suicide subjects (FC: 1.14, $qv = 0.015$) shown in **Figure 4B**. Each microarray study has three probes for *PRKCI* and two studies (Study ID 2 and 3) did not show any changes in *PRKCI* expression while the rest of studies showed a tendency toward increase in expression. A combined analysis of microarrays demonstrated small but consensus changes in expression levels of *PRKCI* in the suicide group as compared to the non-suicide group.

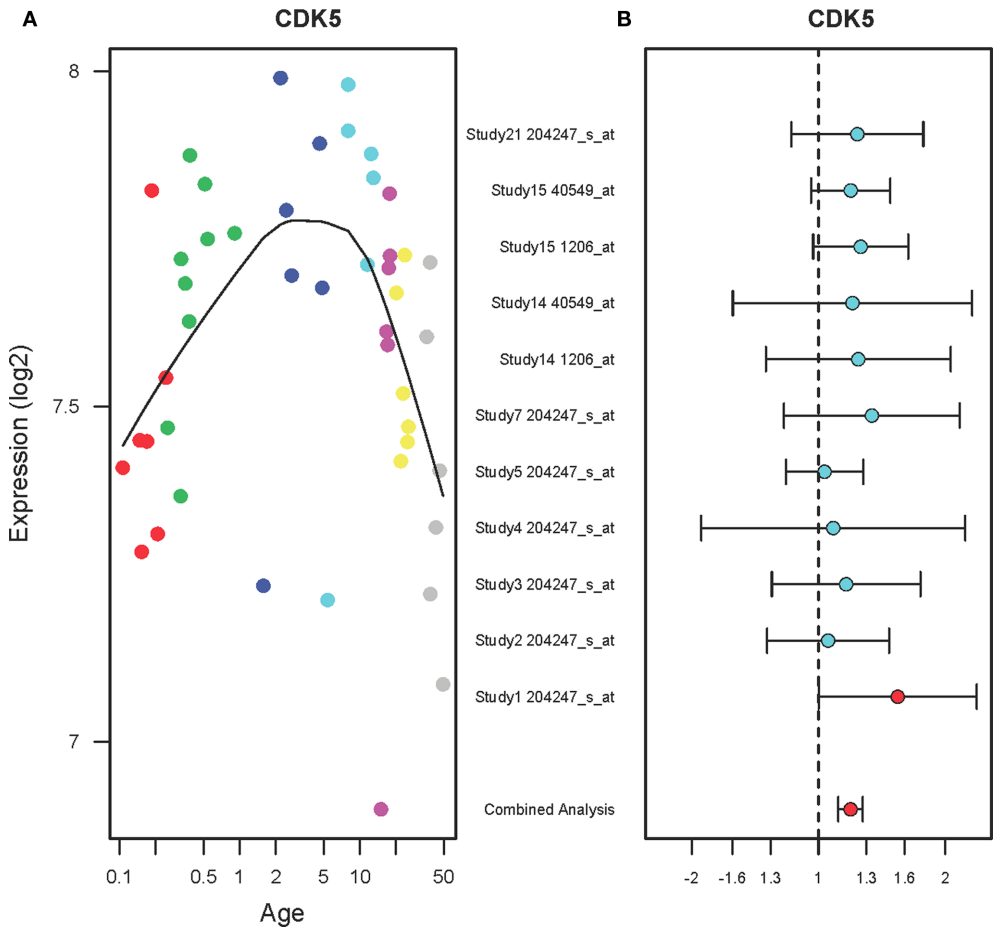

FIGURE 2 | The expression profiles of *CDK5* in the PFC of normal developing individuals and suicide victims. (A) The scatter plot with a locally weighted scatter plot smoothing (LOWESS) line demonstrates inverted U-shape expression pattern of *CDK5* in the PFC of normal humans ranging in age from birth to 49 years. Points are colored by the predefined age groups: red = neonate, green = infant, blue = toddler, light blue = school age, pink = teenage, yellow = young adult, gray: adult. **(B)** The plot with fold changes and 95% confidence intervals shows consistent up-regulation of *CDK5* in the PFC of suicide victims as compared to the non-suicide controls across nine microarray studies. A combined analysis shown on the bottom represents the weighted fold change and 95% confidence intervals. Individual microarray probes for *CDK5* across multiple studies are shown on the left (Study ID and Affymetrix probe ID). Red point: adjusted $p < 0.05$, Cyan point: adjusted $p > 0.05$.

DISCUSSION

The mechanisms of suicide are complex mediated by the interaction of multiple factors including genes and environment (Brezo et al., 2008; Fiori et al., 2011). However, the search for specific genetic factors that contribute to the pathophysiology of suicide has been challenging (Mann et al., 2009; Tsai et al., 2011). Previous studies with rodent models with fear and stress suggested that kinase genes in the brain may play a role in fear and stress-related behavior (Kouzu et al., 2000; Li et al., 2002; Revest et al., 2005; Sananbenesi et al., 2007; Hains et al., 2009). Although fear and stress may contribute to suicidal behavior in humans, expression profiles of those kinases genes in suicide brains have not been well-characterized. Thus, we investigated the expression profiles of the kinase genes using the microarray data from a well-characterized cohort of postmortem brains of mood disorder patients who died with suicide and without suicide. We found that four kinase genes including *CAMK2b, CDK5, MAPK9, and PRKCI* undergo age-dependent changes in expression in the PFC of normal individuals ranging in age from birth to 49 years. The expression levels of the same genes were increased in the PFC of suicide victims as compared to the non-suicide controls. It is important to note that these genes have been implicated in the mechanisms of fear and stress-related disorders. Thus, these genes may also contribute to the pathophysiology of suicide via interactions with the fear and stress circuitry in the brain.

Here, we demonstrated the advantages of combining multiple microarray datasets to detect small but consensus changes in gene expression in the PFC of suicide victims. We found robust changes in gene expression in the PFC of normal individuals ranging in age from birth to 49 years, suggesting that chronological age is one of major factors affecting brain gene expression during development. We attempted to identify the genes that are associated with both chronological age and suicide phenotype in individuals with mood

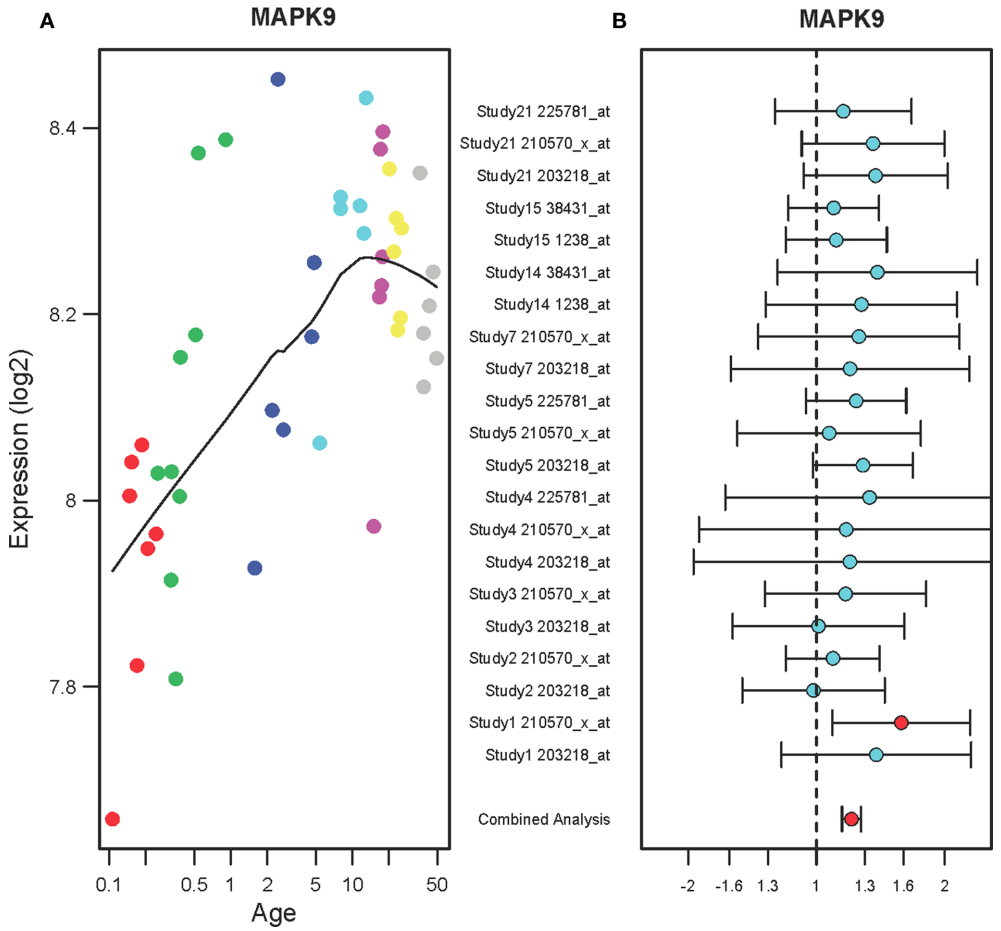

FIGURE 3 | The expression profiles of *MAPK9* in the PFC of normal individuals and of suicide victims. (A) The scatter plot with a locally weighted scatter plot smoothing (LOWESS) line demonstrates gradual increases in expression of *MAPK9* in the PFC of normal humans ranging in age from birth to 49 years. Points are colored by the predefined age groups: red = neonate, green = infant, blue = toddler, light blue = school age, pink = teenage, yellow = young adult, gray: adult. **(B)** The plot with fold changes and 95% confidence intervals shows consistent up-regulation of *MAPK9* in the PFC of suicide victims as compared to the non-suicide controls across nine microarray studies. A combined analysis shown on the bottom represents the weighted fold change and 95% confidence intervals. Individual microarray probes for *MAPK9* across multiple studies are shown on the left (Study ID and Affymetrix probe ID). Red point: adjusted $p < 0.05$, Cyan point: adjusted $p > 0.05$.

disorder. A recent study showed the advantage of performing the genetic analysis using only mood disorder subjects with or without suicide attempts (Perlis et al., 2010). Thus, we have taken a similar approach of comparing suicide vs. non-suicide among the mood disorder subjects. Because we used only mood disorder samples, we were able to minimize the potential confounding effects of comorbid psychiatric disorders in our analysis.

Although many kinases are involved in a complex network of intracellular phosphorylation, previous studies suggested that multiple kinase genes such as *PKC, CAMK, MAPK,* and *CDK* play a significant role in regulating fear memories (Schafe et al., 2000; Li et al., 2002; Frankland et al., 2004; Lepicard et al., 2006; Sananbenesi et al., 2007; Bergstrom et al., 2011). *CAMK2* including two splice variants, *CaMK2A* and *CaMK2B*, control dendritic growth and maturation in neurons, as well as phosphorylation of numerous receptors including GABAA receptor (Churn and Delorenzo, 1998) and NMDA glutamate receptor subunits (Rakic et al., 1994). *CAMK2* modulates catecholamine metabolism via phosphorylation of tyrosine hydroxylase in dopamine neurons (Seeman et al., 1976). Previous studies investigated the role of *CAMK2A and CAMK2B* in the postmortem brains of individuals with mood disorder. For example, a gene expression microarray study showed that *CAMK2A* expression levels were increased in the PFC of individuals with major depression (Tochigi et al., 2008). Another study replicated an up-regulation of *CAMK2A* in the PFC of individuals with major depression but not bipolar disorder using a real-time quantitative PCR (Novak et al., 2006). The authors showed that the expression levels of *CaMK2A and CAMK2B* were elevated in the depression subjects by 29 and 36%, respectively. Moreover, the increased levels in depression subjects were not altered by a history of antidepressant medication in that study. Here, we classified the mood disorder subjects into either the suicide or the non-suicide group and we had a slightly higher number of depression cases in the suicide group. Although the increased levels of *CAMK2B* in the suicide

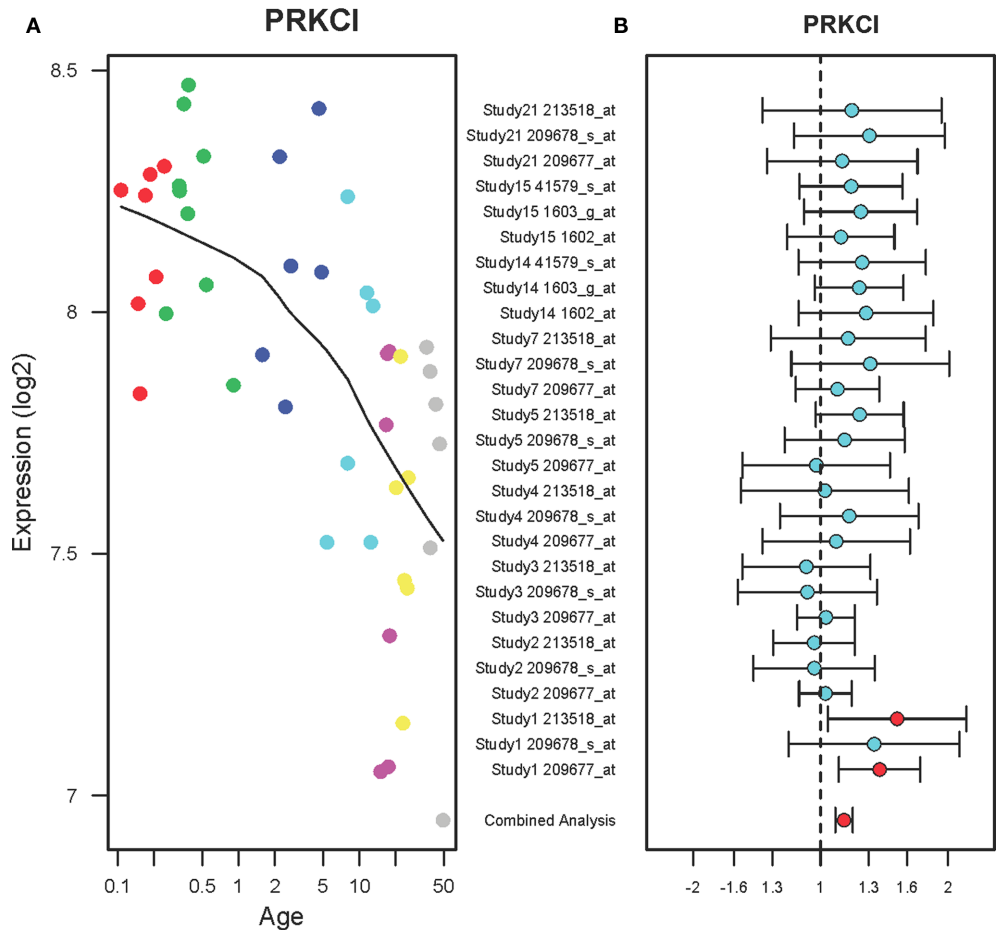

FIGURE 4 | The expression profiles *PRKCI* in the PFC of normal developing individuals and suicide victims. (A) The scatter plot with a locally weighted scatter plot smoothing (LOWESS) line demonstrates gradual decreases in expression of *PRKCI* in the PFC of normal humans ranging in age from birth to 49 years. Points are colored by the predefined age groups: red = neonate, green = infant, blue = toddler, light blue = school age, pink = teenage, yellow = young adult, gray: adult. **(B)** The plot with fold changes and 95% confidence intervals shows consistent up-regulation of *PRKCI* in the PFC of suicide victims as compared to the non-suicide controls across nine microarray studies. A combined analysis shown on the bottom represents the weighted fold change and 95% confidence intervals. Individual microarray probes for *PRKCI* across multiple studies are shown on the left (Study ID and Affymetrix probe ID). Red point: adjusted $p < 0.05$, Cyan point: adjusted $p > 0.05$.

group may have been influenced by higher number of depression samples in the suicide group, this is unlikely because we adjusted for the mood disorder effects in the multiple regression analysis of suicide. Given that *CAMK2B* could phosphorylate and influence the activity of many neurotransmitter receptors as well as neuronal growth and pruning, its altered expression in both developing and suicide brains suggests an important role in fear and stress.

CDK5 has been implicated in anxiety and stress-related disorders that may require the promotion of the fear extinction process, which is defined as the learned reduction of fear. Some of the roles of *CDK5* during neurodevelopmental processes, such as interactions with distinct cytoplasmic and synaptic target molecules, may be related to the synaptic plasticity underlying memory consolidation (Fischer et al., 2003). A study using genetic and pharmacological approaches showed that extinction of fear memory requires the down-regulation of *CDK5* in mice (Sananbenesi et al., 2007). The authors demonstrated that several key proteins associated with the *CDK5* pathway play a critical role in extinction of fear memory. Here, we found age-dependent changes in *CDK5* expression: up-regulation until 10 years of age and then gradual down-regulation until 49 years of age. This suggests that there is a sensitive period of *CDK5* expression in the PFC during postnatal development. Thus, any disruptions in normal gene expression changes during development may result in dysfunction of stress and fear mechanisms. Increases in *CDK5* levels in the PFC of suicide victims further suggest that fear learning and extinction mechanisms may be disrupted in the individuals with suicide.

MAPK signaling pathway is critical for cell division and differentiation in the hippocampus as well as subsequent synaptic plasticity and memory formation (Sweatt, 2001). Importantly, *MAPK* has been shown to be critical for the formation of new fear memories, as well as for extinction and reconsolidation of fear memories (Schafe et al., 2000; Herry et al., 2006; Bergstrom et al., 2011). These studies also support the role of neuronal plasticity

in the PFC for fear extinction (Herry et al., 2006). Stress, acting via glucocorticoids, may directly interact with *MAPK* mediated neuronal plasticity. A study showed that activation of glucocorticoid receptors increased the expression and enzymatic activity of proteins associated with the *MAPK* signaling pathway in mouse hippocampus (Revest et al., 2005). Inhibition of the *MAPK* pathway in the hippocampus abolished the increases in contextual fear conditioning induced by glucocorticoids. These results suggest a functional interaction between glucocorticoids receptors and the *MAPK* system in fear learning and memory. An epigenetic study showed that *MAPK1* regulates hippocampal chromatin remodeling in memory formation (Chwang et al., 2007). The authors identified the mitogen- and stress-activated protein kinase 1 (MSK1), a nuclear kinase downstream of ERK, as an important regulator of chromatin remodeling in long-term memory formation. Here, we found that the expression levels of *MAPK9* in the PFC of normal individuals gradually increased until 20 years of age then stabilized during postnatal development. This suggests that there is an increased demand on *MAPK9* function in the PFC during the sensitive period of brain development. Moreover, abnormal increases in *MAPK9* levels in the PFC of suicide victims indicate a critical role of this gene in the pathophysiology of suicide.

Previous studies suggested that the protein kinase C (*PRKC*) gene is involved in fear memory and suicide. For example, a chronic administration of *PRKC* inhibitor staurosporine (0.1 mg/kg, for 14 days) significantly reduced the acquisition of conditioned fear in rats (Li et al., 2002). This supports the notion that *PRKC* plays a key role in synaptic plasticity underlying emotional learning and memory. However, it is important to note that the staurosporine is not a selective inhibitor of *PRKC* and therefore other kinases are also inhibited by this compound. A postmortem brain study examined potential association between the pathogenesis of teenage suicide and *PRKC* using 17 teenage suicide victims and 17 non-psychiatric control subjects (Pandey et al., 2004). Enzymatic activity, protein and mRNA levels of various *PRKC* isozymes (including *PRKC* alpha, beta, and gamma) were measured in the PFC and hippocampus of suicide and non-suicide groups. There were significant decreases in protein and mRNA levels of *PRKC* alpha, beta, and gamma isozymes in the PFC and hippocampus of suicide victims as compared to the non-psychiatric controls. However, potential confounding effects of comorbid psychiatric disorders in the suicide victims have not been controlled in that study. Moreover, another *PRKC* isozyme, *PRKC* iota (*PRKCI*), was not measured. Here, we showed that the expression levels of *PRKCI* in the PFC were gradually decreased across age from birth to 49 years of age and increased in the PFC of mood disorder patients with suicide as compared to the non-suicide mood disorder subjects. Consistent with our findings, a study demonstrated that inhibition of *PRKC* signaling protected dendritic spines in the PFC and restored working memory impairment caused by chronic stress (Hains et al., 2009). This suggests that stress and anxiety may disrupt *PRKC* signaling in the PFC and inhibition of *PRKC* function may be neuroprotective and beneficial for the treatment of anxiety disorders. Many biological functions are mediated through phosphorylation by *PRKC* in the brain and therefore, *PRKC* may be a potential target for therapeutic intervention in individuals with anxiety disorders and suicidal ideation.

Postmortem brain studies of psychiatric patients are often challenging because many known and unknown factors can affect gene expression profiles in the brain tissue. Biological effects are often hindered by relatively small sample sizes, small effect sizes and comorbid psychiatric disorders. Available clinical information from each patient is typically sparse so that unknown covariates may either confound or confuse gene expression findings in postmortem brains. Thus, appropriate statistical adjustment is critical to improve inferences in determining gene expression changes in the brain of suicide victims. Although gene expression studies using postmortem brain tissue may reveal valuable information related to suicide, this approach alone is limited in terms of being able to distinguish between changes reflecting the primary disease etiology from those reflecting compensatory mechanisms and many potential confounding influences such as medication and substance use. Thus, it is important to study the functional role of these kinase genes that are being differentially expressed in the PFC of suicide victims as compared to the non-suicide subjects. In order to allow for a conclusive evaluation of the current findings, replication studies using independent samples as well as functional *in vitro* assays and pharmacological studies in animals are warranted. More research is needed to better understand the significance of the kinase genes that may be involved in the pathophysiology of suicide.

CONCLUSION

We identified four kinase genes including *CAMK2B, CDK5, MAPK9, and PKCI* that show both age-dependent changes in normal individuals as well as pathological changes in suicide victims. Importantly, these kinases are critical for fear and stress associated neuronal plasticity. Given that these genes play a critical role in the mechanisms of fear and stress, abnormal changes in expression in the PFC of suicide victims further suggest overlapping biological pathways between fear, stress, and suicide. Thus, any disruptions in normal expression changes during the sensitive period of postnatal development may result in dysfunction of those genes that contribute to the pathophysiology of suicide in adulthood. A better understanding of the kinase genes and their interaction with environmental factors may help to develop novel strategies for suicide prevention.

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Differential regulation of neuropeptide Y in the amygdala and prefrontal cortex during recovery from chronic variable stress

Jennifer L. McGuire[1]*, Lauren E. Larke[2,3], Floyd R. Sallee[3], James P. Herman[2,3] and Renu Sah[2,3]

[1] *Center for Neuroscience and Regenerative Medicine, Department of Psychiatry, Uniformed Services University, Bethesda, MD, USA*
[2] *Neuroscience Graduate Program, University of Cincinnati, Cincinnati, OH, USA*
[3] *Department of Psychiatry, University of Cincinnati, Cincinnati, OH, USA*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Phillip R. Zoladz, Ohio Northern University, USA
Amanda Sharko, University of South Carolina School of Medicine, USA

****Correspondence:***
Jennifer L. McGuire, Department of Psychiatry, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD 20814, USA.
e-mail: jennifer.mcguire.ctr@usuhs.mil

Accumulating evidence from clinical studies and pre-clinical animal models supports a role for neuropeptide Y (NPY) in adaptive emotional response following stress. The long-term impact of stress, particularly chronic stress, on availability, and function of resilience factors such as NPY may be critical to understanding the etiology of stress-related psychopathology. In these studies, we examined expression of NPY during recovery from a chronic variable stress (CVS) model of repetitive trauma in rats. Due to the importance of amygdala and prefrontal cortex in regulating emotional responses, we predicted chronic changes in NPY expression could contribute to persistent behavioral deficits seen in this model. Consistent with the hypothesis, ELISA for NPY peptide identified a significant reduction in NPY at the delayed (7 days) recovery time-point. Interestingly, a significant increase in prefrontal NPY was observed at the same recovery time-point. The mRNA expression for NPY was not changed in the amygdala or PFC, although there was a modest but not statistically significant increase in NPY mRNA at the delayed recovery time-point in the prefrontal cortex. The observed changes in NPY expression are consistent with maladaptive coping and enhanced emotionality, due to the nature of NPY signaling within these respective regions, and the nature of reciprocal connections between amygdala and prefrontal cortex.

Keywords: neuropeptide Y, amygdala, resilience, prefrontal cortex, chronic variable stress

INTRODUCTION

Accumulating evidence from pre-clinical and clinical studies implicates neuropeptide Y (NPY) as an important stress resiliency factor/hormone. NPY acts directly in limbic forebrain structures, antagonizing the actions of pro-anxiety hormone, corticotropin-releasing hormone (CRH) (Heilig, 2004; Giesbrecht et al., 2010) working to maintain balance between pro- and anti-anxiety signaling and helping to regulate emotional state (Sajdyk et al., 2004). Additionally, NPY in the amygdala regulates the expression of fear responses (Fendt and Fanselow, 1999). Animals over-expressing NPY in forebrain regions (Thorsell et al., 2000) or exclusively in the amygdala (Primeaux et al., 2005) are resistant to anxiogenic stress as measured in pharmacologically validated behavioral tests of rodent anxiety. Recent studies in humans corroborate the data from animal studies. A variant allele in the promoter region of NPY is linked to higher trait anxiety (Zhou et al., 2008), and increased psychopathology after adversity in analyses of gene × environment interaction (Sommer et al., 2010). Interestingly, lower haplotype-driven NPY expression predicted higher emotion-induced activation of the amygdala, as wells as higher neuroticism scores and diminished resiliency (Zhou et al., 2008). Reduced concentrations of NPY are observed in cerebrospinal fluid of posttraumatic stress disorder (PTSD) patients (Sah et al., 2009) and in plasma of trauma exposed individuals (Morgan III et al., 2003). Increased plasma NPY levels are correlated with symptom improvement in individuals with past PTSD, supporting an association of NPY with coping and resilience (Yehuda et al., 2006). Collectively, a considerable body of evidence supports the relevance of NPY as an important regulator of stress and fear responses.

Stress-associated psychopathologies are often associated with inadequate stress coping and failure to recover from traumatic life events. Optimal function of putative resiliency factors such as NPY may be essential for adequate reactivity to and recovery from stress. In this regard, it is important to investigate how stress impacts long-term expression of NPY. This is particularly relevant for repeated stress exposure, where depletion of stress buffering systems are likely.

In this report we investigated regulation of NPY expression after cessation of chronic stress, where factors influencing resilience would be most critical for recovery. We used a chronic variable stress (CVS) paradigm recently developed by our group as a model of chronic traumatization and posttraumatic-like phenomena (McGuire et al., 2010). Exposure to CVS produces a delayed expression of enhanced fear reinstatement and fearful arousal, behaviors that may be impacted by a dysregulation in NPY. To test the hypothesis that repeated stress would dysregulate neural NPY systems, NPY mRNA, and peptide expression were measured at early and delayed recovery time-points in the amygdala and prefrontal cortex, brain regions implicated in posttraumatic pathophysiology (Shin et al., 2006; Liberzon and Sripada, 2008).

In support of our hypothesis, central NPY systems manifest alterations in mRNA and protein expression during recovery from CVS in both amygdala (down-regulation) and PFC (up-regulation), both of which are consistent with exaggerated stress responsiveness observed in this model.

MATERIALS AND METHODS

THE CVS MODEL

Subjects were male Long–Evans rats between 225 and 250 g (Harlan, Indianapolis, IN, USA). Animals were housed in a climate-controlled vivarium on a 12:12 light dark cycle, lights on 6:00 a.m. All procedures were reviewed and approved by the University of Cincinnati animal care and use committee.

