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# UNDERSTANDING STRESS RESILIENCE

Topic Editors
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Michael V. Baratta and Steven F. Maier

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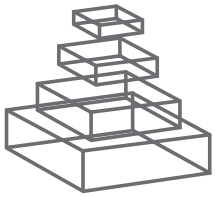

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# UNDERSTANDING STRESS RESILIENCE

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Traumatic and chronic stressors can impact brain structure and function and are considered primary sources of risk for depression, anxiety, and other psychiatric disorders. Interestingly, the majority of individuals who encounter stressful life events do not develop untoward outcomes. As successful adaptation relies on an organism's effective response to environmental and homeostatic challenges, a greater understanding of the factors that promote resistance to the deleterious effects of stress is of great clinical relevance. This Research Topic focuses on advances in understanding how genetic and experiential factors mitigate the consequences of stressful events, and the neural mechanisms that mediate their effects. Additionally, this Research Topic seeks to highlight recent efforts to identify conditions during an initial stress experience that can alter an organism's response to aversive events later in life.

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# Understanding stress resilience

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**Keywords: resilience, stress, trauma, coping, depression, anxiety, posttraumatic stress disorder, neuroimaging**

Adverse events can impact brain structure and function and are considered primary sources of risk for depression, anxiety, and other psychiatric disorders. However, the majority of individuals who encounter adverse or stressful life events do not develop untoward outcomes, and so an understanding of the factors that promote resistance to the deleterious effects of stress is of clinical importance. At the level of basic research, there has been considerable effort directed at identifying experimental parameters that blunt/augment outcomes from an adverse event, but even when parameters are held constant, there is inter-subject heterogeneity in behavior. This has shifted the focus to understanding how genetic and experiential factors can shape an organism's resistance to future adversity. The articles collected in the present Research Topic provide an overview of recent efforts directed at elucidating the neural mechanisms underlying resilience, and utilizing such information to mitigate vulnerability.

Achieving genetic, epigenetic, and neural circuit-level insight into the causal mechanisms underlying stress resilience has come from a variety of disciplinary approaches. Wu et al. (2013) lead this special issue by providing a comprehensive overview of recent progress in each of these units of analysis. At the human level, much of what is known about the biological determinants of resilience has increasingly come from neuroimaging studies. In this issue van der Werff et al. (2013) examine the structural and functional alterations related to resilience, particularly contrasting findings of individuals who either have, or have not, developed posttraumatic stress disorder (PTSD) following trauma.

One of the consequences of long-term stress exposure is to disrupt processes involved in successful adaptation to environmental threat. The endogenous opioid peptide, β-endorphin, has been shown to facilitate recovery following stress and here Barfield et al. (2013) directly investigate its role in anxiety-like behaviors using transgenic mice with varying capacities to synthesize the peptide. Vander Weele et al. (2013) examine the contribution of another endogenous peptide, growth hormone (GH), in hippocampal dysfunction following prolonged stress. The authors demonstrate that chronic stress regimens reduce hippocampal GH and that stress-induced hippocampal-dependent learning impairments are restored following site-specific viral-mediated overexpression of GH. Their findings implicate GH signaling in the hippocampus as a novel target for promoting resilience following prolonged stress regimens.

Environmental conditions during development also contribute to the heterogeneity of an individual's response to adversity encountered as an adult. As Macrì (2013) outlines, in an opinion piece, developmental conditions may be harnessed to promote resilience in experimental settings. One such experiential factor in humans, access to regular physical activity, has long been known to positively modulate an individual's adaptive capacity in the face of stress, an outcome that is readily observed with voluntary wheel running in rodents (Greenwood and Fleshner, 2011). Here Loughridge et al. (2013) combine laser capture microdissection with microarray expression analysis to investigate novel molecular targets of exercise-induced stress resistance. In addition to several genes that participate in neural mechanisms previously shown to be critical in the impact of exercise on stress, their innovative approach revealed sets of immune- and circadian-related genes that deserve further investigation.

In many organisms, prior exposure to repeated stressors often potentiates the neural and behavioral responses to later adverse events, a phenomenon termed stress sensitization, which is thought to be an important process involved in the susceptibility to anxiety disorders. Conversely, a reduction in autonomic, neuroendocrine, and behavioral responses is observed following repeated exposures to identical stressors (homotypic stress), a phenomenon termed stress habituation. Herman (2013) discusses the mechanisms underlying habituation and the challenges of identifying neural processes that represent a transition from adaptive to maladaptive responding during conditions of chronic challenge.