The CVS model was as previously described (McGuire et al., 2010). Subjects were randomly assigned to weight matched control and chronic stress groups. Briefly, experimental animals underwent two stressors a day, morning, and afternoon, for 7 days. Morning and afternoon stressors were administered between 0900–1100 and 1400–1600 hours, respectively. Stressors were selected to include both primarily anxiogenic and primarily physiologic stressors, including restraint, hypoxia, forced swimming, cold, temporary crowding, and agitation of the cages. In addition to the daily stressors, twice during the CVS period the animals were housed overnight in a confined space (a mouse shoebox cage). Overnight stressors began immediately after cessation of afternoon stressors and terminated at the initiation of the next day's morning stressor. Within the CVS and control groups, animals were further subdivided in early and delayed recovery time-points and sacrificed at either 24 h (early) or 7 days (delayed) after termination of CVS. Brains were rapidly isolated: a midline sagittal incision was made to divide the brain into two equal halves that were then rapidly flash frozen in isopentane on dry ice. One half was processed for NPY ELISA for peptide concentrations while the other was subjected to *in situ* hybridization for mRNA levels. The samples were randomized between the two procedures to overrule any lateralization effects.

ELISA FOR MEASUREMENT OF NPY PEPTIDE CONCENTRATION

The brains were kept frozen until transfer into acid for extraction of the NPY peptide. The amygdala and prefrontal cortex were dissected from cryostat-sliced sections using bregma −2.12 to −3.6 (amygdala) and 3.20 to 2.20 (PFC) as stereotaxic coordinates (Paxinos and Watson, 1998). Dissected tissue was homogenized in 200–300 μl of 0.2 M HCl. The homogenates were boiled for 5 min and cooled on ice. Ten microliter aliquots were removed for later analysis of total protein concentrations. Remaining supernatants were then lyophilized overnight in a speed vac to ensure complete drying. Dried extracts were stored at −80°C until ELISA assay.

Frozen samples were re-constituted with ELISA buffer and used for NPY ELISA (Peninsula Laboratories, San Carlos, CA, USA) as described previously (Sah et al., 2009). Homogenate volumes for ELISA were optimized in preliminary runs for each region such that OD readings were obtained within the linear section of the NPY standard curve. Peptide concentration was determined from plotting optical density of unknown samples against a 10 point standard curve for NPY. Total protein was determined by Bradford protein assay. Data was calculated for nanogram NPY per mg protein. Samples from Control and CVS exposed animals were tested for post-CVS early and delayed recovery.

IN SITU HYBRIDIZATION

Brain samples were coronally sectioned at 14 μm on a Leica 3050 cryostat, mounted on Fisherbrand Superfrost-Plus-charged glass slides (Hampton, NH, USA), and stored at −20°C until further analysis. Prior to hybridization, sections were thawed to room temperature and fixed for 15 min in 4% paraformaldehyde. Sections were then rinsed 2 × 5 min in 5 mM DEPC-treated potassium phosphate buffered saline (KPBS), 2 × 5 min in PBS containing 0.2% glycine, followed by 2 × 5 min in KPBS. Sections were acetylated for 10 min in triethanolamine (0.1 M, pH 8.0), containing 0.25% acetic anhydride, rinsed twice in SSC buffer (0.25 M sodium chloride, 0.015 sodium citrate, pH 7.2) for 5 min, followed by dehydration in a graded ethanol series. Sections were re-hydrated to 70% ethanol and then air-dried. Antisense rat NPY riboprobes were generated (complimentary to bp 20–532 of the NPY sequence Accession #M15880) by *in vitro* transcription using ^{35}S-labeled UTP. Riboprobe ^{35}S percent incorporation was determined with TCA precipitation. Labeled probes were added to a hybridization buffer containing 50% formamide, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 335 mM NaCl, 1× Denhardt's solution, 200 μg/ml fish sperm DNA, 150 μg/ml yeast transfer RNA, 20 mM dithiothreitol, and 10% dextran sulfate. Probes were denatured for 15 min at 65°C and 50 μl (1×10^6 cpm) of diluted probe applied to each slide. Slides were coverslipped, placed in moistened chambers, and incubated overnight at 55°C. After hybridization, coverslips were removed in 0.2× SSC, and rinsed in fresh 0.2× SSC for 10 min. Sections were then treated with RNase A (50 μg/ml) for 30 min at 37°C, and transferred to fresh 2× SSC and then rinsed three times in 0.2× SSC (10 min/wash), followed by a 1-h wash in 0.2× SSC at 65°C. Finally, sections were dehydrated in a graded ethanol series, dried at room temperature, and exposed for 4–6 days to Kodak BioMAX film (Eastman Kodak, Rochester, NY, USA).

IMAGE ANALYSIS

Film images of brain sections were captured by digital camera. Semi-quantitative microdensitometry analysis for autoradiograph images was performed using Scion Image (Alpha 4.0.3.2; Scion, Frederick, MD, USA) software. Brain regions were identified using the Paxinos and Watson rat brain atlas. Each identified region of interest was analyzed by subtracting the non-hybridized tissue (background) from the hybridized signal within the same brain section, and data were expressed as corrected gray level (CGL). Multiple brain sections were analyzed per region per animal. Average CGL values were calculated in series for the amygdala and prefrontal cortex. ^{14}C standards were developed with each film and analyzed for CGL to confirm that all measured gray levels were within the linear range of the film.

STATISTICAL ANALYSIS

Data for NPY ELISA and *in situ* hybridization for each region was analyzed by unpaired *t*-test for the early and delayed recovery time-point using stress as the variable. Data is expressed as mean ± standard error of the mean (SEM). Criterion for statistical significance was $p < 0.05$.

RESULTS

To investigate the regulation of NPY mRNA and peptide in the same animals, brains were bisected in the midline. As a predicted stress resilience factor, it was hypothesized that NPY would be regulated in the amygdala and PFC following traumatic stress, and that these alterations may persist into later recovery. Therefore tissue was collected at timepoints early and later in recovery as depicted in **Figure 1A**. Consistent with this hypothesis, NPY content in the amygdala showed a significant reduction (38.4%) at the delayed recovery time-point in the amygdala (**Figure 1B**). Unpaired t-test with stress as the variable revealed significant depletion at this recovery point ($t = 2.258$; $p < 0.05$). There was a reduction NPY content at the early time-point (32.2%), but that did not reach statistical significance. In contrast to the amygdala, NPY peptide concentration in the PFC was significantly upregulated (128.3%) at delayed recovery ($t = 2.761$; $p < 0.05$ by unpaired t-test), while no changes were noted at early recovery (**Figure 1B**). To reveal whether alterations in NPY peptide were accompanied by changes in NPY synthesis within the region, NPY mRNA expression was measured in contralateral sections from the same animals (**Figure 2**). No significant changes in NPY mRNA density were observed at the early recovery time point in the amygdala or the PFC. However, the change in PFC NPY at the 7-day delayed recovery time-point approached statistical significance ($p = 0.09$).

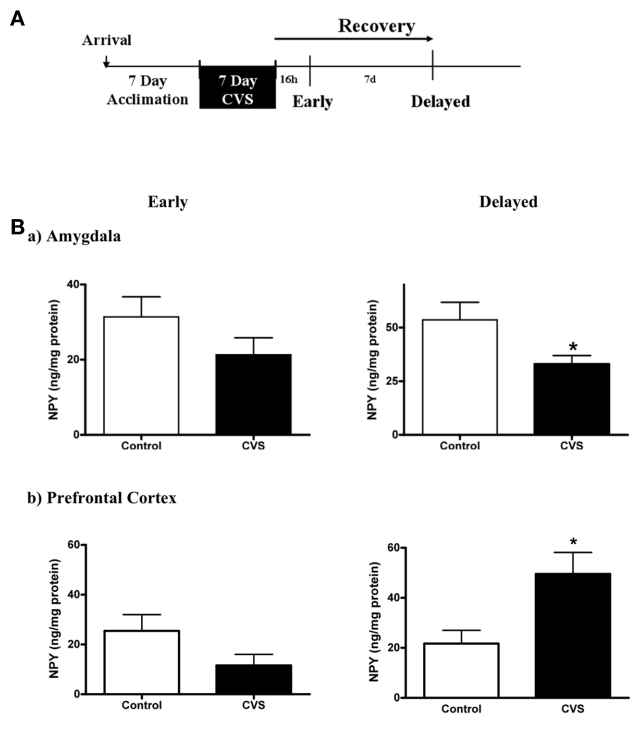

FIGURE 1 | Changes in NPY protein in early and delayed recovery from chronic variable stress (CVS) (A). Schematic of experimental design and tissue collection. **(B)**. Significant changes in NPY tissue content emerge during recovery from CVS. NPY is decreased in amygdala **(a)** and increased in prefrontal cortex **(b)** 7 days after cessation of CVS as measured by ELISA. NPY content is not different in either region when tested 16 h after CVS completion. "*" Indicates $p < 0.05$. Data is represented as mean ± SEM.

DISCUSSION

Investigating neural factors associated with recovery and resilience constitutes an important scientific priority for developing treatments for stress-induced disorders, especially PTSD. Here we report that exposure to chronic intermittent stress in an unpredictable fashion can induce long-term alterations in the putative resiliency factor NPY, in limbic brain areas that regulate behavioral, physiological, and cognitive effects of stress and trauma. There are two main findings of our study: first, that significant NPY dysregulation was noted well into the recovery period when restoration and normalization would be expected, and in some cases (PFC) NPY dysregulation appears to be emergent over the recovery period. Second, the amygdala and PFC elicit differential NPY responses to chronic stress that may be caused by different mechanisms. Importantly, dysregulation of NPY is temporally coincident with the expression of enhanced fear recall and emotional arousal that we previously reported in this model (McGuire et al., 2010).

The trajectory of NPY regulation following stress was investigated in the amygdala and PFC based on (a) their well established role in regulation of stress homeostasis and relevance in stress-induced disorders such as PTSD (Shin et al., 2004;

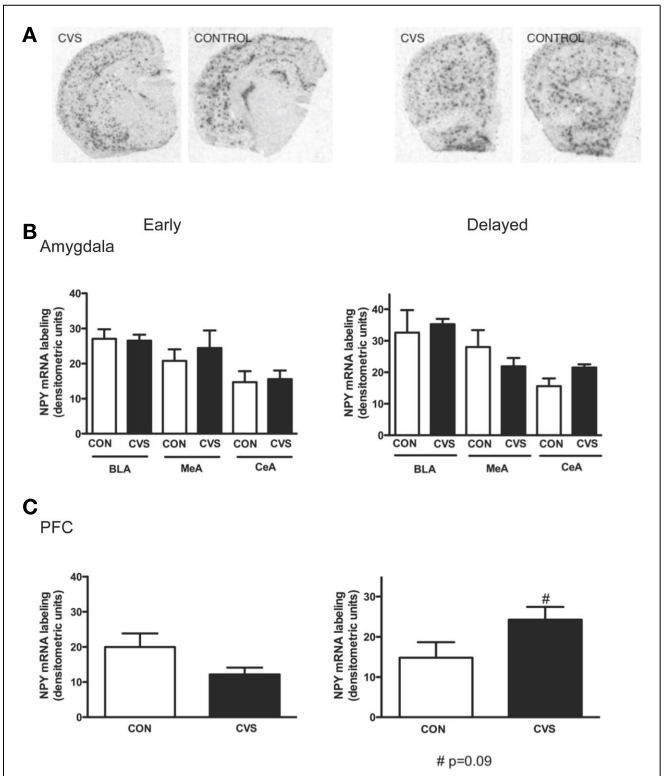

FIGURE 2 | *In situ hybridization for NPY mRNA in amygdala and prefrontal cortex during recovery from CVS.* (A). Representative images of NPY *in situ* hybridization from control and CVS animals. **(B)**. Levels of NPY mRNA did not differ between CVS and control brains in basolateral, medial, or central amygdala in either early or delayed recovery. **(C)**. NPY mRNA in the prefrontal cortex did not differ between control and CVS animals in early and delayed recovery. However there was a trend toward increase in NPY mRNA in later recovery when NPY peptide was also increased. "#" Indicates $p = 0.09$. Data is represented as mean ± SEM.

Eaton et al., 2007), (b) a defined role of NPY in the control of excitability and pro-stress transmitters in these regions (Bacci et al., 2002; Chung and Moore, 2009; Giesbrecht et al., 2010), and (c) preliminary experiments revealing the absence of persistent CVS-induced regulation of NPY content in other limbic regions such as the hippocampus and hypothalamus (data not shown).

Decreased NPY concentration in the amygdala was observed at delayed post-stress recovery, accompanied by no changes in NPY mRNA synthesis. Since intra-amygdalar NPY mRNA remained unaffected by CVS, reduced NPY peptide content may be a potential outcome of reduced transport via afferent projections to the amygdala. NPY innervation from extra-amygdalar sources has been proposed, although the exact source of afferent inputs are not yet identified (Leitermann et al., 2009; Rostkowski et al., 2009). Reduction of NPY peptide content in the absence of reduced synthesis could also be due to an increase in proteolytic degradation. Previous studies have reported that NPY effects in the CNS are modulated by dipeptidyl peptidase IV. DPPIV-like enzymatic activity is responsible for the cleavage of NPY (Karl et al., 2003). A previous study reported increased NPY mRNA and protein in the amygdala following repeated restraint stress for 9–10 day (Thorsell et al., 1999). This increase was described as an adaptive functional response that coincided with the absence of behavioral and neuroendocrine deficits that were evident after acute restraint episode. It is possible that paradigms supporting habituation may produce enhanced NPY expression in the amygdala and possibly NPY function. On the other hand, CVS paradigms favor sensitized responses without habituation. This is supported by the delayed expression of sensitized emotional and neuroendocrine responses evoked by CVS in our paradigm (McGuire et al., 2010). Other studies have reported increased NPY concentrations and NPY-immunoreactive fibers in the amygdala 7 day following single prolonged stress exposure (Cui et al., 2008) and elevated NPY mRNA at 2 weeks following single session of multiple footshocks (de Lange et al., 2008). Thus, it is possible that engagement of the putatively "pro-adaptive" NPY system is dependent on stressor modality, duration, and intensity.

We also observed significant increases in NPY peptide and trend toward increase in mRNA expression in the prefrontal cortex at 7 day post-CVS cessation. Impact of chronic stress on the expression of NPY in the PFC has not been investigated previously. Interestingly, modulation of stress on NPY content in the PFC was in the opposite direction as observed in the amygdala. While the exact mechanism for this differential regulation is unclear, we also observed a modest increase in NPY mRNA expression at the same time-point suggesting that increased NPY synthesis may contribute to this effect. Acute stress-induced decrease in cortical NPY mRNA has been reported earlier, however this normalized at 10 h post-stress (Thorsell et al., 1998). The delayed up-regulation of NPY thus appears to be a result of long-term plasticity within the PFC. Exposure to chronic stress has been shown to induce structural and functional plasticity in this area (Goldwater et al., 2009). These long-term neuroplastic alterations may be accompanied by altered synthesis and content of transmitter systems such as NPY.

IMPLICATIONS OF CVS-EVOKED NPY DYSREGULATION

In recent years, a prominent role of neuropeptides such as NPY in integrating stress and emotion has emerged (Sajdyk et al., 2004; Alldredge, 2010). Using genetic, behavioral, electrophysiological, and pharmacological approaches, previous studies have determined that NPY in the amygdala promotes successful adaptation to the acute and cumulative effects of stress, anxiolysis, and attenuation of fear (Sajdyk et al., 2008; Fendt et al., 2009; Giesbrecht et al., 2010; Tasan et al., 2010). Persistent reductions in chronic stress evoked NPY in this region would therefore compromise both resiliency to stress as well as induce potentiated fear responses. Exposure to CVS gives rise to exaggerated fear responses and recall following 1 week of recovery (McGuire et al., 2010). These effects are evident at a time when compromised NPY may promote increased excitatory tone in the amygdala leading to sensitized fear responses.

The physiological consequences of NPY expression in the PFC are less well understood. Classification of NPY-expressing cells in the PFC reveals a diverse population of interneurons that are exclusively GABAergic (Karagiannis et al., 2009). NPY elicits a long lasting decrease in evoked excitatory postsynaptic currents through calcium-dependent increase in GABAergic signaling as well as a delayed long lasting increase in inhibitory postsynaptic current (Bacci et al., 2002). Each of these NPY actions would decrease excitability in cortical circuits and output. Significant decrements in synaptic function and neural activity have been reported in the PFC by chronic stress (Wilbur et al., 2011). Increased NPY expression in prefrontal circuits may induce persistent inhibition and reduced excitability leading to dampened PFC output. Given the relevance of PFC in modulating behavioral and neuroendocrine consequences of stress, reduced PFC activity is expected to result in emotional arousal as well sensitization of the hypothalamic pituitary adrenal axis (HPA) responses (Radley et al., 2009; Sotres-Bayon and Quirk, 2010). In agreement with this, we have observed exaggerated fear responses, as well as sensitized HPA responses at the delayed recovery time-point post-CVS (McGuire et al., 2010).

The CVS-recovery paradigm was developed by our group to model chronic traumatization insults and posttraumatic-like outcomes. As described before, this paradigm produces selective effects related to fear memory reinstatement as well as fearful arousal while no significant effects on anxiety are observed (McGuire et al., 2010). NPY in the amygdala has been reported to regulate fear-associated behaviors in several paradigms (Heilig et al., 1992; Britton et al., 2000; Gutman et al., 2008; Fendt et al., 2009) Since NPY has been reported to counteract and contain the effects of stress mediators like CRH (Sajdyk et al., 2004; Giesbrecht et al., 2010) in limbic regions such as the amygdala, it is likely to be released during acute stress responses. However, long-term exposure to stress may dysregulate the NPY system, resulting in reduced inhibition of pro-stress transmitters, and vulnerability to the effects of stress. Although we did not measure CRH or NE in our current studies, others have reported an up-regulation of amygdalar CRH expression following chronic stress (Gray et al., 2010; Wang et al., 2010). Chronic stress induces lasting changes in catecholaminergic neuron structure, and function, particularly in the forebrain (Goldstein et al., 1996; Miner et al.,

2006; Aborelius and Eklund, 2007; Goldwater et al., 2009; Lee et al., 2011), Additionally, increased tonic expression of CRH and NE is associated with a reduced threshold for arousal (van Gaalen et al., 2002; Dierssen et al., 2006). Persistent reduction in amygdalar NPY in the face of enhanced CRH and NE tone will promote exaggerated fear and arousal-associated behaviors that were reported in this model by our group. Likewise, control of excitatory versus inhibitory balance in the cortical output by NPY would be impacted by derangements in NPY that emerge and persist well after stress cessation.

By comparing early and delayed expression of NPY message and protein it is evident that regional disparity exists in how NPY responds to chronic stress. While early decrements in amygdalar NPY are exacerbated with recovery (suggesting depletion), there might exist delayed neuroadaptive changes in the PFC. It is interesting to note that even though these changes are in opposite directions the net outcome may result in enhanced emotional reactivity and sensitized neuroendocrine responses. Another implication of the current study is that dysregulation of limbic NPY may lead to increased vulnerability to subsequent stress or reduced resilience.

In conclusion, persistent dysregulation of NPY, that exists well after cessation of repeated stress may lead to impaired emotional homeostasis and confer vulnerability to subsequent trauma given the stress buffering role of NPY.

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Interpersonal stress regulation and the development of anxiety disorders: an attachment-based developmental framework

Tobias Nolte[1,2]*†, Jo Guiney[3†], Peter Fonagy[1,2], Linda C. Mayes[2,4] and Patrick Luyten[1,5]

[1] Research Department of Clinical, Educational and Health Psychology, University College London, London, UK
[2] Developmental Neuroscience Unit, Anna Freud Centre, University College London, London, UK
[3] Royal Holloway, University of London, London, UK
[4] Yale Child Study Center, Yale University, New Haven, CT, USA
[5] Department of Psychology, University of Leuven, Leuven, Belgium

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
René Hurlemann, University of Bonn, Germany
Kwang Choi, Uniformed Services University of the Health Sciences, USA

***Correspondence:**
Tobias Nolte, Anna Freud Centre, 12 Maresfield Gardens, London NW3 5SU, UK.
e-mail: tobias.nolte@annafreud.org

†Tobias Nolte and Jo Guiney share joint first authorship.

Anxiety disorders represent a common but often debilitating form of psychopathology in both children and adults. While there is a growing understanding of the etiology and maintenance of these disorders across various research domains, only recently have integrative accounts been proposed. While classical attachment history has been a traditional core construct in psychological models of anxiety, contemporary attachment theory has the potential to integrate neurobiological and behavioral findings within a multidisciplinary developmental framework. The current paper proposes a modern attachment theory-based developmental model grounded in relevant literature from multiple disciplines including social neuroscience, genetics, neuroendocrinology, and the study of family factors involved in the development of anxiety disorders. Recent accounts of stress regulation have highlighted the interplay between stress, anxiety, and activation of the attachment system. This interplay directly affects the development of social–cognitive and mentalizing capacities that are acquired in the interpersonal context of early attachment relationships. Early attachment experiences are conceptualized as the key organizer of a complex interplay between genetic, environmental, and epigenetic contributions to the development of anxiety disorders – a multifactorial etiology resulting from dysfunctional co-regulation of fear and stress states. These risk-conferring processes are characterized by hyperactivation strategies in the face of anxiety. The cumulative allostatic load and subsequent "wear and tear" effects associated with hyperactivation strategies converge on the neural pathways of anxiety and stress. Attachment experiences further influence the development of anxiety as potential moderators of risk factors, differentially impacting on genetic vulnerability and relevant neurobiological pathways. Implications for further research and potential treatments are outlined.

Keywords: stress, anxiety, anxiety disorders, mentalization, attachment, hyperactivation, allostasis

INTRODUCTION

Contemporary perspectives in developmental psychopathology conceptualize attachment relationships as part of a complex network of epigenetic factors which interact to confer risk of or resilience to the development of stress-related psychopathology (e.g., Fonagy and Luyten, 2009; van Ijzendoorn et al., 2010; Luyten et al., submitted for publication). This paper presents a model of the influence of attachment relationships on the development of stress regulation strategies and discusses the role those relationships play in the development and maintenance of anxiety disorders, particularly the neurobiological alterations that underpin them.

Anxiety disorders are characterized by a pronounced dysfunction of systems underpinning stress regulation and fear responses (Mineka and Zinbarg, 1996; Rosen and Schulkin, 1998). It has been hypothesized that individual styles of threat response and stress regulation develop within the context of early caregiving experiences (Luyten et al., submitted for publication). These styles are thought to persist throughout the life cycle, providing a theoretical framework for linking early attachment to later anxiety disorders (Gunnar and Quevedo, 2007; Sbarra and Hazan, 2008). From this perspective, stressful experiences, physiological stress regulation, and attachment relationships are inherently linked, as the activation of the attachment system invariably follows the early stages of detecting and processing fear-related cues, stress-responses, and states of anxiety (e.g., Murgatroyd and Spengler, 2011; Luyten et al., submitted for publication). Specifically, the biologically based activation of a child's attachment system following distress entails coordinated behaviors that aim to address the stress response by eliciting the attention, and by ensuring the proximity and protection of attachment figures. When effective, this process leads to a co-regulation of the child's distress (Sbarra

and Hazan, 2008). Hence, at least some of the mechanisms by which stress-regulatory strategies are acquired are inherently interpersonal in nature and, it is argued, these strategies persist into adulthood and are relevant for the understanding of the nature and development of anxiety disorders. In this paper we review evidence from prospective human behavioral studies showing that a history of insecure–anxious infant–caregiver attachment, characterized by an excessive intensification of distress signals to elicit caregiver responsiveness and maintain proximity, is a risk factor for developing anxiety disorders in middle childhood and adolescence (Bosquet and Egeland, 2006), even when maternal anxiety and temperament are controlled for (Warren et al., 1997). Based on these and similar studies, we propose a theoretical model arguing that the characteristic up-regulation of stress reactivity observed in individuals classified as insecure–anxious may be an important risk factor for the development of anxiety disorders (Vasey and Dadds, 2001; Muris et al., 2003; Shaw and Dallos, 2005; Lee and Hankin, 2009). Thus, we argue that an understanding of the normative and pathological development of stress-regulatory systems within an attachment context is likely to be important for elucidating the etiology and may also directly inform the treatment of anxiety disorders.

In this context, the multiple levels of the stress response must be taken into account. Research into stress and anxiety disorders in past decades has tended to consider factors such as behavioral, genetic, or physiological phenomena in isolation. Recently, however, more comprehensive developmental formulations have been proposed that are notable for their integration of findings from genetic and social neuroscience research in adults (e.g., Heim and Nemeroff, 2001; Pine, 2007; Martin et al., 2009). With convergent findings in adult research indicating that neurobiological underpinnings are broadly shared by most anxiety disorders (Martin et al., 2009; Etkin, 2010) there have been calls for a comprehensive integration of research findings from multiple levels of analysis utilizing a developmental perspective (e.g., Gross and Hen, 2004; Murray et al., 2009; Cicchetti, 2010).

The developmental model proposed in this paper attempts to respond to such calls by highlighting, within an integrative account, the role of early attachment relationships. In this framework, individual differences in parental stress regulation are conceptualized as impacting upon the stress regulation strategies developed by the child. This process is proposed to confer both risk of or resilience to the development of anxiety-related psychopathology. More specifically, we propose that stress- and fear-triggered co-regulatory processes between parent and child within the attachment relationship are evolutionarily vital and neurobiologically pre-wired adaptations to the child's specific early caregiving environment. These early adaptations are preserved to ensure the superior adaptation of the organism to likely environmental challenges over the course of the life cycle.

Behavioral and physiological aspects of stress regulation have been comprehensively described in adults (McEwen, 2007; Chrousos, 2009), and this is increasingly the case in children (Gunnar and Quevedo, 2007). However, research focusing on the intergenerational transmission of dysfunctional stress regulation, and the role of this transmission in the subsequent emergence of anxiety disorders, is relatively underdeveloped. Stress in a child's early caregiving environment has been linked to lasting adverse effects on both physiological and psychological domains of development (National Scientific Council on the Developing Child, 2005; Gunnar and Quevedo, 2007; Nugent et al., 2011). These findings are congruent with population-representative studies that have shown that early adversity, and particularly attachment-related trauma, is related to increased risk for anxiety disorders throughout the life span (Green et al., 2010; Luyten et al., submitted for publication). For example, early adversity, such as maltreatment and neglect, have been consistently shown to lead to chronic alterations in the function of the hypothalamic-pituitary–adrenal (HPA) axis, both at a the level of basal activity and in response to stress (De Bellis et al., 1999; Heim and Nemeroff, 2001; Lupien et al., 2009). Hyperresponsiveness of this system is also implicated in the anxiety disorders (Kallen et al., 2008; Etkin, 2010). Further theory-driven research is required to link the interpersonal mechanisms involved in the emergence of childhood anxiety problems with studies of the heterogeneity in stress responsivity (Luyten et al., submitted for publication). This paper is an attempt to provide a theoretical framework for such research and outlines implications for intervention strategies. While the psychopharmacological treatment of anxiety is beyond the scope of this paper, its central role in intervention is acknowledged. Rather, the model presented here suggests how attachment-based interventions may enhance a range of other treatments, including pharmacological approaches.

PAPER OUTLINE

This paper covers three literatures. We begin by reviewing literature on anxiety disorders from the perspective of a number of disciplines. Next, an account of the role of attachment in the development of both normative and aberrant stress regulation is explicated. Finally, an integrative developmental model for the etiology of anxiety disorders is proposed based on constructs drawn from the interface of modern attachment theory and neuroscience.

Differences in the presentations of the various anxiety disorders may reflect variations in etiological pathways, but we suggest that the unregulated fear responses and subsequent hyperactivation of the attachment system is shared by these diagnostic entities. For this reason, the focus of this paper is on the anxiety disorders as a group. This is in line with studies concerning a tripartite model showing that anxiety disorders empirically cluster together, separate from depression for instance (Clark and Watson, 1991; De Bolle and De Fruyt, 2010; Luyten and Blatt, 2011), and with findings from recent studies based upon multivariate statistical analyses indicating that anxiety disorders can be hierarchically ordered as part of a spectrum of internalizing disorders (Krueger et al., 2007).

ANXIETY DISORDERS

While fear is an evolutionarily preserved response to environmental threat and enables appropriate defensive behaviors such as escape and avoidance (Rosen and Schulkin, 1998) the excessive fear responses that characterize pathological anxiety have been conceptualized as a dysfunctional variant of these originally adaptive processes. (Mineka and Zinbarg, 1996; LaBar and Phelps, 2005; Shin and Liberzon, 2009). While resulting symptomatology can

range from the persistent and non-specific apprehension in Generalized Anxiety Disorder to the overwhelming terror present in Panic Disorder, the presence of fear and stress states is common to these presentations (Craske et al., 2009).

PREVALENCE

Lifetime prevalence data consistently shows anxiety disorders to be the most commonly occurring class of mental disorders (e.g., Lépine, 2002; Kessler and Wang, 2008), usually with a chronic-recurrent course (Kessler et al., 2010). Several population studies attest to the high prevalence of anxiety disorders occurring before adulthood (Breton et al., 1999; Canino et al., 2004) and though estimates vary, at any given time approximately 2.5–5% of children and adolescents meet criteria for an anxiety disorder (Ford et al., 2003). Evidence from longitudinal studies suggests that the life-interference associated with shyness and anxiety disorders in childhood persists into early adulthood (Caspi et al., 1996; Last et al., 1996), while studies demonstrating the longitudinal stability of features predicting anxiety disorders from childhood to adolescence (Bittner et al., 2007) and from adolescence to early adulthood (Pine et al., 1998) emphasize the importance of a developmental approach.

COMORBIDITY

Estimates suggest that 40–60% of children and adolescents with a specific anxiety disorder meet criteria for at least one other anxiety disorder (Benjamin et al., 1990; Kendall et al., 2001). Such a high level of comorbidity within the anxiety disorders likely reflects both shared risk factors and common underlying fear processes maintaining the presentation (Rapee et al., 2009).

Population studies also indicate high levels of comorbidity with other psychiatric disorders (Angold et al., 1999), with significant associations existing between anxiety disorders and the subsequent onset of other psychiatric (Beesdo et al., 2007) and substance use (e.g., Zimmerman and Chelminski, 2003) disorders. However, the most striking and consistent finding of population studies is the marked comorbidity of anxiety disorders and depression. It is estimated that anxious children are between 8 and 29 times more at risk of developing subsequent depression than non-anxious children (Angold et al., 1999; Costello et al., 2003; Ford et al., 2003).

Given the early onset of anxiety disorders, they commonly represent the temporally primary disorder in comorbid profiles. It is on this basis that some commentators have suggested that early interventions to treat anxiety disorders might attenuate risk for the onset, persistence, or severity of secondary disorders such as depression and substance abuse (Wittchen et al., 2000; Kessler, 2004) underscoring the value of developmental accounts of etiology and course.

FACTORS CONTRIBUTING TO THE DEVELOPMENT OF ANXIETY DISORDERS

Before outlining the key role of the attachment relationship in the development of stress-regulatory processes in the face of threat and anxiety, a review of the critical factors that have been implicated in the development of anxiety disorders is presented. These factors have been investigated across multiple disciplines and are examined in turn.

GENETIC INFLUENCES ON THE DEVELOPMENT OF ANXIETY DISORDERS

A growing body of research supports the familial aggregation of anxiety disorders (Hettema et al., 2001) with findings consistently demonstrating that children with anxiety disorders are more likely to have a parent with an anxiety disorder (Last et al., 1987, 1996; Cooper et al., 2006; Schreier et al., 2008).

Twin studies have allowed for estimates of the actual contribution of genetic factors to the pathogenesis, or heritability, of anxiety disorders and consistently report a genetic influence of a moderate magnitude (Thapar and McGuffin, 1995; Hettema et al., 2001, 2005; Ehringer et al., 2006; Gregory and Eley, 2007). In considering transdiagnostic overlap of anxiety psychopathology accounted for by genetic and environmental influences, estimates have varied according to the form of anxiety investigated (Eley et al., 2003, 2010; Ehringer et al., 2006), with Obsessive–Compulsive and Shyness/Inhibition behaviors most consistently indicated as highly heritable and Separation Anxiety as more strongly influenced by shared environmental factors.

This evidence for phenotypic and genetic overlap in the various behaviors associated with the anxiety disorders has provided the basis for their common consideration as a group. However, while the differentiation in genetic contributions can partially account for the heterogeneity of presentations in the anxiety disorders, environmental influences remain substantial in each diagnostic entity. Defining the nature of the early experiences that may interact with genetic risk factors to produce anxiety-related phenotypes is therefore crucial (Norrholm and Ressler, 2009).

Furthermore, it has been argued that genetic and environmental factors may be more or less influential depending on a subject's developmental stage, and that factors relating to the primary caregiver will account for more variance during early to middle childhood, when parents exert the strongest influence on their children (Rapee and Spence, 2004). In line with this, variations in a polymorphism of the serotonin transporter gene have been associated with anxiety sensitivity, but only in the presence of childhood maltreatment (Stein et al., 2008).

TEMPERAMENT AND ANXIETY DISORDERS

Various nosologies of temperament have described a style in infancy characterized by inhibition, shyness, withdrawal, and distress in response to novelty, and a tendency to stay within close proximity to attachment figures (Windle and Lerner, 1986; Kagan et al., 1988; Hirshfeld et al., 1992; Sanson et al., 1996; Chorpita and Barlow, 1998). As a result, although studies examining childhood anxiety have utilized different classification criteria for temperament, nearly all employ measures of behavioral inhibition (BI) and proneness to distress reactions when faced with novelty. For purposes of clarity and because it is the most commonly used term across disciplines, we refer to this temperament style as BI.

It has been suggested that BI might serve as a potential endophenotype in research into anxiety disorders (Smoller et al., 2005; Norrholm and Ressler, 2009). Indeed, associations have been found between BI in children and anxiety disorders in their parents (Biederman et al., 1993; Rickman and Davidson, 1994), and longitudinal studies have shown that BI in childhood predicts later anxiety disorders (Hirshfeld et al., 1992; Turner et al., 1996; Prior et al., 2000).

Although BI features have much in common with those observed in children with an insecure–anxious attachment classification (Calkins and Fox, 1992), meta-analyses investigating this overlap have indicated that individual differences in attachment style cannot be explained by temperament constructs (Vaughn and Bost, 1999). Rather, contemporary accounts posit that temperament and attachment are distinct but interacting influences on the child's development (for a review, see Vaughn et al., 2008).

In relation to the model proposed in the current paper, a BI temperament style is conceptualized as one potential risk for the development of anxiety disorders (Rapee and Coplan, 2010), and a factor interacting with an individual's attachment status.

ENDOCRINOLOGICAL, NEURAL, AND COGNITIVE MEDIATORS OF STRESS REGULATION AND ANXIETY DISORDERS

HPA-axis sensitivity programming

The concept of developmental programming (Andrews and Matthews, 2004; Meaney et al., 2007; Seckl, 2008) has been proposed in response to a large body of research demonstrating that environmental cues at sensitive periods of development can result in permanent alterations in the functioning of the HPA-axis (Matthews, 2002; De Kloet et al., 2005; Oitzl et al., 2010). Preclinical and clinical evidence suggests that this programming is relevant to an understanding of the etiology of anxiety disorders in humans (Heim et al., 2004, 2008; Capitanio et al., 2005) with a growing number of studies in human samples indicating that stressors within the early caregiving environment are associated with alterations in the functioning of the HPA-axis and an increased risk of heightened anxiety and psychopathology later in life (Graham et al., 1999; Rinne et al., 2002; Heim et al., 2008).

It has been suggested that maternal care plays a key mediating role in the regulation of the HPA-axis in offspring (e.g., Gunnar and Donzella, 2002; Taylor et al., 2011). Adequate care has been associated with reduced cortisol levels and an attenuation of HPA-axis responsiveness in children, together with a greater cortisol recovery post-stress (Albers et al., 2008). These findings are highly pertinent to the attachment framework applied in the current paper as they underscore the interpersonal nature of stress regulation. The regulation of the HPA-axis as a primary function of the attachment relationship is a key component of the current model.

The effects of prenatal anxiety on HPA-axis function. A number of studies have demonstrated associations of antenatal maternal anxiety with cognitive, behavioral, and emotional problems in the child (Van den Bergh and Marcoen, 2004; O'Connor et al., 2005; Bergman et al., 2010). In investigating potential physiological mediators underpinning the sequelae of prenatal maternal anxiety, attention has largely focused on its effects on the HPA-axis of the offspring. Based on the evidence for overactive and dysregulated HPA axes in the offspring of prenatally stressed animals (Weinstock et al., 1992; McCormick et al., 1995; Huizink et al., 2004), it has been hypothesized that exposure to anxiety and stress in the prenatal environment may result in susceptibility to psychopathology, such as anxiety disorders and/or depression, in humans (Van den Bergh et al., 2008).

In a recent study examining outcomes associated with prenatal stress and the impact of attachment, Bergman et al. (2010) documented that levels of maternal prenatal cortisol measured in amniotic fluid were linked with impaired cognitive development in children. However, mother–infant attachment moderated these *in utero* effects: the negative outcome only held true when early caregiving was characterized by attachment insecurity. Further, it has been shown that prenatal stress is associated with reduced hippocampal volume only when combined with inadequate levels of post-natal care from the mother (Buss et al., 2007).

Taken together, this body of research suggests that although prenatal stress can confer risk for anxiety disorders through altering the set-point of the HPA-axis, this risk can be attenuated by the early caregiving environment and attachment experiences in particular.

Neural basis of anxiety

Current understanding of fear conditioning and threat responses at a neural level derives mainly from animal research and subsequent translational efforts that apply these animal models to study fear and anxiety processes in normal human populations (LeDoux, 2000; Schiller et al., 2010; Schiller and Phelps, 2011). Phenomenologically, the arousal and avoidance responses of subjects with anxiety disorders resemble the reactions of normal subjects to conditioned fear cues (Grillon, 2002). Crucially, both groups of subjects display the same accompanying changes in the neural substrates that coordinate their defensive responses to threats. Responses in humans with anxiety disorders are therefore likely to represent extreme manifestations of the normal, context-appropriate responses to stress and fear that have proven evolutionarily successful (Rosen and Schulkin, 1998; Gray and McNaughton, 2000; Rauch et al., 2000; Shekhar et al., 2005).

Any account of the neurobiological underpinnings of anxiety disorders should therefore be based on an understanding of the neural circuitry underlying normal processing of fear and subsequent normative regulatory mechanisms. Neuroscientific evidence has converged to delineate a well-established limbic-medial prefrontal system comprising three functionally interacting groups of brain structures (Etkin and Wager, 2007; Kober et al., 2008; Martin et al., 2009; Etkin, 2010). The complex interaction of these structures is summarized here in brief.

First, detection of and early response to fear cues and/or negative emotional stimuli occur within the phylogenetically ancient limbic structures of the amygdala and insula. The result is a first integration of sensory, affective, and interoceptive processes (see Etkin and Wager, 2007 for a quantitative meta-analysis of the involvement of these areas in anxiety-relevant emotional processing). In turn, these regions initiate and modulate activity in several target structures (including the hypothalamus, periaqueductal gray, sensory cortices, and the hippocampus) to carry out coordinated physiological and behavioral responses. The hippocampus exerts an important regulatory function via negative feedback to the HPA-axis (Pruessner et al., 2010). Hippocampal volume and neurogenesis have been implicated in stress resilience and in the stress sensitivity associated with anxiety disorders (Lupien et al., 2009; Roozendaal et al., 2009).

Subsequent appraisal of the registered fear cues occurs in the dorsal anterior cingulate and dorsomedial prefrontal cortices. This detailed evaluation of the emotional stimulus has a potential gate-keeping function that may admit the stimulus to conscious awareness and may trigger the context-dependent inhibition or enhancement of limbic activation. Finally, the engagement of a third part of the circuit (involving rostral subregions of the anterior cingulate and ventromedial prefrontal cortex) is responsible for top-down-regulation of negative emotions and limbic processing. Furthermore, executive regions within the lateral prefrontal cortex activate medial prefrontal regulation of emotion processing.

The complexity of the interdependent functions of the regions within the limbic prefrontal circuit suggests that anxiety processing and responses do not rely on specific areas that perform unique functions. Rather, anxiety processing and response should be conceived of as emergent functions of interacting brain areas (Morgane et al., 2005). Furthermore, these circuits are under the modulatory influence of several other neural systems and neuropeptides (e.g., Mathew et al., 2008; Joels and Baram, 2009). Oxytocin, in particular, has been studied widely during the last decade (Insel, 2010). Its crucial role in mediating attachment as well as its influence on the neural circuits underpinning anxiety are discussed further below.

Accruing evidence suggests that the neural correlates of anxiety disorders involve an abnormally elevated activation pattern in the limbic structures. This leads to hypoactivation in prefrontal regions aimed at normalizing limbic response, and thus to regulatory failures. In a normal population, the neuronal processing of participants who scored higher on an anxiety measure, already appears to involve, via activation of the basolateral amygdala, a more generalized dysregulation and distorted detection of negative affect (Etkin et al., 2004; Campbell-Sills et al., 2010). Studying the regulation of negative affect in a sample of older adults, Urry et al. (2006) reported an inverse coupling of amygdala and ventromedial prefrontal cortex activation. This association also predicted diurnal cortisol secretion.

These findings, in particular the role of increased amygdala activation provoked by anxiety-producing unpredictable or ambiguous stimuli, are indicative of "hyperarousal and hypervigilance" (Etkin et al., 2004). These states are similar to behavioral responses found in anxiety disorders. This is of particular relevance, since most anxiety disorders are characterized by intolerance of uncertainty or ambiguity (Holaway et al., 2006; Boelen and Reijntjes, 2009) and a bias toward negative interpretations of ambiguous cues (Bishop, 2007). Additionally, success in interpreting negative stimuli as less threatening is associated with increased PFC and decreased amygdalar activity (Bishop, 2007) implying the central role of interpretation of experience. These processes reflecting normal social cognition or mentalizing capacities will be explained in detail below. Investigating the structural integrity of the amygdala–prefrontal pathway with diffusion tensor imaging, Kim and Whalen (2009) found evidence for an inverse correlation with participants' trait anxiety levels. This linked higher pathway strength with lower anxiety. In addition, studies on the resting brain showed that the level of anxiety can dissociate ventromedial prefrontal cortex functional connectivity with the amygdala, resulting in compromised interactions between these two brain regions (Kim et al., 2011). This may partly explain the failure to downregulate anxiety-provoked stress states, especially when these occur in interpersonal contexts as our model will show.

Taking these findings together, it appears likely that the prefrontal–amygdala circuit mediates basic mechanisms involved in human anxiety. These mechanisms include: "attention to threat, interpretation of stimuli, and acquisition and extinction of conditioned fear" (Bishop, 2007). Ultimately, this mediation can lead to a pathological bias in favor of negative representations of external and internal cues and to a failure to activate alternative non-threatening representations.

Evidence from recent functional neuroimaging research in clinical populations suggests commonalities in the functional anatomy underpinning most anxiety disorders (van den Heuvel et al., 2005; Pine, 2007; Ressler and Mayberg, 2007; Martin et al., 2009; Etkin, 2010; Shin and Liberzon, 2009 for review of the overlap with neural circuits of depression). Additionally, there are disorder-specific features in pathologies such as obsessive–compulsive disorder (Martin et al., 2009; Etkin, 2010). In the most comprehensive meta-analysis on negative emotional processing, Etkin and Wager (2007) demonstrated that limbic hyperactivation in patients with PTSD, social anxiety, or specific phobia was similar to anxiety experimentally induced through fear conditioning in healthy individuals. The finding that amygdala and insula hyperactivation is common to all three anxiety disorders is suggestive of patients' "excessive engagement of fear- or negative emotion-related circuitry" and reflects a neural phenotype of anxiety (Etkin and Wager, 2007) as well as of alterations in interoceptive processing of anxiety-induced affect (Paulus and Stein, 2006; Stein et al., 2007). Future research, however, is needed to address whether these functional perturbations represent acquired characteristics of anxiety disorders or reflect vulnerability factors that precede the onset of psychopathology. A growing body of developmental research investigates neuro-structural correlates of exposure to stressors in the early environment, finding for example, corticostriatal-limbic gray matter reductions in adolescents reporting maltreatment in childhood (Edmiston et al., in press) and decreases in corpus callosum volume in maltreated children and adolescents compared to their non-maltreated peers (Jackowski et al., 2008). Preliminary evidence suggests such structural differences in response to early life stress might be mediated by gender (Teicher et al., 2004).

Attentional bias to threat

Cognitive accounts have suggested that development of an attentional bias to threatening stimuli is both a mechanism by which early experience shapes an individual's stress responsivity and a risk factor for the development of anxiety disorders (MacLeod et al., 2002). It is now well-established that attentional biases are present in individuals diagnosed with a range of anxiety disorders (Bar-Haim et al., 2007) as indexed by heightened and sustained vigilance for visual stimuli conveying threat (Mogg and Bradley, 2002). Attentional biases have also been associated with heightened HPA-axis activity (Ellenbogen et al., 2002; Roelofs et al., 2007) providing a basis for cognitive-biological accounts of mood disorders (Beck, 2008). Furthermore, a genetic mechanism for attentional biases has emerged through its association with variations in the serotonin transporter gene (Perez-Edgar et al., 2010).

It has, however, been proposed that a child who is genetically vulnerable to anxiety may or may not develop an attentional bias toward threat depending upon the early caregiving environment (Fox et al., 2007). This research demonstrated that caregivers who highlight or identify negative events in their child's environment are contributing to the child's own development of a negative bias. It is interesting therefore that recognition and modification of attentional biases has been a central aspect of Cognitive Behavioral approaches to the treatment of the anxiety disorders (e.g., Beck, 1976; Beck and Emery, 1985).

ENVIRONMENTAL INFLUENCES

Early adversity

Although there is evidence indicating that children diagnosed with an anxiety disorders experience more negative events preceding diagnosis when compared to non-anxious controls (Goodyer et al., 1988; Phillips et al., 2005), such findings are called into question by studies indicating reciprocal influences. For example, it has been demonstrated that childhood anxiety predicts the occurrence of subsequent negative events (Swearingen and Cohen, 1985). Similarly, longitudinal research by Kim et al. (2003) demonstrated that internalizing problems such as anxiety and depression followed, but were also followed by, negative life events.

The processes whereby adverse events lead to the development of an anxiety disorder are therefore likely to be mediated by multiple factors, including attachment experiences (Cicchetti and Rogosch, 1997). Studies have in fact demonstrated that, in the presence of risk factors such as early adversity (Carlson and Sroufe, 1995) and stressful events (Heinrichs et al., 2003; Powers et al., 2006), secure attachment can act as a protective factor moderating the potential for development of psychopathology via the impact on stress regulation (Gunnar et al., 1996; Nachmias et al., 1996).

Parenting influences

Modeling and information transfer. A child's observation of anxiety in others has been proposed as a route for the intergenerational transmission of anxiety disorders (Mineka, 1985). Such learning-theory accounts posit that caregiver modeling allows the child to vicariously acquire behaviors, and that this is likely to be evolutionarily advantageous because it prepares the child for environmental challenges without exposing him to direct threat (Mineka, 1988). For example, in a sample of mothers without anxiety disorders and their 15 to 20-month-old infants, fear modeling by the mothers was found to be associated with the subsequent fear responses of the infants (Gerull and Rapee, 2002). In a more recent longitudinal study comparing mothers with and without an anxiety disorder (Murray et al., 2008), the level of anxiety expressed by the mother toward a stranger in front of their 10-month-old infant predicted the infant's subsequent avoidance of the same stranger at 14 months.

Research examining features of child–parent discussions has shown that anxious mothers are more likely to make comments of a catastrophic nature to their children (Whaley et al., 1999; Moore et al., 2004) and less likely to refer to positive emotions (Suveg et al., 2008). Further, compared to discussions in non-clinical families, discussions regarding ambiguous situations within families of anxious children appear to be characterized by reciprocal reinforcement of comments regarding risk and have been shown to magnify the extent of a child's anxiety and avoidance behavior in subsequent situations (Barrett et al., 1996).

This body of literature links parental behavior to anxiety in children via parental displays of anxiety or verbal behaviors emphasizing threat in the environment. These instances of "modeling" may also in part be seen as failures of the attachment system since the parent does not (or is unable to) show appropriate caregiving behavior within a stressful situation, and thus fails to effectively co-regulate the child's stress. Rather, the caregiver models to the child their own strategies for evaluating and responding to threat.

Parenting styles. A related body of research has considered the impact of a range of parenting practices on the development of anxiety disorders in children. The two major facets of parenting considered in these studies are lack of warmth (or parental rejection) and overcontrol. Within a cognitive framework, lack of warmth and rejecting behaviors can be seen as likely to reinforce a child's expectations that the world and others are hostile and unsupportive (Bögels and Tarrier, 2004). Overcontrol and the concomitant discouragement of independence are likely to limit the child's sense of agency and competence and to reinforce avoidance of potentially threatening situations (Parker, 1983; Chorpita and Barlow, 1998).

Evidence for associations of such parenting factors with childhood anxiety has been mixed (Wood et al., 2003; DiBartolo and Helt, 2007; McLeod et al., 2007), but with stronger and more reliable associations generally found for overcontrolling parenting. Inconsistencies in findings may partly reflect different study designs and measurement contexts and methods. Direct observation of parenting, in samples of children with diagnosed anxiety disorders rather than proxy symptoms, produces the most robust associations. A meta-analysis of studies accounting for these factors suggested that of all aspects of parenting style, a low level of autonomy granting (a feature of overcontrol) was the one most reliably associated with anxiety disorders in children (McLeod et al., 2007).

These associations can be considered in relation to quality of parent–child attachment. Attachment theory has long held that rejecting and overcontrolling parental behaviors are related to the child's level of attachment security. This general hypothesis is well supported in observations of mothers and children (Crowell and Feldman, 1991). More specifically, attachment theorists have hypothesized that limited autonomy granting and/or rejecting parental styles engender an anxious style of attachment in the child (Ainsworth and Bell, 1974; Sroufe et al., 1983). This specific hypothesis has been consistently supported in observational research (e.g., Sroufe et al., 1993).

Child-driven effects and parenting factors

While studies examining parenting styles have established that certain features are more commonly found in the context of childhood anxiety and insecure–anxious attachment (for review see Bögels and Brechman-Toussaint, 2006), researchers have attempted to establish whether such styles *cause* anxiety in the

child, are the *effect* of having an anxious child, or result from an *interaction* between parent and child.

Studies pertaining to support a causal model of parenting styles that use sibling controls are problematic (Hudson and Rapee, 2002; Barrett et al., 2005) given that the controls often have considerable levels of anxiety themselves. This leaves open the possibility that parenting style is provoked by a child's anxiety. Evidence for child-driven effects was provided in a study by Moore et al. (2004) in which parenting styles such as lack of warmth and catastrophizing were found to show a main effect of child diagnosis. Further, Ghera et al. (2006) found that 4-month-old infants who responded negatively to novel stimuli and were viewed by their mothers as "difficult to soothe" received low levels of maternal sensitivity (see also Hane and Fox, 2006). The same group reported that 9-month-old infants who showed high levels of behavioral avoidance to ominous stimuli and a corresponding pattern of right frontal electroencephalogram (EEG) asymmetry (itself a correlate of continued inhibition across early childhood; see Fox et al., 2001), received low levels of maternal sensitivity (Hane et al., 2008). This result was replicated in a follow-up study of the same sample in early childhood (Hane et al., 2010).

There is therefore some evidence that child-driven effects can potentially influence the quality of the early caregiving environment by provoking a certain style of parenting response. Other than child-driven effects, however, these studies do not adequately address potential influences on maternal behavior that could be impinging on mothers' abilities to provide sensitive care. Social support and maternal anxiety are two examples of such factors and are considered in turn.

It has long been established that reported level of social support correlates with quality of caregiver behavior (e.g., Crockenberg and McCluskey, 1986), with the level of social support being of particular importance for mothers of distress-prone infants (Crockenberg and McCluskey, 1986). One study demonstrated that maternal insensitivity was predicted by the joint effect of infant distress-proneness and low social support (Pauli-Pott et al., 2004) while Hirshfeld et al. (1997) demonstrated that parenting styles associated with anxiety disorders in children emerged only in anxious mothers with BI infants. Taken together, these studies suggest that infant temperament (BI) predicts later child anxiety only when accompanied by certain anxiogenic parenting styles. These styles are more readily provoked in mothers who are themselves anxious, an interaction that is more likely to occur against a background of low social support. Recent longitudinal research offers support for this complex pattern of interaction effects (Warren and Simmens, 2005; Murray et al., 2008).

Regarding parenting effects, research over the last decade has largely focused on the impact of maternal factors. In order to understand the development more comprehensively the role of fathers should also be considered (Bögels and Phares, 2008). While this is a relatively under-researched area, there is preliminary evidence for the role of paternal anxiety as a moderator of treatment outcomes for children with anxiety disorders (Rapee, 2000). Rapidly changing patterns of parenting in Westerns countries make delineation of the shared and gender specific parenting influences on emotional development an urgent social as well as psychological issue (Grossmann et al., 2005, 2006).

SECTION SUMMARY

Given their high prevalence, associated functional impairments and robust associations with the onset of other debilitating disorders, anxiety disorders warrant continued, multidisciplinary attention. While further elucidation of the genetic substrates and related biological processes by which anxiety disorders are inherited will no doubt offer exciting insights, greater understanding of the processes by which such genetic vulnerabilities may be modulated by the early environment will afford the most comprehensive etiological account.

ATTACHMENT EXPERIENCES AND STRESS REGULATION

Having reviewed literature pertinent to anxiety disorders, we now transition to integrating these findings in an attachment framework. First, we discuss normative co-regulation of stress and threat in the secure attachment relationship. We then propose a model for the dysfunction of regulation in anxious attachment and how this moderates genetic vulnerabilities and biological pathways that underpin subsequent development of anxiety disorders.

Contemporary attachment theory posits attachment as a behavioral and physiological system that is biologically based and dynamically adapting to meet the needs of the individual's particular environment (Mikulincer and Shaver, 2007). It responds to the stress provoked by environmental threats by promoting strategies that best maintain proximity to the caregiver.

Recent literature has conceptualized the stress response as an interpersonal process (Sbarra and Hazan, 2008; Luyten et al., submitted for publication), and has proposed an empirically testable and integrative framework of individual differences in stress regulation and susceptibility to anxiety disorders. Because it provides a developmental account of both normative and maladaptive stress regulation, attachment theory is best positioned to integrate findings that are proliferating in the various fields investigating stress and anxiety disorders (for a comprehensive review, see Luyten et al., submitted for publication).