Psychological and social factors (e.g., coping style, cognitive flexibility, and social support) have long been associated with resilience to adversity (Southwick et al., 2005), although many of these factors are difficult to study in animals where the underlying neural mechanisms can be directly explored. In an animal model of intimate partner violence, Poirier et al. (2013) examine the influence of baseline trait anxiety in female rats on the neural and behavioral consequences of long-term cohabitation with an aggressive male partner. Despite the fact that anxious temperament modulated the behavioral outcome, many of the regional gene expression patterns that are altered by cohabitation did not differ between the "low" and "high" anxiety subgroups, highlighting the growing consensus that mechanisms of resilience are not always the opposite of those mediating vulnerability.

Additional psychosocial factors associated with resilience involve processes that engage coping strategies (Agaibi and Wilson, 2005). Active coping is generally conceptualized as behavioral or psychological efforts that individuals employ to master or reduce negative circumstances. Actual or perceived behavioral

control over some aspect of the adverse event is central to coping, and Drugan et al. (2013) provide a review of rodent paradigms in which the degree of behavioral control is experimentally manipulated. The authors further discuss the development of continuous non-invasive measurements (e.g., ultrasonic vocalizations) within the original stress episode that may be predictive of later performance during a subsequent challenge. On a related note, Nechvatal and Lyons (2013) review how coping, within the context of stress exposure therapy, impacts functional and structural measures in patients with specific phobias or PTSD. A long-term goal of this line of research, the authors note, is to guide the development of new intervention modalities that enhance the neuroadaptations associated with coping with stress in order to facilitate recovery.

Overall, the perspectives presented in this *Frontiers in Behavioral Neuroscience* Research Topic represent an integrative approach for elucidating the neural mechanisms underlying stress resilience. Extension of these efforts to other experimental paradigms may identify common themes that will very likely inform and enhance current therapeutic modalities.

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# Understanding resilience

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Resilience is the ability to adapt successfully in the face of stress and adversity. Stressful life events, trauma, and chronic adversity can have a substantial impact on brain function and structure, and can result in the development of posttraumatic stress disorder (PTSD), depression and other psychiatric disorders. However, most individuals do not develop such illnesses after experiencing stressful life events, and are thus thought to be resilient. Resilience as successful adaptation relies on effective responses to environmental challenges and ultimate resistance to the deleterious effects of stress, therefore a greater understanding of the factors that promote such effects is of great relevance. This review focuses on recent findings regarding genetic, epigenetic, developmental, psychosocial, and neurochemical factors that are considered essential contributors to the development of resilience. Neural circuits and pathways involved in mediating resilience are also discussed. The growing understanding of resilience factors will hopefully lead to the development of new pharmacological and psychological interventions for enhancing resilience and mitigating the untoward consequences.

**Keywords: resilience, stress, neurobiology, depression, PTSD**

## INTRODUCTION

Resilience is the capacity and dynamic process of adaptively overcoming stress and adversity while maintaining normal psychological and physical functioning (Russo et al., 2012; Rutter, 2012b; Southwick and Charney, 2012). Every individual experiences stressful events and the majority are exposed to trauma at some point during life. Therefore, understanding how one can develop and enhance resilience is of great relevance to not only promoting coping mechanisms but also mitigating maladaptive coping and stress response in psychiatric illnesses such as depression and posttraumatic stress disorder (PTSD). Although the understanding of resilience is overall still at an early stage, recent investigations have identified mechanisms encompassing genetic, epigenetic, developmental, psychological, and neurochemical factors that underlie the development and enhancement of resilience and factors that predict vulnerability to stress and susceptibility to psychiatric disorders in the face of stress and trauma. This review outlines discoveries from recent years from studies that have considerably advanced our understanding of resilience to stress and trauma and will likely move forward the development of pharmacological and psychological interventions for enhancing resilience.

## GENETIC FACTORS IN RESILIENCE

Genetic factors contribute significantly to resilient responses to trauma and stress. A range of human genes and polymorphisms associated with NPY, HPA axis, noradrenergic, dopaminergic and serotonergic systems, and BDNF have been linked to resilient phenotypes (**Table 1**) (Feder et al., 2009; Russo et al., 2012).