SECURE ATTACHMENT AND THE REGULATION OF STRESS AND ANXIETY

Perceived threats and fear activate an individual's attachment system, prompting a series of processes that ultimately aim to regulate the stress response (Mikulincer and Shaver, 2007). These processes include primary attachment behaviors such as separation distress and subsequent proximity seeking (Sbarra and Hazan, 2008). Experimental and naturalistic studies have demonstrated this in children, adolescents, and adults (Sbarra and Hazan, 2008 for review, Mikulincer and Shaver, 2007).

If these behaviors successfully elicit the safety-promoting response of the attachment figure, the attachment system is deactivated. Over time, if the attachment figure is reliably available, attentive, and responsive, a secure attachment develops. This attachment is characterized by experiences of reassurance, a sense of safety and, ultimately, effective affect regulation. These repeated experiences become generalized as experience-expectant predictions of interactions and lead to a reduced reliance on external cues of safety (Mikulincer and Shaver, 2007). Individuals become increasingly capable of effectively regulating their stress-responses

by calling upon mental representations of internalized attachment figures – so-called "internal working models" (Bowlby, 1973; Bretherton and Munholland, 2008). Recently, research studies have operationalized the effects of the working model as support-seeking, self-esteem, and self-worth (e.g., Lee and Hankin, 2009). Thus, securely attached individuals can efficiently regulate stress and anxiety either by seeking proximity to a reliable attachment figure in their actual environment or by mentally drawing upon past experiences in which stress was effectively co-regulated. In this way, stress regulation remains an inherently interpersonal process (Diamond and Aspinwall, 2003; Luyten et al., submitted for publication) with the attenuation of anxiety embedded in all close relationships.

Ganzel et al. (2010) have modeled the stress response in an allostasis framework that accounts for: (a) ongoing evaluations of internal resources and external demands; (b) advance physiological adjustment through anticipatory arousal; and (c) adaptation to environmental circumstances over time. This notion has been greatly enriched in contemporary attachment theory by the elaboration of the concept of mentalization (Fonagy, 1998; Fonagy et al., 2002). The role of mentalization, that is, to conceive of self and others as social agents whose thoughts, feelings, desires, and behaviors are underpinned by intentional mental states (Fonagy et al., 2002), has been highlighted as a potent factor in social cognition and particularly in stress-related interpersonal contexts (Fonagy and Luyten, 2009). There is accruing evidence that effective mentalization that enables infants to regulate negative affect, threat cues, separation anxiety, and the resulting stress states – and thus subjective as well as physiological distress – follows a pattern of intergenerational transmission (Sharp and Fonagy, 2008). A mother's mentalizing ability, that is, the parent's ability to treat the child as an psychological agent with mental states independent of their own (Fonagy and Target, 1997) predicts both secure attachment and their child's own capacity to mentalize (Meins et al., 2002; Slade et al., 2005). For instance, a distinctive marker of secure attachment is the capacity to tolerate negative affect (Sroufe, 1996). Crucial to these processes is the caregiver's capacity to attenuate the child's stress or anxiety once its attachment system has been activated. A child's general sense of a secure base not only enables them to explore their environment freely but, more importantly, enhances their ability to contemplate own mental states and those of others. Studies have demonstrated attachment security to be a predictor of performance on diverse theory of mind (ToM) tasks, including false belief tasks in preschoolers (e.g., Arranz et al., 2002), and of the development of socio-cognitive capacities which support ToM, such as internal state language (i.e., emotion regulation and self-awareness vocabulary) in toddlers (e.g., Lemche et al., 2007). In critical contexts, these mentalizing capacities are online only once the attachment system has been downregulated after a threat or stressor has abated (Luyten et al., submitted for publication). This in turn, creates positive feedback loops for the possibility of the adjustment and regulation of impending stress response. In an attachment-based approach secure attachment is therefore viewed as the interpersonal training ground for the infant in which social cognition or mentalizing and their concomitant neural correlates are developed. These capacities allow for allostatic accommodation by enabling individuals to recognize and to regulate stress-related states (Schulkin, 2010).

As noted earlier, there is increasing evidence that attachment security serves a protective function by promoting resilience to the impact of stress mainly via anxiolytic and trust-enhancing effects mediated by the neuropeptide oxytocin (Heinrichs et al., 2003; Powers et al., 2006; Feldman et al., 2007; Heinrichs and Domes, 2008). Moreover, studies investigating stress responsivity in both human and animals have demonstrated that a secure attachment leads to an "adaptive hypoactivity" of the HPA-axis (Gunnar and Quevedo, 2007). Conversely, in a study of human adults low-quality parenting was found to be linked with elevated salivary cortisol levels during experimentally induced psychosocial stress (Gunnar et al., 2007). Such parenting was also linked to an increased release of dopamine in ventral striatal areas, which is a factor in the response to aversive stressful stimuli (Pruessner et al., 2004).

A further feature of a secure attachment is its encouragement of effective seeking of supportive attachment relationships throughout the lifespan. This is in line with contemporary attachment theory which posits that a attachment security leads to a cyclical process of "broaden and build" (Fredrickson, 2001) in which the individual experiences a sense of personal agency, can effectively regulate emotions and conflicts and engage in exploratory behaviors (Mikulincer and Shaver, 2007). Such behaviors direct the individual into new environments (broaden) that require adapting to new challenges (build). Moreover, broadening experiences have been shown to result in the recruitment of supportive relationships (Hauser et al., 2006) which further enhances resilience in the face of stress (Masten and Obradovic, 2008). Additional evidence for this notion is provided by functional neuroimaging studies that demonstrate an inverse relationship between participants' cortisol levels during social stress and the extent of their supportive social network (Eisenberger et al., 2007), with individual differences in activity of brain areas associated with social separation (Brodmann area 8, dorsal anterior cingulate cortex) found to mediate this relationship indicating a "protective" effect of social support on the neural processing of social threat and subsequent HPA reactivity. Furthermore, secure attachment has been associated with stronger decreases in state anxiety levels following laboratory-induced stress exposure (Ditzen et al., 2008). More interestingly, an interaction effect between combined social support and secure attachment resulted in even lower post-stress anxiety levels. Secure attachment and a normative stress response, in the current model, are closely linked with adaptive allostasis and neural plasticity (Ganzel et al., 2010; McEwen and Gianaros, 2010), a process conceived of as a buffer against future environmental challenge and conferring resilience to the development of psychopathology (Gluckman et al., 2007). The capacity to retain high levels of mentalization when faced with threat or anxiety is supposed to play a key mediating role therein, mainly by keeping regulatory brain regions such as the prefrontal cortex engaged during experiences of stress and attachment activation and by enabling a fast recovery from the momentary loss of this capacity. This, is turn, results in a reinforced feeling of attachment security, a sense

of agency and autonomy during successful affect regulation, facilitated by undistorted perception and representation of self and others (Fonagy and Luyten, 2009).

There is increasing evidence that the interplay between attachment activation, stress-related arousal, and continuing mentalizing is subserved by both the activation of mesocorticolimbic dopaminergic reward circuits and stress attenuating (Neumann, 2008; Fonagy and Luyten, 2009) and anxiolytic effects of oxytocin (Heinrichs et al., 2003; Kirsch et al., 2005; Ditzen et al., 2009; Kubzansky et al., 2009; Quirin et al., 2010). Given that oxytocin has been implicated in the parent–infant attachment relationship (e.g., Gordon et al., 2008; Strathearn et al., 2008), the attachment system may play a key role in the functioning of neural systems involved in anxiety processing. Oxytocin thus provides a link to the attachment system and has been shown to enhance experiences of secure attachment in an experimental setting (Buchheim et al., 2009). Because of the impairments in attachment in insecurely attached individuals, the quality of early experiences seems to have differential effects on the oxytonergic system (Heim et al., 2009; Bartz et al., 2010) and peripheral oxytocin levels in mothers watching cues of their infants (Strathearn et al., 2009).

INSECURE ATTACHMENT, DYSFUNCTIONAL STRESS REGULATION, AND THE DEVELOPMENT OF ANXIETY DISORDERS

Given that the primary evolutionary function of the attachment system is to maintain an infant's proximity to the caregiver, the system has to allow for adaptation to sub-optimal caregiving, as in cases where the caregiver is inconsistently responsive, unavailable, or abusing. Therefore, when faced with stress or threat and the primary attachment strategies have failed to elicit appropriate caregiving behaviors, the infant utilizes so-called secondary strategies in order to promote proximity and regulate anxiety. These secondary strategies are characterized by "hyperactivating" or "deactivating"[1] modes of stress and anxiety regulation (Cassidy and Kobak, 1988; Mikulincer and Shaver, 2007; Roisman, 2007). Hyperactivating strategies are central to an attachment account of anxiety disorders given their initiation in response to anxiety states, and are typically observed in anxiously attached individuals. Such strategies are characterized in infancy by frantic attempts to gain the attention of the attachment figure and develop when the infant's previous interactions have required up-regulation of seeking behaviors in response to an inattentive, preoccupied, or anxious caregiver (Mikulincer and Shaver, 2008). If repeated over time, these experiences serve to consolidate expectations of unreliable and unpredictable responses from the attachment figure and therefore create anticipatory anxiety and heightened vigilance for threat rather than successfully regulating anxiety states. The frantic demanding of support and constant activation of the attachment system may only allow for temporarily effective stress regulation but in the long run "undermine the goal of recruiting a soothing figure" (Luyten et al., submitted for publication) and compromise the establishment of social networks to provide supportive care (Campbell et al., 2005). At the intrapersonal level, these dynamics are characterized by autonomy-dependency conflicts which in turn affect interpersonal functioning Joraschky and Petrowski (2008). Furthermore, Mikulincer and Shaver (2007) demonstrated that secondary strategies impact on the primary attachment strategy of fear and threat appraisal via inhibitory or excitatory feedback loops (with the latter being of particular relevance regarding hypervigilance and HPA-axis functioning in anxiety disorders). Anxiety therefore increases the seeking of proximity, while separation from the attachment figure in turn increases anxiety and withdrawal (Luyten et al., submitted for publication).

While evolutionarily advantageous in early childhood, these strategies are associated with maladaptive outcomes in later life due to their detrimental impact on interpersonal functioning (Mikulincer et al., 2010). The resulting anxious pattern of attachment is then likely to persist into adulthood (anxious–ambivalent attachment) and represents the predominant mode of stress regulation. In individuals who have experienced highly unpredictable and abusive caregiving environments, attempts to regulate anxiety states are observed to be characterized by a chaotic oscillation between both hyperactivating and deactivating strategies – so-called disorganized attachment (Main and Solomon, 1986; Main and Hesse, 1990). Both anxious and disorganized attachment patterns have been associated with the development of anxiety disorders (Manassis et al., 1994; Warren et al., 1997).

ATTACHMENT RESEARCH IN RELATION TO ANXIETY DISORDERS

Whilst not considered inherently pathological, insecure infant attachment patterns, and the reliance upon secondary strategies increases the likelihood of psychopathology. Specifically, anxious attachment has been consistently associated with internalizing problems (e.g., Colonnesi et al., 2011) and a growing body of research lends support to the view that anxious attachment, and therefore the use of hyperactivating strategies, predisposes an individual to various anxiety disorders (Colonnesi et al., 2011 for meta-analysis). For example, it has been shown that a history of anxious attachment measured at 12 months of age puts children at risk of developing anxiety disorders in childhood and adolescence even when maternal anxiety and temperament are controlled for (Warren et al., 1997). Bosquet and Egeland (2006) found attachment history was moderately correlated with self-reports of anxiety at the age of 16. Further, childhood anxiety classification was predictive of negative adolescent peer relationships which in turn predicted anxiety symptoms. In another longitudinal study, Bar-Haim et al. (2007) linked anxious–ambivalent attachment at the age of 12 months with higher levels of school phobia 10 years later. This association was, however, only found in boys.

Hyperactivating strategies therefore hold a relatively unique position of predicting an array of transdiagnostic anxiety behaviors. Anxiously attached children experience constant worry about being abandoned and left alone when fear is experienced (Sroufe, 1996). This response is characterized by chronic hypervigilance toward the social environment which may give rise to

[1]Deactivating (minimizing) strategies are typically observed in individuals with a avoidant style of attachment, and are characterized by attempts to downregulate and suppress the attachment system in times of stress. Behaviorally, deactivating strategies are observable in self-soothing activities, assertions of independence, and the denial of attachment needs (Cassidy and Kobak, 1988). Deactivating strategies are derived from a history of attachment experiences in which the caregiver was rejecting, emotionally distant, or prone to withdrawal when called upon. A reliance upon deactivating strategies strengthens expectations of attachment figures as unavailable, characteristic of a dismissive attachment in adulthood.

the development of anxiety symptoms (Cassidy and Berlin, 1994; Weinfield et al., 1999).

In adulthood, the manifestation of anxiety disorders is linked with ambivalent (anxious) attachment classification (Fonagy et al., 1996; Rosenstein and Horowitz, 1996; Dick et al., 2005; Colonnesi et al., 2011) and negative attachment-related experiences such as overprotective parenting or abandonment and separation distress (DeRuiter and van Ijzendoorn, 1992; Cassidy, 1995; Bandelow et al., 2002). In some of these studies (Manassis et al., 1994; Fonagy et al., 1996) and particular when investigating PTSD (Kobak et al., 2004; Stovall-McClough and Cloitre, 2006 for review), there seems to be a high prevalence of disorganized attachment suggesting that childhood trauma or loss can give rise to particular anxiety disorders.

More importantly, the study by Manassis et al. (1994) demonstrates, albeit with a small sample, that 80% of the children of mothers diagnosed with anxiety disorders were classified as insecurely attached with 65% of them matching their mother's attachment classification.

In line with our framework it has to be noted that anxious attachment itself is, at least in part, influenced by genetic contributions. This seems to be more relevant when the *style* of attachment is assessed via self-reports. Brussoni et al. (2000) found that 25% of the variability in adult attachment measured with the Relationship Scales Questionnaire was accounted for by genes. Using a twin study design, Crawford et al. (2007) demonstrated that 40% of the variance was attributable to heritable factors, a finding recently confirmed by Picardi et al. (2010) who reported 45% heritability. Behavioral genetics studies focusing on attachment classifications obtained with interview-based instruments, in contrast, highlight the role of shared environmental factors with only little influence accounted for by genes in the contribution to the transgenerational transmission of attachment in children (Bokhorst et al., 2003; Fearon et al., 2006; Bakermans-Kranenburg and Ijzendoorn, 2007). These inconsistencies might result from different methodological approaches and the differential effect of gene x environmental interplay depending on the timing of when genetic effects come into play.

CONSEQUENCES OF HYPERACTIVATING STRATEGIES

As indicated earlier, secondary attachment strategies might be temporarily adaptive in a specific context or even at a societal level (Simpson and Belsky, 2008; Ein-Dor et al., 2010), but in the long run fail to attenuate stress effectively and result in increased allostatic load. More specifically, the heightened subjective and physiological stress reactivity found to be associated with hyperactivation has been shown to affect core processes involved in allostatic adaptation on a behavioral, endocrinological, and neural level.

Compromised broaden and build features

The attachment-based coping strategies associated with hyperactivation prohibit the ability of the individual to "broaden and build." Potentially supportive and competent others, especially in close relationships, are experienced as untrustworthy and/or unpredictable in their support and these expectations are combined with chronic worry about abandonment (Campbell et al., 2005; Miculincer and Shaver, 2009). Psychodynamic accounts furthermore highlight the role of conflictuous interpersonal functioning (e.g., Joraschky and Petrowski, 2008). Further, the use of such strategies inhibits motivational systems responsible for exploratory, affiliative, and caregiving behaviors (Mikulincer and Shaver, 2005). Other factors characterizing hyperactivating behavior, such as a negative view of self, a lack of self-efficacy, and the tendency to avoid fears – all inversely correlated with findings regarding resilience (Cicchetti, 2010) – reinforce the systems responsible for hyperreactivity to stress.

Dysfunctional HPA-axis

The excessive use of behavioral hyperactivation has been linked to physiological and neuroendocrinological hyperresponsivity (Lupien et al., 2009). In a large prospective cohort study, for instance, insecure–anxious (resistant) children displayed elevated cortisol levels after being exposed to a separation paradigm (Luijk et al., 2010). Similarly, in adults, hyperactivation has been found to result in an altered and more sensitive HPA-axis (Powers et al., 2006; Diamond et al., 2008; Gordon et al., 2008) and to have direct effects on reducing hippocampal cell density (Quirin et al., 2011) which might reflect a stress-driven neurotoxic impact on the glucocorticoid system. These indicators of allostatic load together with the previously reviewed effects of stress and anxiety on HPA-axis functioning suggest that chronic wear and tear entails that the once regulatory and anticipatory functions of the HPA-axis are rendered to conferring vulnerability to psychopathology (Schulkin, 2010). Most notably, the acquisition of prior allostatic load as observed in attachment experiences characterized by anxiety and ineffective stress regulation might impair the individual's capacity to accommodate to a current or future stressor (Ganzel et al., 2010).

Effects of allostatic load on the neural circuits – mentalization deficits under heightened stress and in the face of anxiety – biobehavioral switch

The core emotional regions of the brain (the fronto-limbic circuit), as outlined above, are the primary and central mediator of allostatic load as they are involved in the immediate stress response but also iteratively update evaluations of stress and threat-related environmental challenge (Ganzel et al., 2010). Together they coordinate physiological and behavioral responses to stress and require the effective recruitment of additional neural resources due to increased attentional and processing load. (Vuilleumier et al., 2001; Davidson et al., 2004). As these neural circuits represent the main interface between changes in the environment and the individual's accommodation to it they have been shown to be vulnerable to accrual of stress load and resulting wear and tear (LeDoux, 1996; Phelps, 2006; McEwen, 2007; Fonagy and Luyten, 2009; Rodrigues et al., 2009; Ganzel et al., 2010) and are most malleable during fetal and early childhood periods (National Scientific Council on the Developing Child, 2005). Arnsten (2009) has drawn attention to the stress signaling pathways and the neuromodulatory alterations that markedly impair PFC functioning, the ventromedial section in particular. More specifically, the impact of allostatic load can damage brain circuits due to an overproduction of neurochemicals involved in the stress response (Bremner et al., 1995;

Gould et al., 1997; Ganzel et al., 2010). These effects of significantly stressful events on neural processing have been studied in great detail in fear consolidation and fear extinction paradigms which are associated with anxiety and affected by hyperactivation strategies (Wellman, 2001; Izquierdo et al., 2006; Milad et al., 2009; Rodrigues et al., 2009).

More importantly, following the biobehavioral model put forward by Luyten et al. (submitted for publication), sustained hyperactivation in the face of anxiety or stress is directly linked to a relative switch in activation from cortical to subcortical brain systems, from slow, reflective regulation to a rapid, reflexive response (see also Mayes, 2006; Fonagy and Luyten, 2009; Johnson et al., 2011). This arousal-dependent switch furthermore affects the capacity to mentalize and modulates the neural network underpinning this faculty. More broadly, on a neural level, what can be observed is a "switch from non-stress to stress conditions" (Arnsten, 2009). Brain areas that have been consistently shown to underpin mentalization include the medial PFC, superior temporal sulcus, and temporal lobes (Gallagher and Frith, 2003; Frith and Frith, 2006; Lieberman, 2007). In keeping with this notion, Fraley et al. (2006) showed that anxious attachment is associated with hypervigilance in perception of emotional expression and poorer affect judgments.

Beyond the well-established effects on neural circuitry underpinning anxiety, there is strong evidence from electrophysiology studies and functional neuroimaging that anxiously attached individuals employing hyperactivating strategies under-recruit prefrontal brain regions involved in emotion regulation, display a neurobiologically supported bias toward memories of negative valence and respond with amygdala hyperactivation to negative social feedback (Gillath et al., 2005; Zilber et al., 2007; Vrticka et al., 2008; Zhang et al., 2008). Moreover, it has been shown that when comparing the effect of a general stress induction versus an attachment-related (interpersonal) stress induction, only the latter results in a relative deactivation of core areas associated with mentalization. In this study of a normal population, when inferring mental states of others during the Reading the Mind in the Eyes Test (Baron-Cohen et al., 2001), Nolte et al. (under review) found that it was only after exposure to *attachment* stress that activation decreased in the inferior frontal gyrus (a part of the prefrontal cortex), the posterior temporal sulcus, and the temporoparietal junction combined with stress-driven alterations of functional connectivity. It can be hypothesized that these stress-related alterations will be more pronounced in anxious individuals, although this has yet to be investigated.

Together, these findings provide preliminary evidence that the mitigating role of mentalizing is reduced in anxious individuals due to excessive use of hyperactivating strategies. Consistent with this theory, Milrod and colleagues (Rudden et al., 2008) report preliminary evidence that individuals with Panic Disorder display no general deficits in mentalizing but markedly impaired mentalization related to threat and anxiety cues.

SECTION SUMMARY

The hallmarks of hyperactivation strategies in response to stress and anxiety states are a low threshold for activation of the attachment system, a low threshold for relative deactivation of brain areas involved in controlled, reflective social cognition, and mentalization as well as amygdala hyperreactivity resulting in neuroendocrinological hyperresponsivity. The current model locates the main "programming" of these circuits and the neural acquisition of allostatic adaptation (i.e., plasticity) in the early attachment experiences. The ineffective down-regulation of stress which is linked with impaired interpersonal functioning and long-term consequences of allostatic load can lead to an exhaustion and dysfunction of the stress response system with increased risk for stress-related psychopathologies such as anxiety disorders.

AN ATTACHMENT-BASED DEVELOPMENTAL FRAMEWORK OF ANXIETY DISORDERS

We conceptualize the attachment system as a central organizer of biological, genetic, and environmental influences on the development of dysfunctional stress-regulatory processes and fear responses that underpin anxiety disorders. The model, based on the preceding review is presented in **Figure 1**. Component sections are discussed in turn.

CHILD FACTORS

Genetic influences have been demonstrated to account for temperamental factors (BI) which may, in some instances, represent child-driven effects in the evocation of certain parenting styles. Individual differences in attachment have also been demonstrated to be influenced by genetic factors, although to a lesser degree. The direct contribution to anxiety disorders accounted for by genes is most likely the result of multiple loci additive and/or interactive gene effects (Norrholm and Ressler, 2009; **Figure 1**, Box 1).

However, as we have seen, stress sensitivity, and temperament at birth, is modifiable by the effects of prenatal environment given that, maternal stress and anxiety during pregnancy can lead to a sensitization of the HPA-axis. Thus, characteristics the infant is born with could represent an *in utero* adaptation to the stress regulation style of the mother.

PARENTAL FACTORS

Multiple parental factors have been outlined as possible contributors to the development of dysfunctional stress regulation of the child. Such factors have been delineated as the presence of an anxiety disorder (or preclinical anxiety, both entailing HPA-axis alterations), low social support, parental insecure attachment, and low mentalization capacity (**Figure 1**, Box 2).

THE ATTACHMENT RELATIONSHIP AS A DIALECTICAL SYSTEM ORGANIZING EARLY EXPERIENCES

Together, or individually, parental factors interact with child factors outlined above, within the context of the early attachment relationship. The proposed mechanisms by which these factors can affect the parent's capacity to effectively co-regulate the child's stress include the modeling of anxiety responses, the reinforcement of threat cues in the environment (and their avoidance) and parenting styles characterized by overcontrol of the child and limited autonomy granting as observed in caregiver responses to anxiously attached children (**Figure 1**, Box 3).

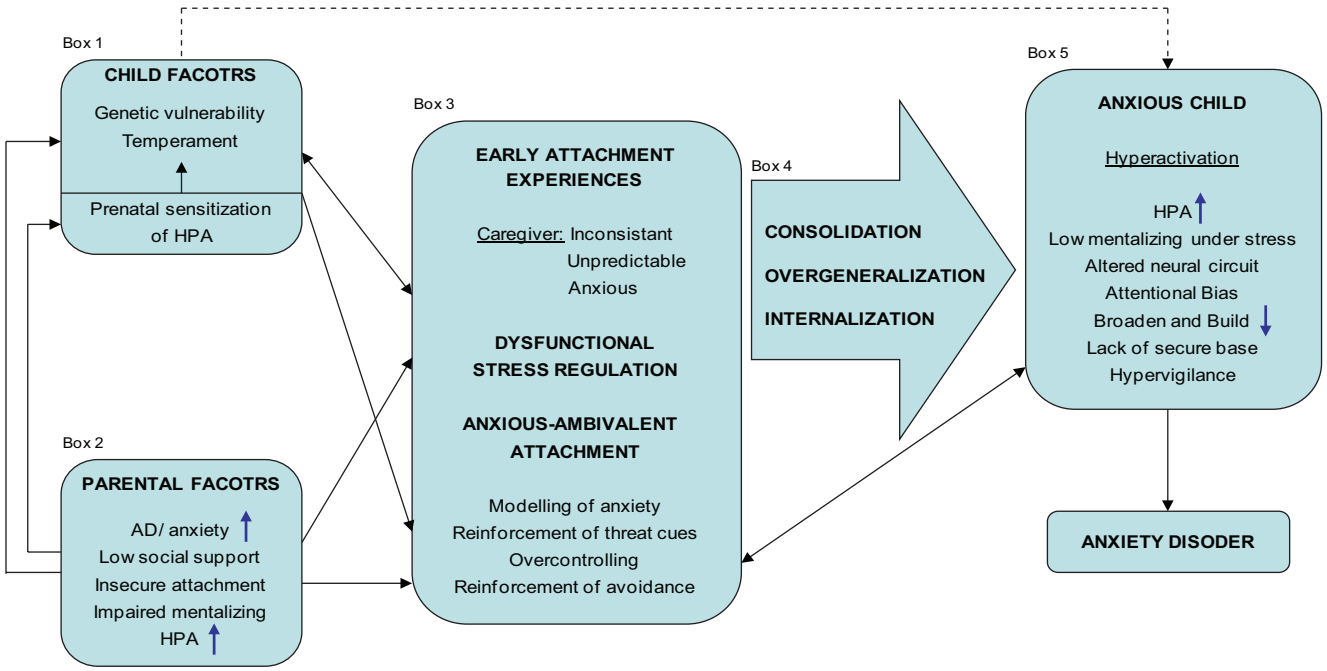

FIGURE 1 | A developmental model of the attachment-moderated interaction of risk factors in the development of anxiety disorders. Black bolts from one box to another indicate either uni- or bidirectional effects and refer to the box as a whole. Blue bolts inside boxes indicate either an increase or decrease of the magnitude of the respective feature.

FROM EARLY ATTACHMENT EXPERIENCE TO A RISK PROFILE

It is proposed that the development of a stress regulation style characterized by hyperactivating strategies is adaptive to the child's particular early environment. However, this pattern of responsivity, once internalized, leads to an overgeneralized application of these strategies in the face of stress, a process that over time becomes consolidated. Consolidation occurs as the individual detects more threat in the environment in the first instance, avoids these perceived threats, and subsequently limits the repertoire of experience that could potentially correct and adjust the internalized expectations of the environment as ultimately unpredictable and threatening (attentional bias to threat). These maintaining factors echo those delineated in both cognitive and learning-theory accounts of the persistence of fear responses in anxiety disorders (Wells, 1997; LaBar and Cabeza, 2006; Mineka and Zinbarg, 2006; Britton et al., 2011; **Figure 1**, Box 4).

These processes are hypothesized, in the current model, to represent pathways from insecure attachment experiences to the presentation of chronic anxiety-related phenomena which, in turn, predispose the child to the development of full anxiety disorder symptomatology depending on vulnerability factors and life events.