### NEUROPEPTIDE Y (NPY)

NPY is a neuropeptide that produces anxiolytic effects and promotes protective responses in the face of stress (Wu et al., 2011). Several studies in humans showed that genetic variations of *NPY* contribute to individual susceptibility to stress. One recent study found that two *NPY* haplotypes represented by three single nucleotide polymorphisms (SNPs) correlated with increased susceptibility to anxiety disorders after childhood adversity, and suggested that such behavioral effects can be mediated by altered NPY expression and subsequently dampened HPA-axis responsiveness under the influence of the genetic variation (Donner et al., 2012). Other studies also demonstrated that NPY release was substantially mediated by genetic variations in the *NPY* locus, especially in the promoter region, and that lower haplotype-driven NPY expression predicted weakened resilient response to stress (Zhou et al., 2008; Zhang et al., 2012).

### HPA AXIS (HYPOTHALAMIC-PITUITARY-ADRENAL AXIS)

Alterations in genes that regulate HPA-axis functions play an important role in shaping resilience. Polymorphisms in two key HPA-axis genes, *CRHR1* [corticotropin-releasing hormone (CRH) receptor 1 gene] and *FKBP5* (FK506-binding protein 5 gene), have been found to interact with early life stress to predict susceptibility to psychiatric illnesses in adults (Gillespie et al., 2009). One study identified, in two independent populations, significant gene × environment interactions with several individual SNPs of the *CRHR1* gene that influenced the risk of developing adult depressive symptoms in individuals with a history of child abuse (Bradley et al., 2008). The *FKBP5* gene, which

**Table 1 | Genetic factors in resilience.**

| CNS systems | Genes related to resilience | Influences of polymorphisms on resilience | References |
|---|---|---|---|
| NPYergic | Neuropeptide Y gene (*NPY*) | Increased susceptibility to anxiety disorders after childhood adversity. | Donner et al., 2012 |
| HPA Axis | CRH receptor 1 gene (*CRHR1*) | Affected the likelihood of developing adult depressive symptoms from child abuse. | Bradley et al., 2008 |
| | FK506-binding protein 5 gene (*FKBP5*) | Predicted severity of adult PTSD symptoms and onset of depression in individuals with childhood trauma. | Binder et al., 2008; Zimmermann et al., 2011 |
| Noradrenergic and Dopaminergic | Catechol-O-Methyltransferase gene (*COMT*) | Influenced the risks of developing PTSD and deficits in stress response and emotional resilience. | Heinz and Smolka, 2006; Skelton et al., 2012 |
| Dopaminergic | Dopamine transporter gene (*DAT1*) | Contributed to susceptibility to PTSD with a history of trauma. | Segman et al., 2002 |
| | Dopamine receptor genes (e.g., *DRD2*, *DRD4*) | Induced differential emotional processing and variability in brain responses to emotional stimuli; Influenced vulnerability to stress and trauma and risk of developing PTSD. | Blasi et al., 2009; Ptacek et al., 2011 |
| Serotonergic | Promoter region of serotonin transporter gene (*5-HTTLPR*) | Short allele strongly associated with increased stress sensitivity and risk for depression upon stress exposure, especially early life stress. | Karg et al., 2011 |
| | Serotonin receptor genes (e.g., *HTR1A*, *HTR3A*, *HTR2C*) | Interacted with environment to mediate stress response and to predict susceptibility to depression. | Gatt et al., 2010; Kim et al., 2011a; Brummett et al., 2012 |
| BDNF | Brain-derived neurotrophic factor gene (*BDNF*) | Interacted with early life stress to predict syndromal depression and anxiety; no clear evidence of association between the $Val^{66}Met$ polymorphism and anxiety disorders. | Frustaci et al., 2008; Gatt et al., 2009 |

is involved in the modulation of glucocorticoid receptor (GR) activity and thereby glucocorticoid signaling, was also found to interact with child abuse through its four SNPs to predict severity of adult PTSD symptoms (Binder et al., 2008). A more recent study showed that interactions between genetic variants of *FKBP5* and early life trauma strongly predicted the onset of depression later in life (Zimmermann et al., 2011).