THE "ANXIOUS CHILD": A PROFILE OF RISK

Following the development of hyperactivating strategies and their persistent utilization over time, the anxiously attached child is characterized by a discernable repertoire of stress-responses. Such phenomena include (1) the lack of a secure base and concomitant poor exploratory behaviors, perception of reduced control, an attentional bias to threat, (2) chronic reliance upon hyperactivating strategies and hypervigilance of the social environment with neurophysiological correlates of a sensitized HPA-axis and altered fronto-limbic neural circuitry, and (3) compromised social–cognitive capacities under stress with slow recovery of mentalization (**Figure 1**, Box 5).

The various aspects of this combined profile of stress responsivity have been shown within this review to associate with the development of anxiety disorders. The mechanisms by which these response characteristics ultimately lead to the expression of a clinical anxiety disorder likely include the chronic sensitization of the HPA-axis and neural systems, or the impact of subsequent stressful life events that trigger a style of responding that, while adaptive in early childhood, proves maladaptive in adult environments.

Furthermore, the various factors that may influence the expression of anxiety disorders likely interact. The interplay between these different risks at different developmental stages, congruent with the developmental principle of multifinality (Cicchetti and Rogosch, 1996; Luyten et al., 2008), may give rise to different clinical presentations of anxiety disorders. Further longitudinal research is needed in this area to investigate these assumptions.

LINKS IN THE CHAIN: THE ROLE OF EPIGENETIC FACTORS IN THE DEVELOPMENT OF STRESS REGULATION

As demonstrated throughout this review, the understanding of interactions between genetic and environmental factors are key to elucidating how early experiences confer risks for anxiety disorders that persist throughout the lifespan (Rutter et al., 2006). Epigenetic processes, through which events in the environment

alter the activity and expression of genes without altering DNA-sequence, are key candidates in explaining how the effects of early attachment experiences manifest beyond the early years (for review see Murgatroyd and Spengler, 2011).

While attention to these epigenetic processes is developing rapidly, many key hypotheses rely on findings in animal research in which early environments can be experimentally controlled. Data from rodent models indicate that the long-term effects of maternal caregiving appear to depend upon alterations in differentiation of those neurons involved in down-regulation of the stress-response (Meaney et al., 1996; Meaney, 2010), a process involving glucocorticoid feedback systems and related levels of corticotropin releasing hormones (CRH; Plotsky and Meaney, 1993). Further, the offspring of mothers providing high quality, attentive care exhibit reduced levels of CRH in the hippocampus and a reduced sensitivity of the HPA-axis when compared with rat pups of mothers who had not provided such sensitive caregiving (Francis et al., 1999). These environmental-dependent effects are hypothesized to be underpinned by epigenetic alterations in DNA methylation processes (Weaver et al., 2004) and are in line with our model which holds the early interactions within the attachment context as the vital component in the development of stress-regulatory capacities that persist into adulthood.

More recent studies that examine these processes in humans have shown that post-mortem hippocampal tissue from individuals who have completed suicide following a history of depression and early adversity, is marked by altered GR promoter methylation (McGowan et al., 2009). In comparing GR promoter methylation in these subjects with those who had committed suicide (with or without a diagnosis of depression) but with no known history of early adversity, Alt et al. (2010) demonstrated that altered GR promoter methylation was characteristic of subjects with early adversity only, thus addressing the question of whether such markers may be correlates of mood disorders irrelevant of early experiences. Such findings represent initial evidence that epigenetic programming in animal models may extrapolate to human studies of psychopathology associated with sub-optimal early caregiving environments. How such epigenetic alterations could contribute causally to the emergence of anxiety disorders is yet to the be understood, and the search for so-called "epigenetic biomarkers" of psychiatric presentations (Murgatroyd and Spengler, 2011) is made complicated by the limited evidence available indicating that psychopathology can be present without the presence of epigenetic markers (Alt et al., 2010).

Epigenetic processes are hypothesized to underpin the developmental plasticity of an organism, characterized by biological adaptations made early in life that remain in order to enhanced biological preparedness for later, similar environments. While the evolutionary function of such plasticity is clear, adaptations made through epigenetic processes may ultimately increase vulnerability for anxiety-related diseases as the strategies they have promoted may prove redundant and pathognomonic in later environments. Thus, the process of overgeneralization and consolidation in the current model could be taken as representative of the inflexible application of strategies initially promoted via epigenetic mechanisms due to their adaptive properties.

CONCLUSION

The model presented conceptualizes anxiety disorders as caused and maintained by a complex interplay between genetic, environmental, and epigenetic contributions – a multifactorial etiology which ultimately results in dysfunctional stress regulation and fear appraisal strategies that are acquired within the early attachment relationship. These strategies, in our model, are maintained by alterations of social–cognitive as well as biological functions. Emotional strategies adopted by a child may be associated with cumulative allostatic load and subsequent "wear and tear" effects. These converge on the neural pathways involved in processing of signals and experiences associated with anxiety and stress. We suggest that chronic anxiety conditions entail the triggering of a biobehavioral switch causing a shift from more controlled, reflective mentalization to more automatic, reflexive modes (McEwen, 2007; Luyten et al., submitted for publication). This in turn increases the individual's vulnerability to further potentially stressful experiences leading to a hyperactivation of strategies that generate salience for somatosensory and perceptual experiences associated with the activation of the attachment system and the potential of an experience of loss.

An attachment-based framework integrates isolated strands of research that successfully characterized processes inherent to anxiety disorders. A further advantage of this framework is its potential to explain individual differences in stress and fear-triggered regulatory capacities. Distinct characteristic patterns of stress responsivity are associated with the different attachment styles, which may go some way toward explaining differences between anxiety-related conditions. In common to the development of anxiety problems, however, is an overgeneralization of predominantly hyperactivating stress-regulatory strategies developed in an individual's specific attachment relationship.

DISCUSSION/IMPLICATIONS

While delineation of disorder-specific etiological pathways has been beyond the scope of the current paper, the framework presented can account for the heterogeneity of presentations of anxiety-related psychopathology, given its focus on the interplay of genetic and environmental factors that potentially contribute to the development of the various anxiety disorders.

The model therefore generates empirically testable hypotheses regarding specific developmental pathways and factors moderating the risk for or resilience to anxiety disorders.

Longitudinal neuroscientific developmental research will be required in order to elucidate the complex interactions that are likely to result in phenomenologically different types of anxiety disorders, and to further understand the role of anxiety and attachment in the emergence of other major psychopathologies, most notably depressive disorders. Research to date has been primarily cross-sectional and the serial sequence of brain changes that characterize the emergence of anxiety problems is not known. Much of the interactional processes discussed in this review are assumed rather than observed to be occurring in chronological time. The limitation this imposes on theorization principally entails limited understanding of the phenomenon of resilience, the processes whereby risks fail to be translated into clinically

significant problems. While we understand more about brain changes that create pathogenic outcomes, far less is known about adaptations that protect the mind of the child, despite adversity (Hauser et al., 2006). Further, future research should devote more attention to individual differences in clinical outcomes. For instance, the extent to which deactivating strategies are used may influence the clinical expression of anxiety problems, may be dependent on contextual factors or could even protect against the development of anxiety-related symptomatology. For example, within the maltreatment literature, findings from studies of HPA-axis responsivity in children and adolescents with histories of early adversity have been mixed (Tarullo and Gunnar, 2006) with hyperresponsivity not consistently found (De Bellis et al., 1994). A study by Kaufman et al. (1997) showed that hyperresponsivity may be dependent upon the child's *ongoing* exposure to a stressful environment. Thus, further maltreatment research might fruitfully examine the insecure attachment relationship as an example of chronic exposure to a stressful environment, potentially differentiated in its pathognomonic effects from more isolated episodes of maltreatment or adversity. The attachment framework might thus shed light on some currently contradictory findings within the maltreatment literature, as well as providing a conceptual framework for understanding mechanisms by which such sub-optimal caregiving can give rise to such differential outcomes. For example. disorganized attachment, where the caregiver's affect regulation is highly inconsistent (Fonagy and Luyten, 2009), may be associated with more severe developmental outcomes related to anxiety and have been shown to impact substantially on neurobiological development (see McCrory et al., 2010 for a review) with evidence for gender-specific stress responsivity (Kirschbaum et al., 1995; Kudielka and Kirschbaum, 2005). The disorganization of attachment possibly entails the loss of the interpersonal underpinning that is at the root of epistemic trust undermining the individuals confidence in exploring their environment and reducing suspicion through testing and exposure of cultural knowledge as well as a physical world (Fonagy, 1998). Modern attachment theory argues that human infants have genetically inbuilt "healthy" social expectations (Baillargeon et al., 2010). Social experience is developmentally "good enough" when it complies with these expectations; in other words it fits in with biologically prepared mechanisms which evolved to transmit human culture and is consistent with neural development (i.e., the capacity to integrate new information; Fonagy et al., 2007). Meeting these basic expectations is inherent to secure attachment and their violation can be toxic because not only does this "teach" the infant inappropriate content but it undermines the biological and psychological mechanisms for the social acquisition of knowledge and the emergence of an effectively biologically functioning agentive sense of self.

Finally, the model may have important implications for clinical interventions. There is already accumulating evidence for the efficacy of interventions informed by attachment theory in a variety of clinical presentations (Brisch et al., 2003; Bakermans-Kranenburg et al., 2008; Dozier et al., 2009; Fonagy and Luyten, 2009; Suchman et al., 2010). Secure attachment is isomorphic with inducing in the infant/child a sense of epistemic trust which may be seen as indicating to the infant/child that the information relayed by the adult charged with conveying key cultural meanings (Tomasello et al., 2005) may be trusted. This has important implications for therapy for children – as the mind is found within the other and not within itself we may say that evolution has "prepared" children's brains for psychological therapy. They are eager to learn about the opaque mental world from those around them and they are prepared to learn most readily about minds in conditions of epistemic trust. Thus, a therapist ignores the persons to whom the child naturally turns for knowledge at their peril. Preliminary support for superior outcomes when treating the parent or family of children with anxiety disorders (compared to treatment focusing on the child only) exists, mainly for cognitive behavioral approaches (Ginsburg and Schlossberg, 2002; Creswell and Cartwright-Hatton, 2007; Creswell et al., 2008; Kendall et al., 2008). Therapy is not just about the *what* but the *how* of learning. It is about opening the child's mind so (s)he once again can trust the social world by changing expectations This review and proposed model emphasizes the importance of considering an attachment-based framework in interventions for the treatment of childhood anxiety disorders in particular in identifying the attachment relationship as a key target of clinical work, alone, or in addition to pharmacological or other treatment components. Further research therefore should address process and outcome factors in relation to increasing attachment security and enhancing mentalizing capacities in combination with the underlying neurobiological substrates.

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Neural mechanisms of impaired fear inhibition in posttraumatic stress disorder

Tanja Jovanovic[1]* and Seth Davin Norrholm[1,2]

[1] *Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, GA, USA*
[2] *Mental Health Service Line, Veterans Affairs Medical Center, Decatur, GA USA*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Steven A. Kushner, Erasmus Medical Center, Netherlands
Bram Vervliet, Katholieke Universiteit Leuven, Belgium

****Correspondence:***
Tanja Jovanovic, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 49 Jesse Hill Jr. Drive, Atlanta, GA 30303, USA.
e-mail: tjovano@emory.edu

Posttraumatic stress disorder (PTSD) can develop in some individuals who are exposed to an event that causes extreme fear, horror, or helplessness (APA, 1994). PTSD is a complex and heterogeneous disorder, which is often co-morbid with depression, substance abuse, and anxiety disorders such as panic or social phobia. Given this complexity, progress in the field can be greatly enhanced by focusing on phenotypes that are more proximal to the neurobiology of the disorder. Such neurobiological intermediate phenotypes can provide investigative tools to increase our understanding of the roots of the disorder and develop better prevention or intervention programs. In the present paper, we argue that the inhibition of fear responses is an intermediate phenotype that is related to both the neurocircuitry associated with the disorder, and is linked to its clinical symptoms. An advantage of focusing on fear inhibition is that the neurobiology of fear has been well investigated in animal models providing the necessary groundwork in understanding alterations. Furthermore, because many paradigms can be tested across species, fear inhibition is an ideal translational tool. Here we review both the behavioral tests and measures of fear inhibition and the related neurocircuitry in neuroimaging studies with both healthy and clinical samples.

Keywords: PTSD, fear inhibition, extinction, fear neurocircuitry, neuroimaging

NEUROBIOLOGICAL INTERMEDIATE PHENOTYPES OF PTSD

Posttraumatic stress disorder (PTSD) can develop in some individuals who are exposed to an event that causes extreme fear, horror, or helplessness (APA, 1994). PTSD is considered the fourth most common psychiatric disorder, affecting 10% of all men and 18% of women (Breslau et al., 1998). The rates of lifetime PTSD are closer to 40% in high trauma populations, such as combat (Kessler et al., 1995) and low-income inner-city populations (Schwartz et al., 2005; Alim et al., 2006). Recent studies have demonstrated a steep dose–response curve between trauma frequency and PTSD symptom severity such that the more traumatic events a person experiences, the greater the PTSD symptoms (Binder et al., 2008; Mcteague et al., 2010). Even at such high prevalence rates, the relationship between trauma exposure and PTSD suggests resiliency in the majority of individuals, indicating the presence of "resilience factors" that allow trauma-related symptoms to diminish over time. These factors can be genetic, as shown by several recent gene by environment interaction studies (Binder et al., 2008; Ressler et al., 2010), or psychological, such positive social support (Charney, 2004; Norrholm and Ressler, 2009).

Delineating these resilience factors is of great importance to the development of improved and personalized treatment approaches to this disorder; however, using the DSM-IV defined disorder as the phenotype under investigation raises many complications. PTSD is a heterogeneous disorder, which presents with different symptom domains, specifically, re-experiencing, avoidance and numbing, and hyper-arousal symptoms. As some patients may present higher symptoms in one domain as compared to another, a one-size-fits-all approach is often inadequate (Norrholm and Jovanovic, 2010). Furthermore, the neurobiological underpinnings of the different symptoms may not overlap (Lanius et al., 2006), suggesting that different "subtypes" of PTSD may have different treatment targets. Finally, PTSD is frequently co-morbid with other disorders, such as depression, substance abuse, and other anxiety disorders (Kessler et al., 1995). Taken together, these issues result in a complex phenotype of PTSD; one that is difficult to model in animal research and does not easily lend itself to treatment outcome studies.

Given this complexity, progress in the field can be greatly enhanced by focusing on phenotypes that are more proximal to the neurobiology of the disorder. Such neurobiological intermediate phenotypes can provide investigative tools to increase our understanding of the roots of the disorder and develop better prevention or intervention programs. Although the narrow focus cannot by definition encompass the entire spectrum of the illness, it can define targets in the neurocircuitry of the illness.

In the present paper, we argue that the inhibition of fear responses is an intermediate phenotype that is related to both the neurocircuitry associated with the disorder, and is linked to its clinical symptoms. An advantage of focusing on fear inhibition is that the neurobiology of fear has been well investigated in animal models providing the necessary groundwork in understanding alterations. Furthermore, because many paradigms can be tested across species, fear inhibition is an ideal translational tool. For example, fear-potentiated startle and inhibition of fear-potentiated startle has been tested in rodents, non-human primates, as well as humans (Myers et al., 2009). Here we review both the behavioral tests and measures of fear inhibition and the related neurocircuitry in neuroimaging studies with both healthy and clinical samples.

INHIBITION OF FEAR AS A NEUROBIOLOGICAL PHENOTYPE

Inhibition of fear responses involves learning and then appropriately responding to safety signals, i.e., the ability to discriminate between danger and safety cues and suppress fear responses in the presence of safety cues (Jovanovic et al., 2011). In the laboratory, fear inhibition can be measured by first using a fear conditioning paradigm to acquire learned fear (termed fear acquisition), which is then followed by training to inhibit learned fear responses (termed fear inhibition). Fear conditioning is based on a simple Pavlovian conditioning model in which a neutral conditioned stimulus (termed the CS; for example, a light) is paired with an aversive unconditioned stimulus (termed the US; for example, cutaneous electric shock). After a number of pairings, the association is formed so that the CS alone elicits the conditioned response (termed the CR; for example, freezing in rodents or fear-potentiated startle in humans; Pavlov, 1927). This basic model is used in animal as well as human research to investigate mechanisms of fear expression (Davis, 1990; Labar et al., 1995; Grillon and Davis, 1997; Fanselow and Ledoux, 1999; Lissek et al., 2005; Jovanovic et al., 2006). The advantage of using these paradigms is that they can be measured with peripheral outcomes such as the skin conductance or startle responses, which are non-invasive but offer physiological measures of fear conditioning. In this review we will describe fear inhibition as the reduction of fear responses in the presence of safety cues which is a manifestation of the underlying inhibitory neurocircuitry.

There are two laboratory models that have been primarily used for behavioral testing of fear inhibition in animals and humans: extinction and differential conditioning. Whereas fear acquisition refers to learning that something is dangerous, extinction is a mechanism by which an individual learns that something that was previously dangerous has become safe. In fear extinction paradigms, a stimulus that was previously paired with an aversive stimulus (the CS+) is then repeatedly presented without the US, so that it no longer elicits a fear response [cf. (Myers et al., 2006; Norrholm et al., 2006), see **Figure 1A**]. In a basic differential conditioning paradigm, the above CS+ pairing is intermingled, at the time of training, with a separate stimulus (CS–). The CS– does not co-occur with an aversive stimulus, and thus represents safety, or inhibition of fear. This paradigm involves a simple discrimination between the danger and safety cues (see **Figure 1B**), and is the one most commonly used in human fear conditioning research (Lissek et al., 2005). More complex tasks, such as conditional discrimination, **Figure 1C** (in which there is an element of the conditioning stimulus that is shared between the CS+ and CS–), and stimulus generalization, **Figure 1D** (in which there is a perceptual gradient of stimuli between the CS+ and CS–), are designed to capture more subtle variation in fear-inhibition processes.

Conditioned inhibition involves a variation of discrimination in which the danger cue (i.e., CS+) is not reinforced when preceded by (or combined with) a second cue, usually termed X, so that CS+, CS/X–). Although the X cue should be conditioned to designate safety, it is vulnerable to second-order conditioning effects and limited by configural processing (Myers and Davis, 2004). This paradigm has been used in several animal studies (Falls et al., 1997; Gewirtz et al., 1997), but rarely in human studies (Grillon and Ameli, 2001) due to the above issues. Conditional discrimination, a modification

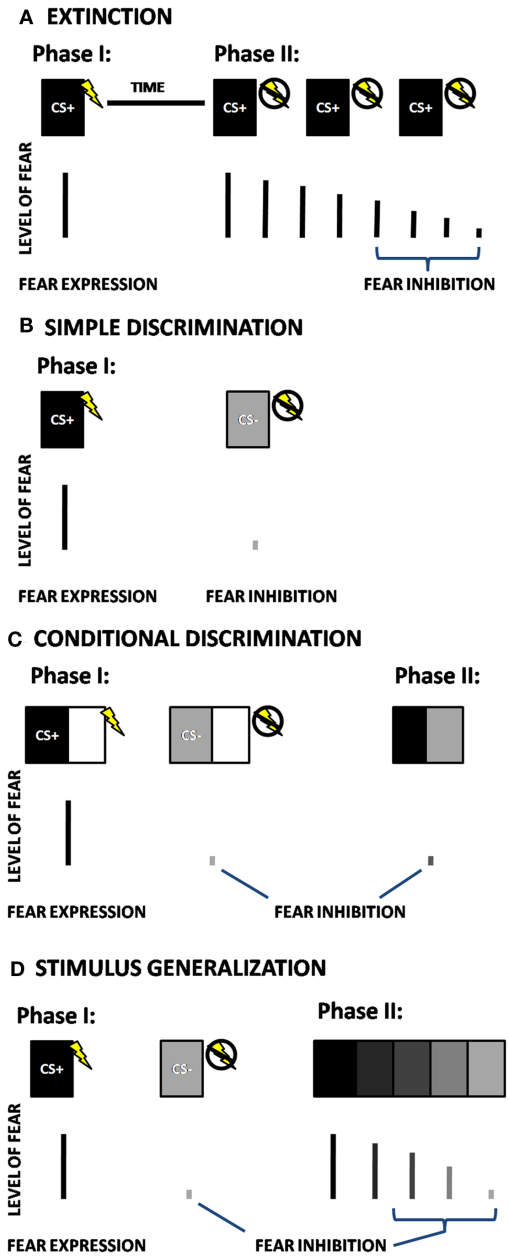

FIGURE 1 | Schematic depictions of four different fear-inhibition paradigms; (A) Extinction, (B) Simple Discrimination, (C) Conditional Discrimination, and (D) Stimulus Generalization. CS+ = Reinforced Conditioned Stimulus; CS– = Non-reinforced Conditioned Stimulus. In fear extinction paradigms **(A)**, a stimulus that was paired with an aversive stimulus (the CS+) during the initial phase is then repeatedly presented without the US in the next phase of the experiment, so that it no longer elicits a fear response. In the simple discrimination paradigm **(B)**, the CS+ is intermingled with a separate stimulus that is not paired with the US (CS–). In a conditional discrimination experiment **(C)**, the US occurs depending on the presence of the CS+ when it is combined with a neutral (X) cue, which is also combined with the CS– to predict the absence of the US. In the next phase of the experiment, presentation of CS+ and CS– together, without the shared cue (X) results in a reduced fear response compared to the response to CS+. In a stimulus generalization experiment **(D)**, after the initial conditioning, the second phase of the experiment presents iterations of CSs that differ from the CS+ in small increments. The number of degrees required for discrimination is an indication of cue overgeneralization.

of the conditioned inhibition design, avoids some of these limitations (Myers and Davis, 2004). In a conditional discrimination experiment, an aversive event occurs depending on the presence of the CS+ when it is combined with a neutral (X) cue (Wagner and Rescorla, 1972). This cue is also combined with the CS– to predict the absence of the US (**Figure 1C**). In a critical subsequent test trial, presentation of CS+ and CS– together, without the shared cue (X) results in a reduced fear response compared to the response to CS+. This is referred to as the transfer test, when the inhibitory properties of the CS– are actively transferred to the combined CS+/CS– cue (Myers and Davis, 2004; Jovanovic et al., 2005).

The current review will focus on extinction, simple discrimination, and conditional discrimination methods of fear inhibition, as these have been more thoroughly investigated using psychophysiological techniques. The final paradigm, stimulus generalization is a very novel approach to studying fear inhibition and has only been recently used in patients with panic disorder (Lissek et al., 2010), and there are no published studies to date that have tested stimulus generalization in PTSD. This paradigm is promising because it assesses both the subjects' ability to detect subtle differences between danger and safety on a continuum, and their ability to show reduction of fear once the discrimination occurs (Lissek et al., 2010).

Both extinction tests and differential conditioning paradigms focus on active suppression of fear responses through learned safety signals; while fear itself may only involve subcortical areas of the brain located primarily in the limbic circuitry, safety signals may require a cognitive, cortical component (Bremner et al., 2005; Weike et al., 2008). This premise is supported by data from our lab showing that awareness of the association between the CS and the US is necessary for inhibiting fear responses (Jovanovic et al., 2006). Furthermore, a recent study by Weike et al. (2008) examined the temporal domain of fear conditioning with a danger and safety signal and found that safety signal processing was slower than danger processing. The authors argued that top-down cognitive processes are involved in responses to safety signals which accounts for the latency in response.

We have recently used extinction, simple discrimination, and conditional discrimination paradigms in a highly traumatized civilian population from inner-city Atlanta (Jovanovic et al., 2010a,b; Norrholm et al., 2011). Data from our study on extinction (Norrholm et al., 2011) suggest that the early phase of extinction is predicted by the level of fear expression to the CS+ (i.e., the danger signal) at the end of acquisition. It is this fear expression during early extinction that is exaggerated in PTSD subjects compared to traumatized non-PTSD controls (see **Figure 2A**). On the other hand, a high degree of fear remaining during late extinction is related to impaired inhibition, as it is best predicted by responses to the CS– (i.e., safety signal) at the end of acquisition (Norrholm et al., 2011). **Figure 2B** shows simple discrimination between the CS+ and the CS– during late acquisition between PTSD subjects and controls. Although PTSD subjects are slower in developing the discrimination, by the final phase of conditioning both groups show higher levels of fear-potentiated startle to the CS+ than the CS–; however, PTSD subjects demonstrate higher levels of fear to both stimuli (Norrholm et al., 2011). The final paradigm, conditional discrimination, measures fear inhibition to the safety signal by testing the reduction of fear-potentiated startle on the transfer test. We used this paradigm in combat veterans (Jovanovic et al., 2009) and traumatized civilian population (Jovanovic et al., 2010b) and have found that individuals who met criteria for PTSD had higher potentiation of the startle response to the CS– than traumatized controls and did not transfer safety on the test trial (**Figure 2C**).

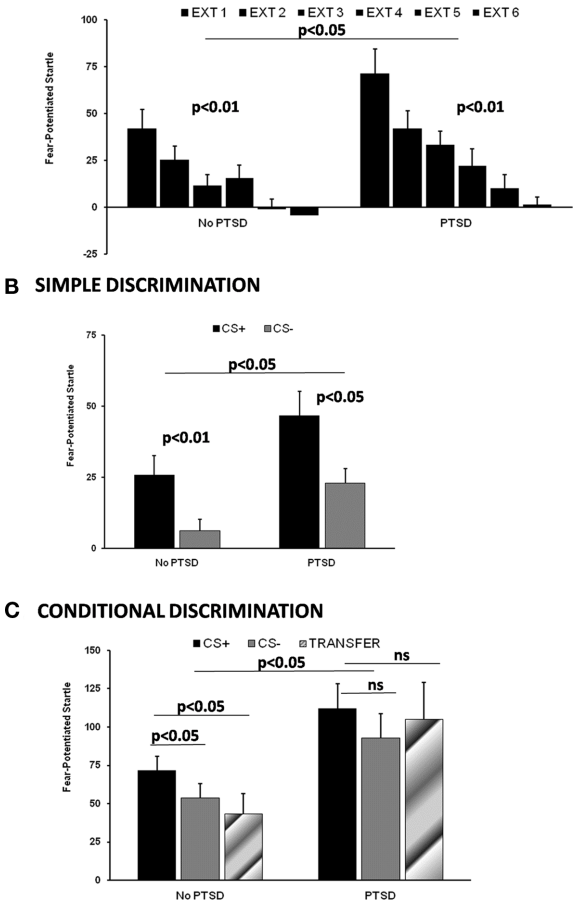

FIGURE 2 | Fear-inhibition data from our studies on a traumatized civilian population. (A) Extinction, adapted from (Norrholm et al., 2011); **(B)** Simple Discrimination, adapted from (Norrholm et al., 2011); **(C)** Conditional Discrimination, adapted from (Jovanovic et al., 2010b). EXT, Extinction Block; PTSD, Posttraumatic stress disorder; CS+, Reinforced Conditioned Stimulus; CS–, Non-reinforced Conditioned Stimulus.

While some data with combat veterans suggest that impaired fear inhibition may be an acquired trait (Milad et al., 2008), that is associated with current symptom severity (Jovanovic et al., 2009), other studies have reported that heightened fear responses and decreased inhibition of fear may be predictors of the disorder. A prospective study of police academy cadets found that greater skin conductance responses to threatening stimuli and slower habituation prior to trauma exposure were predictive of PTSD symptom severity after trauma exposure (Pole et al., 2009). A similar prospective study with firefighters found that reduced extinction of fear conditioned responses examined before the index trauma explained almost a third of the symptoms in later traumatized

individuals (Guthrie and Bryant, 2006). On the other hand, a recent study of Vietnam veterans and their twins found that PTSD subjects did not have impaired extinction learning, but rather had less extinction retention on the day after acquisition and extinction compared to veterans without PTSD (Milad et al., 2008). Furthermore, impaired retention of extinction appeared to be an acquired trait related to the disorder since the twins of the PTSD subjects did not show the same impairment. It is possible that a decreased ability to inhibit fear is a risk factor for developing the disorder and contributes to the maintenance of the disorder, while decreased extinction retention is a state resulting from the disorder – given that these fear-inhibition phenotypes may have different neural underpinnings this would explain the above studies. Extinction learning requires inhibition of the fear circuitry by the prefrontal cortex (PFC; Phelps et al., 2004); whereas discrimination between danger and safety cues, and recall of extinction may also require activation of the hippocampus (Milad et al., 2007b). Given that both extinction and differential conditioning are dependent on the PFC, this review will focus on this region as a primary locus in fear-inhibition neurocircuitry.

INHIBITION NEUROCIRCUITRY AS A TARGET OF INVESTIGATION

The PFC has long been thought to play a role in behavioral inhibition. More than a decade ago, animal studies reported that lesions of the medial PFC (mPFC) prior to original fear conditioning retard extinction to a tone (Morgan et al., 1993). Recent studies have demonstrated that neurons in the PFC may have inhibitory action on the amygdala (Grace and Rosenkranz, 2002; Phelps et al., 2004). The PFC can be subdivided into medial and orbitofrontal PFC. The anterior cingulate cortex (ACC), which is also part of the PFC, has both rostral and dorsal components which may play different roles in the expression and inhibition of fear, as will be discussed in greater detail below. **Figure 3** shows the medial regions of the PFC most involved in inhibitory processes (**Figure 3A**), including the ventral and dorsal PFC and the ACC (**Figure 3B**).

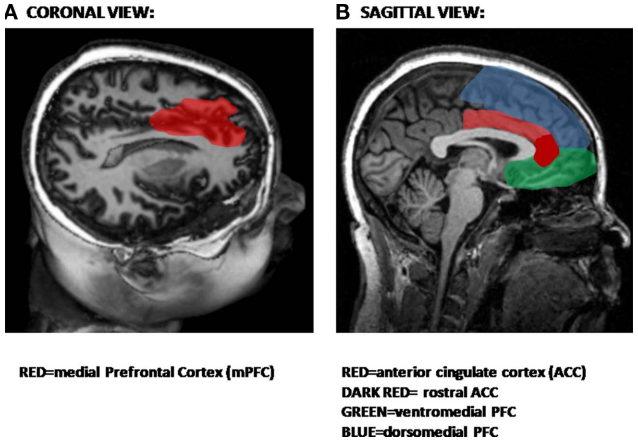

FIGURE 3 | Structural magnetic resonance image (MRI) showing areas involved in inhibition. (A) Coronal view showing location of medial areas in red. **(B)** Sagittal view showing prefrontal cortical areas.

Neuroimaging studies in humans have used several paradigms that activate the PFC; the simplest and most commonly used tasks involve response inhibition. In such tasks, the participant is presented a stimulus indicating that a response is required, for example, to press a button when a letter appears on the monitor. This is referred to as a "Go" signal. On a minority of trials, however, the participant is required to either withhold a response during a "NoGo" signal (the Go/NoGo task) or stop responding once they have begun the execute the action during a "Stop" signal (the Stop task; Hester et al., 2004; Eagle et al., 2008). The Go/NoGo task has been used in subjects with PTSD with electroencephalogram (EEG) evoked-potentials (Wu et al., 2010) and functional magnetic resonance imaging (fMRI) measures (Carrion et al., 2008; Falconer et al., 2008). This task reliably indicates decreased activation in PTSD subjects compared to controls in the rostral ACC, located at the genu of the corpus callosum (see **Figure 3B**). The advantage of this task is that it is very simple to administer in both behavioral and neuroimaging studies, and may provide insight into deficits in inhibiting limbic activity. Although a more general impairment in inhibitory processes mediated by the rACC may very well be an underlying abnormality associated with several psychiatric disorders, the deficits in inhibiting fear responses appear to be uniquely associated with re-experiencing and hyper-arousal symptoms of PTSD (Norrholm and Jovanovic, 2010). Further support for the utility of this paradigm comes from a study predicting positive treatment outcomes in PTSD patients with greater rACC volumes (Bryant et al., 2008).

A well known and frequently used example of a more complex inhibition task is the Stroop effect task, where the meaning of a word (such as the word "red") is in conflict with the color in which it is shown (for example, in blue ink). In this task, the subject is instructed to state the color of the ink while ignoring the interference from the word. Due to the conflict between the color and the word, reaction times are delayed, providing a measure of the cognitive inhibition (Stroop, 1935), and activating the ACC (Pardo et al., 1990; Bremner et al., 2004). The Stroop task can also be adapted to use with emotion-relevant stimuli, in which the emotional content of a word competes with the cognitive content and must be ignored. This task also activates the ACC, but in an area distinct from the strictly cognitive interference tasks (Whalen et al., 1998, 2006). Emotionally relevant stimuli appear to be processed by the rostral or subgenual area of the ACC (Shin et al., 2005), which is anterior to the genu of the corpus callosum. Furthermore, this specific region of the PFC, the rostral ACC, is involved in amygdala regulation (Etkin et al., 2006).

Neuroimaging studies using fear conditioning paradigms demonstrate that fear acquisition and extinction of fear also activate the PFC, specifically the ventromedial (vmPFC; Phelps et al., 2004; Reinhardt et al., 2010). Recent developments in the spatial resolution of neuroimaging techniques have resulted in more fine-tuned examinations of this area of the brain. As mentioned above, the rostral or subgenual regions of the ACC are activated during the presentation of emotional stimuli; these areas are also activated during the regulation of fear (Phelps et al., 2004; Schiller et al., 2008). There are several lines of evidence that this region of the vmPFC is associated with inhibition of fear. For example, fMRI data

indicate increased activation during an extinction recall task that is presented after extinction learning has occurred (Phelps et al., 2004; Milad et al., 2007b). vmPFC is also activated during fear reversal tasks in which the CS contingencies are switched after acquisition so that a previously conditioned danger cue (CS+) becomes the new safety cue (CS–; Schiller et al., 2008). Morphometric data show that the thickness of vmPFC cortical tissue is correlated with extinction retention (Milad et al., 2005; Hartley et al., 2011). Functional and morphometric data support the rostral ACC as an anatomical substrate for fear inhibition, however, similarly acquired functional and morphometric data suggest that dorsal ACC activity underlies fear acquisition and fear expression (Milad et al., 2007a). Given that this area is also implicated in cognitive tasks (Shin et al., 2007), it may be activated by the active learning that occurs during fear acquisition, rather than by the fear itself. However, given that this area has been associated with fear as well as other noxious stimuli such as pain (Vogt et al., 2003), it may be more centrally involved in the expression of negative affect and not simply activated by general learning.

Several studies have indicated that this inhibitory neurocircuit is dysregulated in PTSD patients. Evidence suggests that a hallmark of PTSD neurobiology is exaggerated amygdala activity during fearful stimulation coupled with reduced top-down control of the amygdala by the PFC (Liberzon et al., 1999; Rauch et al., 2000, 2006; Shin et al., 2004; Liberzon and Martis, 2006). Furthermore, functional neuroimaging studies that have examined connectivity between PFC and the amygdala have demonstrated impaired inhibition of the amygdala in PTSD (Lanius et al., 2004). The emergence of MRIs with greater special resolution allows for more precise descriptions of these neural substrates. A recent meta-analyses of imaging studies during emotion processing in PTSD, social anxiety, and specific phobia indicated that the rostral ACC is less active in PTSD patients relative to controls; an effect not found in other anxiety disorders (Etkin and Wager, 2007). Furthermore, deficient activation of the rostral ACC has been observed in women with sexual trauma-related PTSD by coupling the the Emotional Stroop task with neuroimaging techniques (Bremner et al., 2004). Similar effects were also observed in combat veterans (Shin et al., 2001). Decreased activation of this area may be a risk factor for psychopathology: a recent study of children with depressed parents revealed a lack of ACC activation to the Emotional Stroop (Mannie et al., 2008). There has been a paucity of studies investigating fear conditioning in PTSD patients using neuroimaging methodologies. One study using positron emission tomography (PET) during fear acquisition and extinction, demonstrated heightened amygdala activity in PTSD patients relative to controls during the acquisition phase with lower ACC function during the extinction phase (Bremner et al., 2005). The differential involvement of the ACC subcomponents has been further elaborated in more recent studies. For example, a recently published study that tested extinction recall in an fMRI task demonstrated increased activation of the dorsal ACC (associated with learning) and decreased activation of the vmPFC (which includes the rostral ACC) in PTSD patients (Rougemont-Bücking et al., 2011). Finally, as previously mentioned, the rostral ACC is hypo-activated during Go/NoGo inhibition tasks (Carrion et al., 2008; Falconer et al., 2008).

To summarize the neuroimaging findings to date, the rostral ACC is involved in (1) response inhibition tasks, (2) emotion regulation tasks, (3) inhibition of fear, and (4) is hypo-active in PTSD. These data suggest that this specific circuit represents a compelling target for translational investigations of PTSD and anxiety, as a biomarker predictive of PTSD and anxiety disorder vulnerability, treatment response, or as a treatment outcome measure in itself.

INHIBITION NEUROCIRCUITRY AND IMPLICATIONS FOR TREATMENT RESPONSE

Currently, the most effective therapy for PTSD appears to be psychotherapy, such as cognitive-behavioral therapy, which employs imaginal, *in vivo*, or virtual reality exposure to trauma cues (Rothbaum and Schwartz, 2002). The repeated exposure to feared cues without the negative events associated with the trauma, gradually leads to a reduction in symptoms. Clearly, active fear inhibition is critically involved in this treatment type. Although extant treatment approaches are not tailored to an individual patient's symptom profile, future treatment strategies for PTSD may be governed by a shift toward personalized medicine (see Norrholm and Jovanovic, 2010). The identification of particular risk factors such as genotype or gene expression, as well as the assessment of intermediate phenotypes specific to PTSD may dictate which treatment regimens will be most effective for a particular patient.

It now appears that some forms of dysregulated fear inhibition, such as impaired conditioned fear extinction, may be a vulnerability factor for the development of PTSD (Guthrie and Bryant, 2006; Pole et al., 2009), while other forms of impaired inhibition, such as danger/safety signal discrimination, may be associated with current symptom state, (Jovanovic et al., 2009). In addition, impaired fear inhibition that manifests itself as disrupted extinction recall may represent acquired traits of the disorder (Milad et al., 2008). The difference between these paradigms may lie in the involvement of the hippocampus in the latter (Milad et al., 2007c); which early studies have found to be decreased in PTSD subjects (Bremner et al., 1995) possibly as a result of the trauma and associated stress (Bremner, 2001). However, some have argued that smaller hippocampal volume is a risk factor for PTSD (Pitman, 2001; Gilbertson et al., 2002). These issues can only be resolved with a prospective study examining neural volume before and after onset of PTSD. Regardless of whether fear inhibition is impaired prior to PTSD or develops as part of the disorder itself, it may be modifiable with treatment.

Clinically, fear inhibition has not yet been tested with regard to treatment response. The lack of these studies is partly due to the difficulty of testing *de novo* learning in a repeated design. For example, improvements in fear inhibition after treatment may not be due to treatment efficacy, but rather to a practice effect from patients remembering the previously administered training paradigms. A recent study with fear acquisition and fear extinction tests spaced 12 weeks apart demonstrated good test–retest reliability on these measures (Zeidan et al., 2011); this is a crucial first step to developing treatment outcome measures.

Although treatment outcome and psychophysiological fear-inhibition measures have not been actively examined concurrently, several studies have begun to investigate the relationship between pre-treatment neural function and subsequent treatment response. In PTSD, one study revealed that larger rostral ACC

volume predicted positive outcomes to cognitive-behavior therapy (Bryant et al., 2008). Furthermore, this brain area has been linked to treatment response in depression (Pizzagalli et al., 2001; Pizzagalli, 2011). The ventral area immediately below the corpus callosum has been used as a target in deep brain stimulation to relieve depression (Holtzheimer and Mayberg, 2010), with long-term positive outcomes (Kennedy et al., 2011).

There is an emerging body of literature assessing structural and functional changes in the neural underpinnings of PTSD with treatment. An early study using single photon emission computed tomography (SPECT) imaging pre- and post-treatment with selective serotonin reuptake inhibitors (SSRIs), found significant changes in ACC and hippocampus after 12 weeks of treatment (Carey et al., 2004). More recent studies using fMRI before and after psychotherapy for depression have indicated changes in prefrontal areas (Dichter et al., 2010; Ritchey et al., 2011).

FUTURE DIRECTIONS: CAN WE INCREASE NEURAL INHIBITION OF FEAR?

Several exciting and novel avenues have been revealed for the further exploration and development of neurobiologically based, translational studies of PTSD and trauma-related disorders. We are currently well-poised to investigate these avenues as a means of developing better diagnostic tools based on novel neurobiological intermediate phenotypes. An obvious first step is to test fear inhibition before and after treatment in order to improve existing predictors of treatment response. This will also allow for the exploration of potential individual differences that contribute to positive treatment outcomes and aid in personalization on treatment strategies. Another goal is to track treatment efficacy in responders.

In addition to increased exploration of the putative fear-inhibition phenotype with respect to treatment, we also need to investigate treatment-related changes in brain neurocircuitry and structure. As previously described, fMRI has been successfully employed in depressed patients with significant treatment results (Ritchey et al., 2011). Given the specificity of the brain areas that are related to fear inhibition in PTSD, namely, the rostral ACC, simple inhibition tasks such as the Go/NoGo can be administered before and after treatment to detect changes in the volume, activity, and connectivity of this area. Furthermore, the use of fMRI tasks in concert with fear-inhibition paradigms (e.g., extinction and differential conditioning) with PTSD patients is in its infancy and we anticipate several emerging studies using these methods.

Finally, future studies should be focused on strengthening the activation of the rostral ACC, especially during the presentation of fear-related stimuli. The aforementioned fMRI studies from the depression literature suggest that the PFC represents a relatively neuroplastic brain region with the potential for treatment-related modifications in activity (e.g., successfully attenuating amygdala-driven fear responses); these findings offer great promise to improving on the available treatments for PTSD. Novel treatment approaches have employed computerized tasks in order to train patients to generate the anticipated (or therapeutic) response. For example, attention bias training has been used successfully with many anxiety disorders to train patients' attention away from negative stimuli and, as such, significantly decrease symptom severity (Reese et al., 2010; Rozenman et al., 2011). Similar computerized tasks or games can be devised to bolster fear-inhibition training. For example, exposure therapy, which is one of the most successful psychotherapeutic approaches to PTSD, may in fact produce its effects by increasing fear inhibition. Given that exposure therapy is based on extinction learning, which activates the vmPFC, this premise would provide a likely neural mechanism of action. Facilitating extinction, either pharmacologically or behaviorally, may produce therapeutic, beneficial modifications to underlying neural connectivity and thus increase inhibition of fear.

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Revealing context-specific conditioned fear memories with full immersion virtual reality

***Nicole C. Huff*[1], *Jose Alba Hernandez*[1], *Matthew E. Fecteau*[1], *David J. Zielinski*[2], *Rachael Brady*[2] *and Kevin S. LaBar*[1]*

[1] *Psychology and Neuroscience Department, Center for Cognitive Neuroscience, Duke University, Durham, NC USA*
[2] *Pratt School of Engineering, Duke University, Durham, NC USA*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA
Fred J. Helmstetter, University of Wisconsin – Milwaukee, USA

***Correspondence:**
Kevin S. LaBar, Center for Cognitive Neuroscience, Duke University, Box 90999, B247 LSRC Building, Research Drive, Durham, NC 27708-0999, USA. e-mail: klabar@duke.edu

The extinction of conditioned fear is known to be context-specific and is often considered more contextually bound than the fear memory itself (Bouton, 2004). Yet, recent findings in rodents have challenged the notion that contextual fear retention is initially generalized. The context-specificity of a cued fear memory to the learning context has not been addressed in the human literature largely due to limitations in methodology. Here we adapt a novel technology to test the context-specificity of cued fear conditioning using full immersion 3-D virtual reality (VR). During acquisition training, healthy participants navigated through virtual environments containing dynamic snake and spider conditioned stimuli (CSs), one of which was paired with electrical wrist stimulation. During a 24-h delayed retention test, one group returned to the same context as acquisition training whereas another group experienced the CSs in a novel context. Unconditioned stimulus expectancy ratings were assayed on-line during fear acquisition as an index of contingency awareness. Skin conductance responses time-locked to CS onset were the dependent measure of cued fear, and skin conductance levels during the interstimulus interval were an index of context fear. Findings indicate that early in acquisition training, participants express contingency awareness as well as differential contextual fear, whereas differential cued fear emerged later in acquisition. During the retention test, differential cued fear retention was enhanced in the group who returned to the same context as acquisition training relative to the context shift group. The results extend recent rodent work to illustrate differences in cued and context fear acquisition and the contextual specificity of recent fear memories. Findings support the use of full immersion VR as a novel tool in cognitive neuroscience to bridge rodent models of contextual phenomena underlying human clinical disorders.

Keywords: fear conditioning, virtual reality, contextual fear, memory retention, hippocampus

INTRODUCTION

In the emotional learning literature, it is well established that the extinction of conditioned fear to a discrete cue is context-specific (for review, see Bouton et al., 2006). Experimental and clinical findings of fear renewal and relapse demonstrate that extinction learning does not readily generalize to other contexts in rodents and humans (e.g., Mineka et al., 1999; Corcoran and Maren, 2001; Bouton, 2002, 2004; Schiller et al., 2008; Huff et al., 2009). Therefore, it has been argued that the original fear memory is less context-specific than the competing extinction memory because extinguished fears return when an organism is put back into the acquisition context or a novel context (Bouton, 2004).

However, recent rodent studies reveal that there is a sharp contextual gradient for the original fear memory, which challenges the notion that fear extinction is more context-specific than the fear memory itself. For example, Wiltgen and Silva (2007) demonstrated that contextual fear memory is initially specific but becomes generalized over time when memory for a footshock is tested 1, 14, 28, or 36 days after context exploration. Moreover, mice that can discriminate between fearful and safe contexts rely on the hippocampus, whereas generalized fear memories are hippocampus-independent (Wiltgen et al., 2010). Winocur et al. (2007) employed a comparative contextual fear and food preference conditioning paradigm by testing rats in a new context or the conditioning context at 1 and 8 days for food preference memory, or 1 and 28 days for fear memory. Responding to both the food and fear cue was context-specific at the short intervals but not at the long intervals. This decrease in the learned response outside of the original context is known as the *context shift effect* (reviewed in Riccio and Joynes, 2007) and suggests that both conditioned fear and food preference memory retention is initially context-specific due to the incorporation of background stimulus attributes into the memory (Perkins and Weyant, 1958; McAllister and McAllister, 1963; Feinberg and Riccio, 1990; Zhou and Riccio, 1996; Anderson and Riccio, 2005).

It has been argued that contextual specificity in rodent memory models provide an evolutionary basis for more complex forms of episodic memory in humans. The *transformation hypothesis* argues that such memories change from an initially hippocampus-dependent representation to a more neocortical framework

through systems-level consolidation processes (Winocur et al., 2007, 2010). Contrary views, such as multiple memory trace theory (Nadel and Moscovitch, 1997), diverge from the transformation hypothesis and predict long-term hippocampal involvement for episodic and detailed spatial memories whereas long-term semantic memories reside in the neocortex (Nadel et al., 2000). Both theoretical positions, however, would predict that humans should express a context-specific fear memory soon after fear acquisition. Yet, direct comparisons of cued fear conditioning both in and out of the original learning context are lacking in order to evaluate this predicted context-specificity of recent fear memories. Here we investigate 24-h delayed recall of a conditioned fear memory in healthy humans using a manipulation that varies the testing context in order to assess the spatial specificity of recent cued fear memories.

A major challenge to addressing this research question is the ability to evoke stable contextual fear retention in humans. Previous studies have moved participants from one physical room to another (LaBar and Phelps, 2005; Huff et al., 2009), but this method is limited in the number and type of contexts that can be manipulated as well as their salience. An alternate method uses a single-cue context manipulation, such as changing the color of a background light in a scene (Milad et al., 2005), but this method is known in animal models to not engage the same hippocampal-dependent mechanisms as exploration of a multisensory complex environment (O'Keefe and Nadel, 1978; Squire, 1992; Wiltgen et al., 2010; Winocur et al., 2010). Measurement of conditioned fear to a context rather than a discrete conditioned stimulus (CS) has only recently been explored in humans (e.g., Baas et al., 2004; Grillon et al., 2006) because it has been technically difficult to create a context that is more salient to the participant than the laboratory in which a study is being conducted. Several recent fear conditioning studies have also employed 2-D virtual reality (VR) in which participants view a computerized scenario through a head mounted display (e.g., Baas et al., 2004; Grillon et al., 2006; Alvarez et al., 2008; Marschner et al., 2008). However, the VR literature (Sanchez-Vives and Slater, 2005) suggests that head mounted displays presenting flat 2-D representations do not create the same level of "presence" or subjective feelings of "being there" that a 3-D immersive VR experience does. Moreover, these initial studies on contextual fear conditioning have focused on the acquisition processes rather than fear retention.

To overcome these methodologic challenges, the present investigation implemented a contextually rich, fully immersive 3-D VR preparation in the Duke immersive virtual environment (DiVE). The unique technology utilized in these studies simulates a life-like experience by guiding participants through 3-D worlds that are back-projected onto movie screens surrounding them, including ceiling and floor projection (**Figure 1**). Dynamic CSs are inserted into the environments and are viewed through VR goggles, providing a fully immersive virtual experience (Huff et al., 2010). This setup simulates how CSs are encountered in the real-world using rich contextual manipulations and brings human studies closer to rodent preparations in which subjects explore a novel conditioning chamber.

Evidence from the learning and memory literature suggests that, in an intact neurobiological system, fear learning to a context and cue naturally occurs in a *conjunctive* or holistic manner (Rudy and O'Reilly, 2001; Rudy et al., 2004). That is, a rodent rapidly acquires a representation of the context and the features of the context, such as a fear-predicting cue, in a unitary, hippocampal-dependent representation. Therefore, we hypothesized that fear retention to the CS in the original context would be superior to that tested in the original context without the CS present or to the CS in a novel context. To test whether conditioned fear is initially context-specific and retrieved as a function of a combined cue and context representation, we implemented a differential fear conditioning paradigm conducted over 2 days. Skin conductance responses (SCRs) to a compound audio–visual CS paired with a mild wrist shock unconditioned stimulus (US) were analyzed 24 h after fear conditioning in a novel VR context, the same VR context, and to the context alone during the interstimulus interval (ISI). This approach to assessing context effects is derived from the rodent literature (e.g., Phillips and LeDoux, 1992; Huff and Rudy, 2004; Rudy et al., 2004) to allow for dissociation of three aspects of fear memory retention: *contextually cued* (CS + original context), *cued* (CS + novel context), and *contextual* (context alone). Given the rich feeling of presence in a fully immersive virtual environment (Sanchez-Vives and Slater, 2005), this novel application of VR technology permits a strong assay of contextual influences on fear memory in human participants.

FIGURE 1 | Schematic of the control room and Duke's immersive virtual environment (DiVE) with a human participant viewing a virtual scene.

MATERIALS AND METHODS

PARTICIPANTS

Subjects consisted of 58 young adults (28 male and 30 female; mean age = 19) who were recruited from the Duke University community. Participants were randomly assigned to either the *Same Context* or *Different Context* groups. *Same Context* participants experienced the same VR setting on Days 1 and 2 whereas *Different Context* participants experienced a context shift between Day 1 and Day 2. Participants completed a questionnaire assessing attitudes toward snakes and spiders (Klorman et al., 1974). No subjects scored within 1 SD of the mean of patients with

specific phobia. Participants received either psychology course credit or were compensated at a rate of $10/h. All participants provided written informed consent and experimental procedures were approved by the Duke University Institutional Review Board.

CONDITIONING PROCEDURE AND DESIGN

Participants were seated in the center of the DiVE, a six-sided, fully enclosed 10 ft^3 cube, facing forward with head tracking on the 3-D headset (**Figure 1**). During each learning phase, participants were taken on a fixed virtual walk through the designated environment in which dynamic virtual snakes and spiders (CS+ /CS−) were encountered. The onset of each CS was also paired with an auditory cue (rattle sound for snake and tapping sound for spider) to orient the participant to the presence of the CS in the environment. These postural constraints were made to avoid dizziness, account for variability in height, control for amount of context and stimulus exposure between participants, and ensure that the visual display is realistically updated according the participants' movement through the scenario. For a video demonstration of the methodology, see Huff et al. (2010).

PSYCHOPHYSIOLOGICAL RECORDING IN THE DiVE

Skin conductance, the dependent measure of fear, was collected on the middle phalanges of the second and third digits of the non-dominant hand using Ag–AgCl electrodes attached to velcro straps. A wrist band was secured along the median nerve on the dominant forearm for transmission of the electrical stimulation pulses that constituted the US. Recording and stimulating leads reached the BIOPAC (Goleta, CA, USA) physiological recording system and shock generator, respectively, just outside the DiVE in the control room where this equipment interfaces with the stimulus presentation computer running Virtools software. The BIOPAC's digital input was connected to the control computer's parallel port. SCR data was continuously monitored and data was collected on a laptop computer connected to the BIOPAC system via a parallel port. The Virtools software program triggered the shock generator via a National Instruments DIO-24 data acquisition card (Austin, TX, USA).

TRAINING AND TESTING PHASES

Fear acquisition followed an initial habituation period on Day 1 to allow for acclimation to the experimental environment and reduction of orienting responses to the CS. Habituation consisted of four trials of each CS type presented without reinforcement in a gray screen virtual background. The fear acquisition phase consisted of 16 intermixed trials of each CS type (5 of the 16 CS+ trials were reinforced with the US). Approximately 24 h later, fear retention was tested in an extinction session that consisted of 16 unreinforced trials of each CS type in a pseudorandomized order. Participants experienced the fear retention test in either the same virtual context as the fear acquisition context the day before, or they were shifted to a novel context (randomized across participants). Three contexts were utilized and counterbalanced across participants – an interior of a furnished apartment, an outdoor suburban neighborhood scene, and a forest (see **Figure 2**). The path length and navigation course were matched between virtual worlds, as were the number and placement of objects in the different environments.

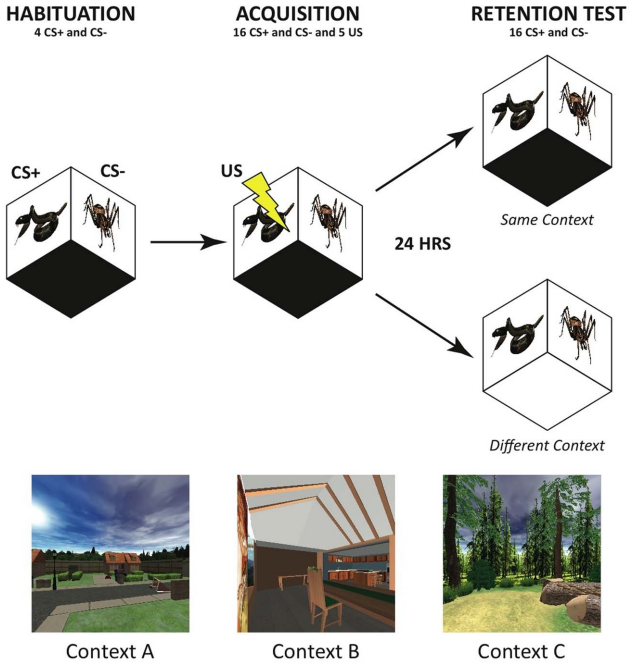

FIGURE 2 | Time line of fear conditioning and retention testing. Participants were seated in the DiVE during habituation/acquisition and extinction sessions on two consecutive days. Conditioned Stimuli (CS+) and (CS−) indicate the phasic reinforced and non-reinforced snake and spider images. The unconditioned stimulus (US), mild wrist shock, was paired with the CS+ on 40% of acquisition trials. Participants were tested in either the same or a different VR context (bottom panel) on the following day during extinction trials to measure fear retention.

STIMULUS PARAMETERS

The dynamic snake and spider CSs were created using Maya graphic design application and imported into Virtools software (Virtools SA, The Behavior Company, Paris, France), which individually appear in the middle and center of the front screen of the DiVE, for a duration of 4 s. The ISI was 12 ± 2 s. The sequence of CSs was pseudorandom, subject to the constraint that no more than two trials of the same CS occur consecutively (to avoid confounding inductions of state anxiety and cognitive expectancy). Partial reinforcement of the CS+ was used to delay rapid extinction that normally occurs in human participants following 100% CS+ reinforcement (LaBar et al., 1998; Phelps et al., 2004). In addition, partial reinforcement provides a more realistic conditioning contingency in that aversive consequences do not always occur following a threatening stimulus.

The US was a brief electric shock (200 ms duration delivered at 30–50 Hz) administered transcutaneously by a bipolar surface-stimulating electrode with 21-mm electrode spacing (Grass-Telefactor Model F-E 10S2, West Warwick, RI, USA). The electrode leads were secured by a rubber strap and are attached to a Grass-Telefactor SD-9 stimulator via coaxial cable leads that were shielded and grounded through a radiofrequency filter. A saline-based gel (Sigma Gel, Parker Laboratories, Fairfield, NJ, USA) was used as an electrolyte conductor. Electrical stimulation

was adjusted prior to the start of the experiment according to each subject's tolerance level in order to facilitate group comparisons and eliminate confounding influences of overall arousal level differences across groups (LaBar et al., 2004; LaBar and Phelps, 2005). The stimulation level was chosen by each participant to be his or her perception of "highly annoying but not painful" using an ascending staircase procedure. Voltage was initially set at a low level of 30 V and increased in increments of 5 V until participants indicated that their tolerance level had been reached without inducing pain.

TASK INSTRUCTIONS – US EXPECTANCY

Prior to each experimental phase, participants were informed that they would encounter snakes and spiders in the virtual environment and that they may receive electrical stimulation at the level that was set prior to conditioning at any time throughout the study. Participants were instructed to press a button on a VR hand wand using their dominant hand to indicate their expectancy of a shock occurring at the onset of each CS presentation (1 = least likely, 4 = most likely). They were instructed to face directly forward and attend to the snake and spiders images presented on the front screen. Subjects were also instructed to keep their hand still to avoid movement artifacts in the SCR recording electrode. They were reminded that they did not have any control over their own movement through the world, nor could they control the occurrence of electrical stimulation. They were also informed that they could terminate at any time without penalty.

PSYCHOPHYSIOLOGICAL MEASUREMENTS

Skin conductance was sampled at 250 Hz, amplified, and stored for offline analysis using AcqKnowledge software (BIOPAC Systems). The recorded waveforms are lowpass filtered using a Blackman window (cutoff frequency = 31 Hz) and smoothed over three successive data points. SCR amplitudes were time-locked to the onset of each CS relative to the pre-stimulus baseline to derive a dependent measure of cued fear (LaBar et al., 1998, 2004; LaBar and Phelps, 2005; Zorawski et al., 2005). For inclusion in the data analysis, the following criteria were established: latency = 1–4 s, duration = 0.5–5 s, and minimum amplitude = 0.02 μS. Responses that do not meet these criteria were scored as zero. Context fear in the absence of CS+, CS−, or US presentation was computed as the mean skin conductance level during the ISI (12 ± 2 s) during which participants navigated the environment but no explicit CS was presented. ISIs immediately following a US presentation were discarded for analysis due to potentially confounding residual influences of the unconditioned response.

DATA ANALYSIS

Three dependent measures were analyzed for evidence of differential fear learning on Day 1: SCR to the CS+ and CS− defined as *cued fear*, SCR to the context during the ISI defined as *context fear*, and *US Expectancy* defined as a declarative measure of the fear contingency with a button press response to the CS+ and CS−. Three dependent measures of fear retention were extracted on Day 2 (long-term memory): *contextually cued fear* was defined as SCR to the CS viewed within the same context as acquisition training (*Same Context* group); *cued fear* was defined as SCR to the CS in a novel context (*Different Context* group); and *context fear* was defined as skin conductance level to the Same or Different Context during the ISI. Across all three measures *fear retention* was computed by extracting data collected during the first half (16 mixed stimuli presentations or ISIs) of extinction training trials on Day 2. The second half of trials on Day 2 was not analyzed due to confounds with extinction processes. Repeated measures MANOVAs were conducted to determine how the dependent measures of fear changed within each training phase and CS type across groups. Fischers PLSD and Bonferroni–Dunn *post hoc* analyses were conducted on fear acquisition and fear retention data. US Expectancy was not extracted on Day 2 due to technical errors in data collection. An alpha level of 0.05 was established for all statistical contrasts.

Because SCR data is typically skewed toward zero, the data were square-root transformed prior to statistical analysis to attain a normal distribution. The data from each CS type (virtual snakes or spiders) were collapsed into "Early" (first half – 16 mixed stimuli presentations) and "Late" (second half – 16 mixed stimuli presentations) trial blocks of each phase (Acquisition on Day 1 or Retention on Day 2), as learning typically varies across time within each learning phase. Data were normalized by dividing each value by the participants' own maximum US response to account for individual variations in responding and minimize group differences in overall arousal levels. US Expectancy responses were also normalized to the maximum response of four in order to statistically compare all three dependent variables. Due to technical errors during data collection, the final statistical analysis included 58 participants for cued fear, 54 participants for context fear, and 28 participants for US Expectancy.

RESULTS

FEAR ACQUISITION

Repeated Measures MANOVA was computed using the factors Fear Acquisition Block (Early, Late) by Dependent Variable (US Expectancy, Cued Fear, Context Fear) by CS Type (CS+, CS−). Analysis revealed a main effect of CS Type, $F(1, 272) = 33.793$, $P < 0.001$, indicating greater responding to the CS+ across variables, as expected. A main effect of Dependent Variable, $F(2, 272) = 538.288$, $P < 0.001$, indicated a difference in response magnitude across measures, with Context Fear and US Expectancy exhibiting the largest differentiated responses at both Early and Late Acquisition. Fischers PLSD *post hoc* tests revealed Context Fear responding to be greater then Cued Fear at both Early and Late Acquisition, $P < 0.001$; $P < 0.001$. Likewise, US Expectancy responses were greater than Cued Fear responses, $P < 0.001$; $P < 0.01$. Conversely, Bonferroni–Dunn follow up tests revealed that Context Fear and US Expectancy were not different from each other at either time point, $P = 0.967$; $P = 0.248$. Accordingly, there was a significant interaction of CS Type × Dependent Variable, $F(2, 272) = 12.457$, $P < 0.001$. However, the relatively lower number of subjects' data available for US Expectancy analysis should be taken into consideration in all analyses.

As predicted, there was a significant interaction of Fear Acquisition Block and CS Type, $F(1, 272) = 12.756$, $P < 0.001$, indicating greater responding to the CS+ in Late Acquisition across all dependent measures. Finally, there was a significant three-way

interaction of Fear Acquisition Block × CS Type × Dependent Variable, $F(2, 272) = 4.575$, $P < 0.02$, which revealed when differential fear emerged in the learning phase across dependent measures. Follow up *post hoc* tests indicated that Context Fear and US Expectancy were differentiated by CS Type in Early Fear Acquisition and maintained in Late Acquisition, whereas differential Cued Fear emerged only in Late Acquisition (**Figure 3**). *Post hoc* Bonferroni–Dunn analysis revealed differential Context Fear ($P < 0.002$; $P < 0.001$) and US Expectancy ($P = 0.035$; $P = 0.001$) during both Early and Late Acquisition. However, differential Cued Fear emerged only during Late Acquisition ($P = 0.043$).

FEAR RETENTION

A Repeated Measures MANOVA was computed for Early Extinction SCRs on Day 2 as an index of fear retention using factors CS Type (CS+, CS−), Context Group (Same, Different), and Dependent Variable (Cued Fear, Context Fear). Analyses revealed a significant effect of CS Type, $F(1, 108) = 18.859$, $P < 0.001$, indicating stronger fear memory retention for the CS+ relative to the CS− across groups. Consistent with the primary prediction, there was a significant interaction of CS Type × Context Group, $F(1, 108) = 9.158$, $P < 0.03$, indicating that differential fear was greater in the Same Context participants than the Context Shift participants. Finally, further supporting our primary hypothesis, there was a significant interaction of CS Type × Context Group × Dependent Variable, $F(1, 108) = 4.174$, $P < 0.05$. This three-way interaction signifies that Cued Fear was specific to the CS+ in the Same Context group, revealing contextually cued fear memory for this group only, whereas fear measured to the context during the ISI reflected a generalized fear memory across CS Type in both Same and Different Contexts (**Figure 4**). *Post hoc* Fischers PLSD confirmed that there was differential responding only to the Cue stimuli in the Same Context, (CS Type by Dependent Variable), $P < 0.01$. Importantly, Bonferroni–Dunn tests further revealed that responding to CS− was different by Dependent Variable and Context Group, $P < 0.02$, but not to the CS+, $P = 0.052$. This pattern suggests, as seen in **Figure 3**, that the Context served to reduce generalized fear to the non-reinforced stimulus (CS−).

DISCUSSION

Characterizing how environmental contexts guide the expression of acquired fears has important implications for understanding mechanisms that promote maintenance of anxiety disorders. The current study used fully immersive VR in a novel way to bridge animal models of contextual fear conditioning and real-world expression of human fears to biologically prepared stimuli. By simulating how fears are acquired and retained to dynamic snakes or spiders encountered in real-world settings, the present study extended prior human research on contextual fear conditioning (e.g., Baas et al., 2004; Kalisch et al., 2006; Alvarez et al., 2008) to reveal, for the first time, context-specific cued fear retention after a 24-h delay. In addition, the results indicate that context fear and US Expectancy occurred early in learning whereas differential cued fear became specified later in learning. The fear acquisition findings are consistent with empirical evidence in rodents and computational models demonstrating that the hippocampus

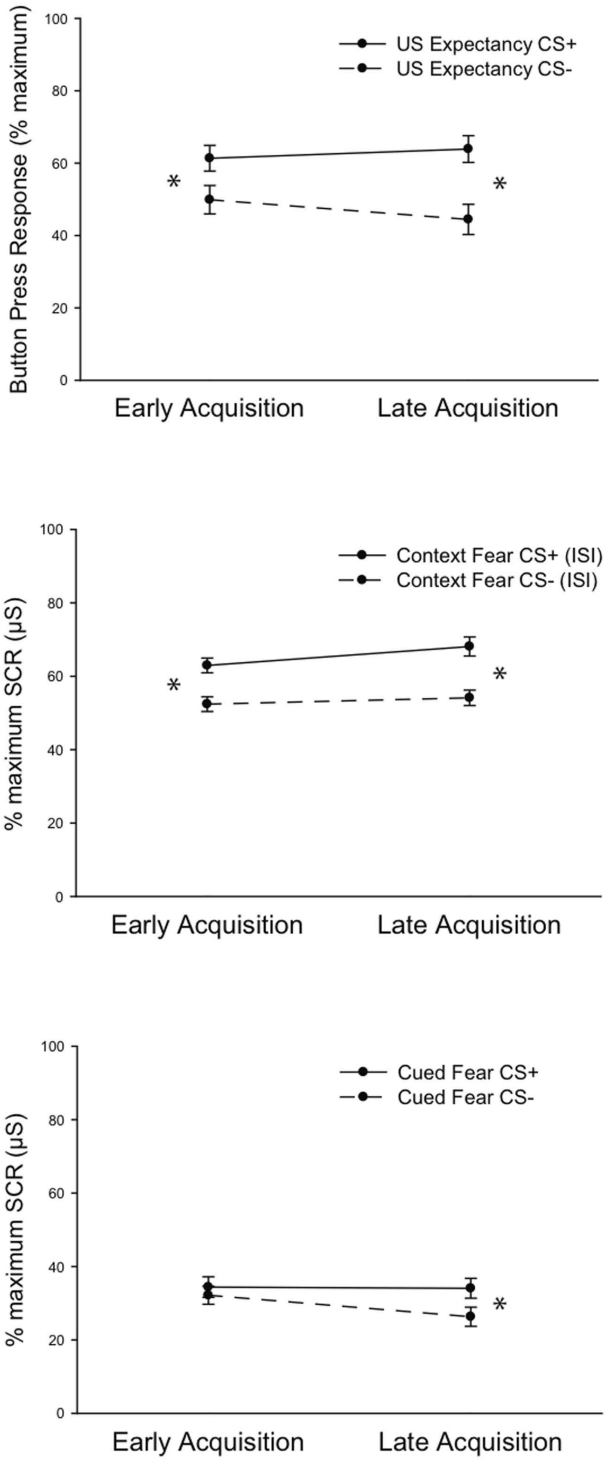

FIGURE 3 | Fear acquisition results. Data depict mean values (±SEM) across the three dependent measures all normalized to the maximum response on Day 1 when the differential fear contingency is initially learned. In early acquisition, differential fear is reflected in US Expectancy ratings (Top Panel) and Context Fear (mean skin conductance level during the interstimulus interval; Middle panel). Differential Cued Fear (skin conductance response to the CS+ relative to the CS−) emerges in late acquisition (Bottom Panel). *$P < 0.01$. SCR = skin conductance response.

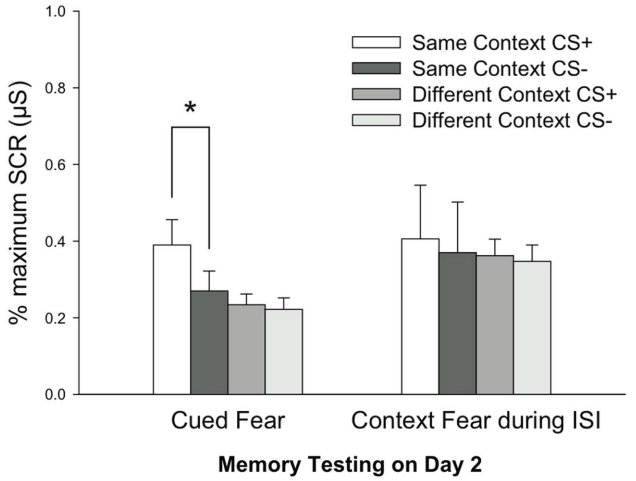

FIGURE 4 | Fear retention results. Data depict mean values (±SEM) across Contextually cued, Cued, and Context Fear measurements on Day 2 during retention testing trials. Data are normalized to the maximum response on Day 2. Fear was specified to the CS+ (relative to the CS−) in the Same Context group only, indicating significant contextually cued fear retention, $*P < 0.001$. These group differences were not due to differences in baseline Context Fear expressed during the interstimulus interval. SCR = skin conductance response.

rapidly and automatically stores a context memory (Rudy and O'Reilly, 2001; Rudy, 2009), and that medial temporal lobe activation to predictive CSs in humans emerges early during training (LaBar et al., 1998; Lang et al., 2009). In rodent studies, contextual conditioning can be more protracted than cue learning (e.g., LaBar and LeDoux, 1996); however, these studies rarely use differential training procedures for which the discrimination between the CS+ and CS− takes time to emerge. Altogether, the findings suggest that there is rapid short-term memory consolidation of context fear and US Expectancy early in learning whereas differential fear to the reinforced cue is slower to emerge but is strongly retained in long-term memory. The US Expectancy results should be treated with caution, given that the data included fewer participants than the other measures. Finally, by using multiple 3-D environmental contexts encountered in a fully immersive VR setting, the current study establishes feasibility of this innovative method for dissociating contextual and cued fear in humans that is more analogous to rodent paradigms of fear conditioning (Phillips and LeDoux, 1992; Rudy et al., 2004; Fanselow, 2010) and dynamic, real-world encounters of CS and reinforcers.

The context-dependent fear retention findings challenge the assumption that fear conditioning to a cue is not initially context-specific relative to extinction memories, and suggest that retrieval of either the fear or extinction memory is possible depending on the organism's state at the time of testing (e.g., Bouton, 2002, 2004; Bouton and Moody, 2004). From a theoretical perspective, the current results support the transformation view of memory storage, which posits that initial storage of an episodic event is context- and hippocampal-dependent and has a specific spatiotemporal representation (e.g., Gardiner and Java, 1991; Knowlton and Squire, 1995; Tunney and Bezzina, 2006; Wiltgen and Silva, 2007; Winocur et al., 2007). Furthermore, according to the two-process theory of contextual fear conditioning (O'Reilly and Rudy, 2001; Rudy et al., 2004), fear memories to the CSs are encoded in a conjunctive hippocampal – dependent manner that should yield better fear retention to the CS in the original context compared to a novel context that shares fewer features of the learning context. This perspective is also in accordance with the role of the hippocampus in pattern completion functions in that the degree of contextual feature similarity across acquisition and retention testing should cue pattern completion, yielding recovery of the original fear memory. The findings support increasing evidence in the rodent literature that context fear is initially specific (Biedenkapp and Rudy, 2007; Riccio and Joynes, 2007; Wiltgen and Silva, 2007; Winocur et al., 2007).

We suggest that the dearth of comparable findings in the human literature is due to weak context manipulations compared to those implemented in animal studies for which rats physically navigate multisensory environments. A previous study (Effting and Kindt, 2007) found greater verbal reports of US expectancy during extinction training in a group that remained in the same context compared to those who were shifted to a novel extinction context. However, this effect generalized to both the CS+ and CS−, and no physiological indices of differential fear retention were taken. Our prior study that examined differential SCR conditioning and retention to fear-relevant stimuli across 2 days of testing using a virtually identical paradigm failed to show context-specific retention effects when participants physically moved from one lab room to another (Huff et al., 2009). Using a 2-D VR fear conditioning paradigm with a head mounted display in a fMRI scanner, Alvarez et al. (2007) reported a slight loss of fear response (startle magnitude) in Context B as well as in the 24-h re-test in Context A, suggesting that flattened displays are not powerful enough to generate a lasting representation of Context A. It is possible that these kinds of laboratory manipulations that are commonly employed are not effective enough to engage a conjunctive representation, but rather the paradigms only supported a feature-based representation of the context (Rudy et al., 2004). The use of fully immersive 3-D VR environments appears to be more effective than standard laboratory context manipulations in generating robust contextual fear memory effects. Human participants can thus acquire and retain a strong contextual representation associated with a conditioned cue when provided with sufficient sensory input in an experimental setting that more closely simulates real-world experiences.

In light of generating translational research, it is important to determine why it is rare to find robust long-term contextually cued fear in humans whereas rodent studies readily demonstrate that fear to a conditioned cue is well remembered in the original context (e.g., Phillips and LeDoux, 1994; Corcoran and Maren, 2001, 2004; Maren and Chang, 2006). One difference is that rodent research tends to employ separate tests of cued and contextual fear. In many human studies, training parameters consist of presenting an unpaired shock US and CS in 2-D VR contexts so as to generate contextual fear in only one environment, or alternatively pairing shock with *either* a cue *or* a context (Baas et al., 2004; Alvarez et al., 2008; Marschner et al., 2008). The strength of the US may also

play a role in the emergence of conditioned responding (see Morris and Bouton, 2006), since the use of shock in human research is ethically limited to tolerance levels of the participant. However, across human studies electrical stimulation varies in strength, and even when normalized scoring is used to address individual differences in overall reactivity, context effects are minimal (e.g., Grillon, 2002; Huff et al., 2009). The rate of reinforcement also varies across studies as well as the type of CSs employed. The current paradigm used a partial reinforcement schedule and a multisensory fear-relevant CS, both of which may engage additional memory storage processes to create a stable context representation. Finally, the role of navigation in an environment should be considered. Whereas the rodent studies and the current immersive VR human study present the stimuli and reinforcers while participants navigate their environment, typical human studies require no navigation in the environment, and thus the use of navigation-based idiothetic cues and encoding of spatial relationships of the stimuli with respect to background context features is not necessarily undertaken. With these methodological issues taken into consideration, the use of rich full immersion VR contexts that can be manipulated across training phases may enhance engagement of the relevant neural circuitry (e.g., Marschner et al., 2008; Lang et al., 2009) to support long-term contextual and cued fear associations in humans.

Since evidence in rodents indicates that fear memory is initially context bound but becomes more generalized over time (Riccio et al., 1992; Biedenkapp and Rudy, 2007; Wiltgen and Silva, 2007), it would be important in future human studies to vary the retention interval between acquisition and extinction training to test different theoretical perspectives regarding mechanisms supporting remote fear memory. It will also be important to determine to what extent the findings presented here are specific to fear-relevant CSs or whether they generalize to stimuli that are not biologically prepared to be associated with aversive outcomes. In addition, humans can use higher-order cognitive processes to generalize from an emotional learning experience (Huff and LaBar, 2010) and the contribution of such generalization processes should be evaluated further. Finally, this novel VR paradigm could be used to determine whether anxiety disorders are characterized by less context-specificity of fear retention, even at short delays.

CONCLUSION

Together, data from the present study suggest that it is possible to evoke robust contextually cued fear retention in humans over 24 h with fully immersive VR. In summary the findings implicate that: (1) context fear learning occurs rapidly in humans, consistent with rodent findings, (2) in a rich environment, differential cued fear learning in humans is slower to occur than context fear but is retained in a context-specific manner 24 h after training, (3) contextually cued fear memory retention recently after learning supports the transformation view implicated in rodent memory research; and (4) stronger contextually cued fear retention than cue or context alone supports a conjunctive representation account of conditioning. Taken together, the findings indicate that putative hippocampal-dependent learning processes can be engaged by fear conditioning and memory retention testing using fully immersive 3-D VR. This study represents a paradigm shift in the way human Pavlovian fear conditioning may be implemented in future studies, with important applications for understanding how context effects on fear expression are dysregulated in anxiety disorders.

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The neurological ecology of fear: insights neuroscientists and ecologists have to offer one another

Michael Clinchy[1]*, Jay Schulkin[2], Liana Y. Zanette[3], Michael J. Sheriff[4], Patrick O. McGowan[5] and Rudy Boonstra[5]

[1] *Department of Biology, University of Victoria, Victoria, BC, Canada*
[2] *Department of Neuroscience, Georgetown University, Washington, DC, USA*
[3] *Department of Biology, University of Western Ontario, London, ON, Canada*
[4] *Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK, USA*
[5] *Centre for the Neurobiology of Stress, University of Toronto at Scarborough, Toronto, ON, Canada*

Edited by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Luke R. Johnson, Uniformed Services University of the Health Sciences, USA
June-Seek Choi, Korea University, South Korea

***Correspondence:**
Michael Clinchy, Department of Biology, University of Victoria, PO Box 3020 STN CSC, Victoria, BC, Canada V8W 3N5.
e-mail: mclinchy@uvic.ca

That the fear and stress of life-threatening experiences can leave an indelible trace on the brain is most clearly exemplified by post-traumatic stress disorder (PTSD). Many researchers studying the animal model of PTSD have adopted utilizing exposure to a predator as a life-threatening psychological stressor, to emulate the experience in humans, and the resulting body of literature has demonstrated numerous long-lasting neurological effects paralleling those in PTSD patients. Even though much more extreme, predator-induced fear and stress in animals in the wild was, until the 1990s, not thought to have any lasting effects, whereas recent experiments have demonstrated that the effects on free-living animals are sufficiently long-lasting to even affect reproduction, though the lasting neurological effects remain unexplored. We suggest neuroscientists and ecologists both have much to gain from collaborating in studying the neurological effects of predator-induced fear and stress in animals in the wild. We outline the approaches taken in the lab that appear most readily translatable to the field, and detail the advantages that studying animals in the wild can offer researchers investigating the "predator model of PTSD."

Keywords: animal model of PTSD, indirect predator effects, post-traumatic stress disorder, predation risk, predator stress

INTRODUCTION

Post-traumatic stress disorder (PTSD) represents arguably the most salient example of how fear and stress shape the mind. Because controlled prospective studies cannot be conducted on humans, it is necessary to use an "animal model" to help elucidate the etiology of PTSD and explore the associated neurological changes (Cohen et al., 2010). A suitable animal model should utilize stressors that emulate as closely as possible the relevant stressors in humans; the behavioral, physiological, and neurological responses elicited in the animal must reflect clinical symptomatology; and pharmacological agents known to affect symptoms in human patients should correct, with equal efficacy, comparable symptoms in the animal (Rosen and Schulkin, 1998; Roseboom et al., 2007; Stam, 2007; Armario et al., 2008; Masini et al., 2009; Mitra et al., 2009; Cohen et al., 2010).

Many researchers have adopted utilizing exposure to a predator (e.g., showing a rat a cat; Adamec and Shallow, 1993), or predator odor, as a stressor, in exploring the animal model of PTSD (Cohen et al., 2010; Mackenzie et al., 2010). Predator exposure was initially seized upon for practical reasons as this permits the researcher to utilize a (1) psychological stressor, that is (2) life-threatening, but (3) does not involve pain; all consistent with the etiology of PTSD in humans (Adamec and Shallow, 1993; Roseboom et al., 2007; Campeau et al., 2008; Takahashi et al., 2008; Staples et al., 2009; Cohen et al., 2010; Mackenzie et al., 2010). Of greatest importance with respect to understanding PTSD, the hallmark of which is the long-lasting or "transformational" change in the patient in response to a trauma (Yehuda and Bierer, 2009), predator exposure has been demonstrated to have long-lasting effects on: anxiety-like behaviors, glucocorticoid levels, dendritic morphology, gene expression, and the release of the neuropeptide corticotrophin-releasing hormone (CRH) in the amygdala (the region of the brain most frequently linked to fear), as well as many other phenomena associated with PTSD (Adamec and Shallow, 1993; Schulkin et al., 2005; Roseboom et al., 2007; Stam, 2007; Armario et al., 2008; Campeau et al., 2008; Rosen et al., 2008; Takahashi et al., 2008; Masini et al., 2009; Mitra et al., 2009; Staples et al., 2009). Predator exposure early in life has also been shown to increase vulnerability to developing subsequent long-term behavioral disruptions when exposed to a predator in adulthood (Cohen et al., 2006), consistent with the growing evidence that individual variation in susceptibility to PTSD is influenced by early-life experiences (Yehuda and Bierer, 2009).

Researchers studying the "predator model of PTSD" have increasingly begun to suggest that predator exposure offers an additional advantage in attempting to understand PTSD, because long-lasting predator-induced fear and stress is ethologically and ecologically relevant, and represents a valid experience applicable to animals in their natural environment (Roseboom et al., 2007; Cantor, 2009; Staples et al., 2009; Cohen et al., 2010). Independently, wildlife ecologists have begun to arrive at a similar conclusion, following a line of inquiry that began in the 1990s (Creel and Christianson, 2008). Traditionally, the view of both wildlife ecologists and comparative endocrinologists has been that

predator-induced fear and stress is necessarily acute and transitory: the prey detects a predator; freezes, flees, or fights; survives or does not; the event is over; the animal returns to going about its business; homeostasis is restored (Schulkin, 2003; Sheriff et al., 2009). According to this traditional view, lasting effects are necessarily maladaptive and pathological: since the evolutionary "function" of predator-induced fear and stress is to ensure immediate survival, any further or lasting effect on fitness (i.e., Darwinian fitness), such as an effect on subsequent reproduction, must be maladaptive; and since the "function" of the stress axis is to maintain homeostasis, chronic stress must be pathological (Lupien et al., 2009; Rodrigues et al., 2009; Sheriff et al., 2009). Given this traditional view, the many lasting effects of predator exposure documented by researchers exploring the predator model of PTSD must be an artifact. The most parsimonious explanation being – given this perspective – that such lasting effects stem from the unnatural conditions of captivity, i.e., it is not the fact of predator exposure but the fact the predator is inescapable that must explain these effects, since the animal cannot flee from the predator as it naturally would (Creel et al., 2009; Jöngren et al., 2010).

We propose that the traditional view in wildlife ecology and comparative endocrinology, that the effects of predators on free-living animals are necessarily transitory, is no longer tenable, since the results from a growing number of experimental and observational field studies show that predator-induced fear and stress has long-lasting effects on animals in the wild (Creel and Christianson, 2008; Hawlena and Schmitz, 2010), comparable to those documented by investigators addressing the predator model of PTSD. For animals in the wild that are in peril every moment of every day of being torn limb from limb by any number of predators, responses resembling PTSD in humans may result from necessary trade-offs to stay alive, that are fully adaptive, because dead animals do not reproduce. We suggest that for both, researchers studying the predator model of PTSD, and ecologists, conducting collaborative studies on predator-induced fear and stress on animals in the wild would be of enormous benefit. For investigators addressing the predator model of PTSD, the extremity of the stressors faced by animals in the wild, in a real world context, would appear to much better emulate the circumstances leading to PTSD in humans. For ecologists, building upon the progress that has been made in understanding PTSD in the lab provides the most expedient means of addressing the mechanisms underlying predator-induced fear and stress effects in the field. We briefly review approaches taken to studying PTSD in the lab that appear translatable to the field; and then describe recent field studies on songbirds and snowshoe hares showing that, predator-induced fear and stress affects reproduction in animals in the wild, and the physiological responses involved appear comparable to those documented in response to predator exposure in the lab.

APPROACHES TRANSLATABLE TO THE FIELD

Behavioral responses to predator exposure in the lab include avoidance, reduced activity and increased vigilance (Blanchard and Blanchard, 1989; Stam, 2007; Armario et al., 2008; Takahashi et al., 2008), and similar responses to predator exposure have been exhaustively documented in the field since at least Darwin's time (Caro, 2005). Predator exposure in the lab results in changes in plasma glucocorticoid levels (Blanchard et al., 1998; Roseboom et al., 2007; Takahashi et al., 2008; Masini et al., 2009) and the same has been shown in both birds and mammals in the field (Hawlena and Schmitz, 2010). Measuring glucocorticoid metabolites in feces provides a new, non-invasive means of assessing glucocorticoid responses to predator exposure that is particularly useful in field studies (Sheriff et al., 2009, 2010).

Studying the neurological effects of predator-induced fear and stress in animals in the wild will likely rely primarily on destructive sampling. Though effects on live animals could be studied using pharmacological methods or neuroimaging, there are practical difficulties translating these approaches to the field. The suitability of using predator exposure in exploring the animal model of PTSD has been validated, in part, by the numerous studies showing that pharmacological agents known to affect symptoms of PTSD in human patients also correct comparable symptoms in animals exposed to predators (Cohen et al., 2006, 2010; Stam, 2007; Armario et al., 2008; Nanda et al., 2008). Some of these pharmacological agents can be administered in food (e.g., antalarmin; Zoumakis et al., 2006; Armario et al., 2008), which is of practical advantage for use with free-living animals since it is then not necessary to capture the subject to administer the drug. The principal constraint on using pharmacological agents on animals in the wild is almost certain to be the cost of the drugs, since the intrinsically greater error variation associated with studying any phenomenon in the field necessitates a larger sample size than that required in the lab.

A number of recent neuroimaging studies using magnetic resonance imaging (MRI) have evaluated the neurological effects of exposure to predator odor in lab rats (e.g., Chen et al., 2007; Febo et al., 2009; Huang et al., 2011). MRI has also been used to assess neuroactivity in response to other stimuli in mice and songbirds (Van der Linden et al., 2007). Neuroimaging holds enormous promise as a technique for studying effects on animals in the wild because, being non-destructive, subjects could be returned to the field to determine if differences in brain activity predicted their subsequent behavior and reproduction. However, though MRI is non-destructive it is necessarily invasive and may be very injurious depending upon the method used (e.g., the manganese used in manganese-enhanced MRI is potentially toxic; Silva et al., 2004). At a minimum, neuroimaging requires restraining the subject's head in a scanner for a protracted period. To measure effects in conscious animals requires acclimation to being restrained in this manner, which takes several days in laboratory animals (King et al., 2005), and may be unachievable in many wild-caught animals. Even if anesthetized during the procedure, the trauma of capturing a wild animal and transporting it to wherever the scanner is might render the results uninterpretable (Van der Linden et al., 2007). Nonetheless, we strongly recommend that using neuroimaging to study effects on animals in the wild should at least be attempted.

Because animals in the wild are generally challenging to capture, and limited in number, it is critical to maximize the information extracted from every animal euthanized. Moreover, because free-living animals must be captured, the conditions of capture will vary, meaning the rate at which tissue can be obtained will vary, and the circumstances will often be less than ideal. Measures that respond to an acute trauma or perturbation, such as the trauma of capture, will be largely unsuitable. Several new approaches to

measuring neurological effects, developed in the lab, nonetheless appear amenable to use on animals in the wild, even given these constraints.

Immunohistochemistry has been used to map the expression of genes in response to predator exposure in various brain regions that appear central to the phenomenon of fear (such as the medial amygdala). Whereas a number of lab studies have mapped the expression of the immediate-early gene c-*fos*, in response to predator exposure (Dielenberg et al., 2001; Roseboom et al., 2007; Campeau et al., 2008), c-*fos* is rapidly expressed (within <1 h; Armario et al., 2008) and rapidly down-regulated (Staples et al., 2009), which is problematic for use in the field. Two recent studies (Staples et al., 2009; Mackenzie et al., 2010) have mapped the expression of *fos*B and its protein products FosB/ΔFosB, as an alternative to mapping c-*fos*. ΔFosB can persist in the brain for weeks after chronic stimulus exposure (McClung et al., 2004), and Staples et al. (2009) reported that FosB/ΔFosB expression remained elevated 7 days after repeated predator exposure, making this a much more suitable marker for use in field studies.

Global gene expression has been assessed in response to predator exposure using cDNA microarrays (gene chips) in rats and chickens. Roseboom et al. (2007) euthanized rats 3 h after predator exposure, and found increased CRH-binding protein gene expression in the amygdala, consistent with previous studies (Schulkin et al., 2005). Jöngren et al. (2010) euthanized chickens 2 week after predator exposure and identified 13 significantly differentially expressed genes in the midbrain. Roseboom et al.'s (2007) findings confirm that cDNA microarrays can be used to identify the expression of genes expected to be upregulated in response to fear, and Jöngren et al.'s (2010) study shows that this approach can be used to detect long-lasting effects, even in non-mammalian subjects.

Quantifying dendritic morphology appears ideally suited for indentifying individual variation in susceptibility to predator-induced fear and stress in field studies, and may be useful in evaluating predator-induced changes in neural architecture. Mitra et al. (2009) evaluated behavioral differences in subjects 2 weeks after predator exposure and found differences in the architecture of the neurons in the basolateral amygdala. Total dendritic length, dendritic extent, and total branch points were all greater in individuals that continued to demonstrate anxiety-like behaviors as compared to those that no longer showed anxiety-like symptoms. Though the design of Mitra et al.'s (2009) study did not allow them to determine whether these differences in dendritic morphology were pre-existing or induced by predator exposure, Mitra and Sapolsky (2008) reported changes in dendritic morphology in response to a single day of stress, suggesting that predator-induced fear could indeed induce such changes in neural architecture.

Yehuda and Bierer (2009) recently reviewed the potential role of epigenetic changes in the etiology of individual differences in susceptibility to PTSD. Epigenetic modifications involve long-lasting, often environmentally induced, changes in gene expression and function, that can be inter-generationally transmissible (i.e., heritable), though the DNA sequence itself remains unchanged (Champagne and Curley, 2009; Yehuda and Bierer, 2009). Several lines of evidence point to epigenetic changes as potentially being involved in predisposing individuals to PTSD, including the association of PTSD risk with maternal PTSD, the relevance of childhood adversity to the development of PTSD, and recent evidence of a relationship between childhood abuse, DNA methylation (in gene promoters, an epigenetic marker of gene silencing) and suicide (McGowan et al., 2008, 2009; Yehuda and Bierer, 2009). As noted above, Cohen et al. (2006) reported that early-life predator exposure increased vulnerability to behavioral disruptions in response to exposure in adulthood, though there have been no studies looking specifically at predator-induced epigenetic changes. In the aforementioned suicide study, subjects had been dead an average of 24 h before sampling, suggesting that changes in DNA methylation ought to be detectable in the brains of animals in the wild collected under less than ideal field conditions, as recently corroborated by Pilsner et al. (2010) in a study that examined DNA methylation in the brains of polar bears shot by aboriginal hunters in eastern Greenland.

FIELD STUDIES SHOWING LONG-LASTING EFFECTS OF PREDATOR EXPOSURE

Evolutionarily, the "function" of staying alive is to reproduce, i.e., to transmit genes to the next generation. For ecologists, reproduction is the "currency" that matters. Ecological factors such as food and parasites, with obvious long-lasting effects (malnutrition and disease), have always been considered to be those most likely to affect reproduction, because reproduction (giving birth and rearing young) is a slow process. Traditionally, predators have not been thought to affect reproduction because predator-induced fear and stress has been considered to be far too acute and transitory. Behavioral (e.g., Kotler, 1992) and physiological (e.g., Boonstra et al., 1998) studies began, in the 1990s, to suggest that predator-induced fear and stress could have lasting effects on animals in the wild, but because of the logistical challenges involved the critical experiments necessary to demonstrate effects on reproduction have only very recently been conducted. The principal challenge concerns space. Free-living animals can, and do, simply flee or avoid, a predator in a cage, predator models, or predator odor stations (e.g., Stankowich and Blumstein, 2005). Because sound travels, and thus occupies space, field studies often use playbacks of recorded predator calls and sounds to investigate effects of predator exposure. Moreover, for organisms that rely more on sound and sight than smell, such as birds and humans, auditory stimuli are generally more meaningful than olfactory ones (Jarvis, 2004), and acoustic cues may frequently be more alarming than visual ones (Cohen et al., 2010).

Only one study to date on a bird or a mammal has, to our knowledge, exposed free-living prey to increased predator cues in the field, and demonstrated a resulting effect on the number of offspring produced per year. Zanette et al. (submitted) used an array of speakers spaced over several hectares to expose nesting female song sparrows to playbacks of either predator calls and sounds, or non-threatening calls and sounds. Females exposed to elevated predation threat produced almost 40% fewer offspring than controls (3.8 ± 0.4 vs. 6.0 ± 0.4, mean $\pm$ SE), over the 4-month breeding season, because they laid fewer eggs, fewer of their eggs hatched, and more of their chicks starved to death. These effects on reproduction were most likely mediated in part by predator-threat-induced changes in glucocorticoid levels, because work on the same study populations has

demonstrated lasting effects on glucocorticoid levels associated with variation in predator abundance (Clinchy et al., 2004, 2011), and the probability of suffering nest predation (Travers et al., 2010).

Sheriff et al. (2009) recently reported correlative results suggesting that predator exposure affects glucocorticoid levels and reproduction in free-living snowshoe hares, consistent with the results from Zanette et al.'s (submitted) experiment. To corroborate their findings, Sheriff et al. (2009) presented a live predator (a trained dog) to pregnant hares housed in 4 m × 4 m outdoor pens, and demonstrated that predator-exposed females had dramatically elevated fecal glucocorticoid metabolite (FCM) levels, and were significantly less likely to give birth to live young.

In a subsequent study on snowshoe hares, Sheriff et al. (2010) showed that predator exposure may have very long-lasting effects on animals in the wild, extending from one generation to the next. Sheriff et al. (2010) reported that at a population level, predator exposure, mean maternal FCM levels, and mean juvenile FCM levels, were all correlated, suggesting that predator-induced glucocorticoid changes in mother hares affect their offspring's glucocorticoid levels. To corroborate these findings, Sheriff et al. (2010) measured FCM levels in pregnant hares, housed in 4 m × 4 m outdoor pens, and demonstrated that each mother's FCM level was highly correlated with her offspring's glucocorticoid responses to a hormonal challenge, when the latter was 28 days old. Thus, in animals in the wild, maternal or early-life exposure to predators may increase responsiveness to predators later in life, consistent with Cohen et al.'s (2006) lab results demonstrating that early-life predator exposure increases vulnerability to behavioral disruptions when exposed to a predator in adulthood.

Life-long maternal effects on the glucocorticoid responsiveness of their offspring, resembling the results shown by Sheriff et al. (2010), have been well-studied in relation to stress effects on maternal care in laboratory rodents, and have been shown to be associated with DNA methylation of genes affecting glucocorticoid receptor function in the hippocampus (Weaver et al., 2004; Kappeler and Meaney, 2010). In an example of the kind of collaboration between neuroscientists and ecologists we are herein hoping to encourage, McGowan and Boonstra are currently examining the brains of juvenile snowshoe hares, collected in the field, whose mothers were subject to naturally varying levels of predator exposure, to test if maternal predator exposure affects DNA methylation in their offspring's hippocampus in a manner similar to the way in which childhood abuse evidently affects DNA methylation in humans, as shown in the aforementioned suicide study (McGowan et al., 2008, 2009).

Calisi and Bentley (2009) recently proposed that studying neurobiology and behavior in semi-natural settings may provide a means to merge lab and field approaches. Our focus here is on the lasting effects of predator-induced fear and stress on neurobiology and ecology. As noted above, the principal challenge in studying such lasting ecological effects concerns space, and this applies equally to studying such effects in a semi-natural setting – the subject must have the same amount of space available as it would if it were free-living, to flee or avoid a predator, otherwise any effects seen could be attributed to the unnatural conditions of captivity (Creel et al., 2009). Sheriff et al.'s (2009, 2010) exposure of caged hares to a predator, for example, cannot be considered definitive, for this reason (Clinchy et al., 2011). Moreover, since, as noted above, the ecological "currency" that matters is reproduction, the subject must be able to reproduce as it naturally would. Very large (e.g., several hundred square meter) outdoor enclosures may fulfill these requirements when studying very small animals (e.g., mice or songbirds), whereas housing an animal in a somewhat larger cage than usual in an animal care building (e.g., Blanchard and Blanchard, 1989; Choi and Kim, 2010) does not meet these criteria.

The scope for future collaborations between neuroscientists and ecologists will almost certainly involve studying many more species than just sparrows and hares. As further field experiments on the effects of predator exposure on reproduction are conducted, we have no doubt such effects will be found to be common. Effects on components of reproductive success have already been documented in experiments on several other species. Eggers et al. (2006) reported effects of predator call playbacks on the number of eggs laid by Siberian jays, and Fontaine and Martin (2006) found that where predators were removed songbirds laid heavier eggs. Karels et al. (2000) similarly showed that where predators were removed the proportion of arctic ground squirrel females weaning young was increased. Lasting behavioral and physiological effects pointing to likely effects on reproduction have been shown in an even larger number of species (Creel and Christianson, 2008; Hawlena and Schmitz, 2010). What effect such predator-induced fear and stress has in shaping the minds of free-living birds and mammals is a question that is almost completely unexplored, presenting a wide-open field of study replete with opportunities for new discoveries.

CONCLUSION

Numerous laboratory experiments have shown that predator-induced fear and stress has lasting neurological effects, and wildlife ecologists have begun demonstrating that predator-induced fear and stress has lasting effects on reproduction in free-living animals in the field. We propose that the next two critical questions to answer are: (1) whether predator-induced fear and stress has lasting neurological effects on free-living animals, and if so; (2) which of the effects seen in the lab appear most frequently in wild animals in the field. The insights neuroscientists have to offer ecologists in exploring the effects of predator-induced fear and stress on the minds of wild animals in the field include, where to begin, and what to measure. The insights ecologists, in turn, have to offer researchers studying the predator model of PTSD include, establishing which effects seen in the lab are observed in the greatest number of species and circumstances, and which are most biologically meaningful as gaged by their association with effects on reproduction. We suggest that if, as the predator model assumes, PTSD in humans has evolutionary precursors, then it is virtually certain that collaborations between neuroscientists and ecologists will greatly enhance our understanding of the etiology of PTSD and the associated neurological changes.

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The importance of reporting housing and husbandry in rat research

Eric M. Prager[1], Hadley C. Bergstrom[2], Neil E. Grunberg[1,3,4] and Luke R. Johnson[1,2,3,5]*

[1] *Program in Neuroscience, Uniformed Services University, Bethesda, MD, USA*
[2] *Department of Psychiatry, Uniformed Services University, Bethesda, MD, USA*
[3] *Center for Neuroscience and Regenerative Medicine, Uniformed Services University, Bethesda, MD, USA*
[4] *Department of Medical and Clinical Psychology, Uniformed Services University, Bethesda, MD, USA*
[5] *Center for the Study of Traumatic Stress, Uniformed Services University, Bethesda, MD, USA*
**Correspondence: eric.prager@usuhs.mil*

In 1963, the National Institutes of Health (NIH) first issued guidelines for animal housing and husbandry. The most recent 2010 revision emphasizes animal care "in ways judged to be scientifically, technically, and humanely appropriate" (National Institutes of Health, 2010, p. XIII). The goal of these guidelines is to ensure humanitarian treatment of animals and to optimize the quality of research. Although these animal care guidelines cover a substantial amount of information regarding animal housing and husbandry, researchers generally do not report all these variables (see **Table 1**). The importance of housing and husbandry conditions with respect to standardization across different research laboratories has been debated previously (Crabbe et al., 1999; Van Der Staay and Steckler, 2002; Wahlsten et al., 2003; Wolfer et al., 2004; Van Der Staay, 2006; Richter et al., 2010, 2011). This paper focuses on several animal husbandry and housing issues that are particularly relevant to stress responses in rats, including transportation, handling, cage changing, housing conditions, light levels and the light–dark cycle. We argue that these key animal housing and husbandry variables should be reported in greater detail in an effort to raise awareness about extraneous experimental variables, especially those that have the potential to interact with the stress response.

Rats used in scientific research are usually transported from a breeder to an institution's animal housing facility prior to experimentation. NIH guidelines on animal care do not provide standards on the duration of time to allow for acclimation to the new colony prior to experimentation, even though transportation can be stressful (Van Ruiven et al., 1998; Capdevila et al., 2007). Transporting rats [male Sprague-Dawley (SD), 175–200 g] for 5 h to a new facility has been found to decrease body weight, decrease overall activity levels, and increase heart rate (HR) for up to four days after transportation (Capdevila et al., 2007). Blood corticosterone (CORT), a physiological indicator of the stress response, has also been reported to be significantly lower in male and female Wistar rats 1 day after a prolonged (15 h) transport compared to control animals, but returns to pre-transport values 3 days after arrival in a new environment (Van Ruiven et al., 1998). Therefore, in agreement with Van Ruiven and colleagues, 3–4 days should be a sufficient period for acclimation after which rats' stress parameters return to pre-transport levels.

Although not a component of NIH guidelines for housing and husbandry, most researchers "gentle" or "handle" rats prior to experimentation with the intention of habituating them to human contact, thereby decreasing stress responses. However, evidence to the contrary indicates that handling induces a rapid and significant elevation of physiological stress responses in rats that may persist for 30–60 min or longer (Black et al., 1964; Sharp et al., 2002a,b, 2003; Balcombe et al., 2004). Handling male SD rats for 20 consecutive days significantly increases mean HR, blood pressure, and serum CORT concentrations (Armario et al., 1986a,b; Balcombe et al., 2004). The persisting CORT response after the initial handling may affect performance in subsequent behavioral tests (Brown and Martin, 1974). Interestingly, rats show minimal habituation to these physiological markers of stress (Balcombe et al., 2004). Therefore, daily handling may not reduce stress as commonly thought, but instead, may actually work to increase the stress response. Although it is difficult to estimate a precise timeframe for testing after daily handling, the data cited above, as well as other experimental data (see Sapolsky et al., 1984; Flores et al., 1990) suggest that at least 30–60 min should elapse before conducting stress-sensitive procedures. Given this caveat, we recommended that authors report latency from handling to procedure and consider all handling in their experimental design.

National Institutes of Health guidelines indicate that cages should be changed as often as necessary to ensure that animals are clean and dry, but that cleaning frequency is a matter of the judgment of animal care personnel (National Institutes of Health, 2010, p. 75). Empirical evidence suggests that excessive cage changes may be stressful to rats (Kacergis et al., 1996; Thulin et al., 2002; Balcombe et al., 2004; Burn et al., 2006). Cage cleaning has been linked with increases in cardiovascular parameters and general activity in male SD and Wistar rats (Saibaba et al., 1996; Schnecko et al., 1998; Doerning, 1999; Duke et al., 2001; Burn et al., 2006). Burn et al. (2006) examined the effects of cage cleaning (twice weekly, weekly, or biweekly) across two commonly used rat strains (Wistar and SD). Rats with cage cleanings biweekly displayed fewer defensive behaviors (i.e., biting and audible vocalizations) and struggled less during handling than did rats with cages cleaned weekly or twice weekly. In contrast, it took longer for anxiety-like behaviors to return to pre-stress levels in rats that had cages cleaned less often. Because cage changing may affect behavioral and biological stress responses, it is important for investigators to include this information in experimental reports and to be consistent in frequency of cage changing among treatment groups. Investigators should consider biweekly cage cleaning, if possible, or no more than weekly, if necessary.

Numbers of animals per cage, size of cages, and presence or absence of physical enrichment affect stress responses

Table 1 | Animal housing and husbandry reporting in scientific articles.

Journal	Age/ weight	Light cycle	Light lux	Room temp.	Room humidity	Transport duration	Cage cleaning	Handling	Animals per cage	Cage size	Phys. enrich	Acclimate to facility
Behavior Genetics	70	90	10	80	40	0	10	10	70	20	10	10
Behavioral Neuroscience	100	80	0	30*	20*	0	0	20***	60**	0	0	30
Behavioural Brain Research	50	90	0	50	20	0	10	30***	80**	20	10	10
Biological Psychiatry	80	50	0	10	10	0	0	10	30**	0	0	0
European Journal of Neuroscience	70	50	0	10	0	0	0	0	30**	0	0	0
Frontiers in Behavioral Neuroscience	70	90	0	40*	40*	0	0	0	90**	20	10	30
Neurobiology of Learning and Memory	90	90	0	60	0	0	0	20***	50	20	0	30****
Pharmacology Biochemistry and Behavior	100	90	0	50*	10	0	0	0	80**	20	0	20
Physiology and Behavior	100	90	0	60	20	0	0	10	60**	40	0	0
Psychopharmacology	90	80	0	50*	40*	0	0	0	80	10	10	20
Total %	82	80	1	44	20	0	2	10	63	15	4	15

One hundred articles published in 2010 were chosen, using a random number generator, from 10 different journals that publish scientific papers in the field of behavioral neuroscience. The total percentage of articles reporting the specified housing and husbandry procedures are listed.

**Articles stated that climate was controlled but did not provide temperature/humidity.*

***Articles varied in group housing numbers within experiments (e.g., 2–3 per cage) or stated animals were "group housed" but did not give number.*

****Daily handling but did not specify how many days or duration of handling.*

*****Acclimation to facility was provided, but did not specifically state how many days.*

and a variety of behaviors. This point was reported in the classic work of psychologists Mark Rosenzweig, Marian Diamond, and colleagues who pioneered studies of behavioral and biological effects of enriched environments (Rosenzweig et al., 1962, 1967; Diamond et al., 1972, 1976; Bennett et al., 1974). Socially housing rats ("social enrichment") decreases fearfulness, improves cognitive activities (Hatch et al., 1963; Johnson et al., 1972; Morgan and Einon, 1975; Patterson-Kane et al., 2004), increases locomotor activities (Elliott and Grunberg, 2005; Kim et al., 2007), and shortens recovery after intrusive surgeries (Gornicka-Pawlak et al., 2009). However, space and sex also must be considered. For instance, whereas cage crowding was associated with higher plasma CORT in males, crowded females showed lower CORT (Elliott and Grunberg, 2005). Crowding may induce fighting (especially among males) which may result in increased physiological reactivity. If enough space is available to balance the needs of the rat including sanitation, physical contact, and motor activity, then rats should be housed in groups of two or more (Patterson-Kane et al., 2004), unless the experiment requires individual housing. Group housing in appropriately spaced cages allows for increased socialization and results in a significant decrease in mean arterial blood pressure and HR compared with isolated male and female SD rats (Sharp et al., 2002a,b, 2003). At times, experimental protocols require social isolation. Isolating rats may potentiate the effects of stress as some rats (e.g., male Wistar rats) have been shown to exhibit increased behavioral abnormalities including hyperactivity (Gornicka-Pawlak et al., 2009) and increased substance P levels in the dorsal periaqueductal gray, a midbrain region involved in aversion behavior, pain regulation and the fear response (Brodin et al., 1994). If isolation is necessary, then the use of a cage divider may be employed, which allows for social interactions and enhanced social enrichment without compromising isolation needs (Boggiano et al., 2008). Toys in cages (or "physical enrichment") also can affect behavioral performance in rats (e.g., Elliott and Grunberg, 2005) and should also be considered and reported. It is important to note here that single housing has not consistently been shown to increase stress response, especially in mice (Reber and Neumann, 2008; Singewald et al., 2009).

Light luminance in housing facilities is another variable that has a marked influence on the physiology, circadian rhythm, and behavior of rats (Tucker et al., 1984; Azar et al., 2008). Compared with SD rats housed in a standard 12:12 light–dark cycle with normal illumination (200 lux), the HR of undisturbed male (but not female) rats was decreased under dim lights (10 lux) during a 12:12 photocycle. Increasing the dark cycle to 16 h (8:16 photocycle) under normal light conditions (200 lux) decreased the HR of undisturbed males. Changing the light cycle to be more species specific (e.g., dim light or longer dark period in rats) reduced HR during periods of day and night when rats were left undisturbed (Tucker et al., 1984; Azar et al., 2008). However, neither dim light nor long nights affected HR responses during experimentation (Azar et al., 2008). Behavioral research is commonly carried out during the light phase, an approach that is ethologically incorrect in nocturnal animals. Reverse light cycle allows for measurements during rats' active period, whereas direct light cycles result in measurements during rats' inactive period. Although existing data on the impact of testing phase on the stress response is limited, investigators should consider and report illumination levels, light cycles, and lighting conditions when taking behavioral and biological measurements.

The data reviewed here suggests that rat transport, handling, cage changing, housing conditions, light levels, and the light–dark cycle all have the potential to interact with the stress response. However, these interactions may not always be easily transferred to other rodent models. Therefore we recommend documenting in detail all housing and husbandry procedures as part of standard experimental reporting, so that informed comparisons of experimental results can be made across different laboratories.

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