## NORADRENERGIC AND DOPAMINERGIC SYSTEMS

Polymorphisms in the noradrenergic and dopaminergic systems have also been associated with vulnerability to depression and PTSD. Catechol-O-Methyltransferase (COMT) is an enzyme that metabolizes catecholamines including norepinephrine, epinephrine and dopamine. The *COMT* $Val^{158}Met$ polymorphism has been linked to deficits in stress response and emotional resilience, and was found to influence the risk for development of PTSD (Heinz and Smolka, 2006; Skelton et al., 2012). In an important study, Kolassa and colleagues showed that, predictably, higher numbers of different lifetime traumatic event types led to a higher prevalence of lifetime PTSD but that this effect was, in a typical gene-environment interaction fashion, modified by gene polymorphism (Kolassa et al., 2010). Compared to $Val^{158}Met$ polymorphism, the low-activity Met/Met homozygotes, with higher levels of norepinephrine and dopamine, exhibited a higher risk for PTSD. Children carrying the Met allele showed a higher cortisol response to stress. However, children who had more stressful life events showed a smaller increase in cortisol, implying that they might be more resilient (Armbruster et al., 2012). This study demonstrated differential effects of genetic and environmental factors on reaction to stress. Polymorphisms in the dopamine receptor genes, including *DRD2* and *DRD4*, and in the dopamine transporter gene *DAT1*, have also been implicated in stress responsivity, emotion processing, and susceptibility to PTSD and depression (Segman et al., 2002; Dunlop and Nemeroff, 2007; Blasi et al., 2009; Ptacek et al., 2011; Skelton et al., 2012).

## SEROTONERGIC SYSTEM

Studies of polymorphic traits of the serotonin transporter gene *SLC6A4* and receptor genes have led to several discoveries regarding the effects of gene × environment interactions on resilience. A recent meta-analysis of 54 human studies confirmed that the interaction of stress exposure and the polymorphism in the promoter region of the serotonin transporter gene (*5-HTTLPR*) is strongly associated with stress sensitivity and risk for depression, with the short, less transcriptionally efficient s-allele being

linked to increased stress sensitivity and risk of developing depression upon stress exposure (Karg et al., 2011). A particularly strong association between the s-allele and risk of developing depression was found in the group with a history of childhood maltreatment (Karg et al., 2011; Southwick and Charney, 2012). The s-allele of the *5-HTTLPR* gene was also found, in two independent populations, to interact with childhood and adult traumatic experiences to increase the risk for PTSD (Xie et al., 2009). Polymorphisms in several serotonin receptor genes, such as *HTR1A*, *HTR3A,* and *HTR2C*, have been shown to interact with stressful life environment as well as polymorphisms from other genes (e.g., $Val^{66}Met$ in the *BDNF* gene) to predict susceptibility to depression (Kim et al., 2007, 2011a; Gatt et al., 2010), and to mediate HPA-axis activation and emotional response to stress (Brummett et al., 2012).

#### BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

The role of the *BDNF* $Val^{66}Met$ polymorphism in stress response and resilience has not been clarified. A meta-analysis of seven studies found no significant association between the $Val^{66}Met$ polymorphism and anxiety disorders (Frustaci et al., 2008). Specifically, two case-control studies of PTSD found no significant association between the $Val^{66}Met$ polymorphism and PTSD diagnosis (Rakofsky et al., 2012). One study, however, showed that the $Val^{66}Met$ polymorphism interacted with early life stress to predict syndromal depression and anxiety, with higher depression in Met carriers (Met/Met and Met/Val) and higher anxiety in Val/Val genotype, indicating that both alleles, interacting with exposure to early life stress, may contribute to mechanisms of distinct risks (Gatt et al., 2009).

The field of genetics is now moving rapidly to genome-wide studies on large populations to examine the complex genetic contributions to resilience, with additional genetic polymorphisms, gene-by-gene and gene-by-environment interactions being currently identified. As the genetic underpinnings of resilience become better illuminated, it is anticipated that gene and drug therapies can be developed specifically for genetic profiles of low resilience.

## EPIGENETIC FACTORS IN RESILIENCE

Epigenetics refers to functional modifications to the genome without change in the DNA sequence. Such modifications serve to regulate gene expression and phenotype through mechanisms such as DNA methylation and demethylation, as well as histone modifications including methylation, acetylation, and phosphorylation. Epigenetic differences can be a consequence of exposure to stress-related factors during critical periods of development, and hence contribute to susceptibility to psychiatric disorders (Tsankova et al., 2007; Dudley et al., 2011).

Several animal studies have found that histone acetylation or phosphoacetylation in several subregions of the hippocampus increased after exposure to acute stressors (social defeat stress, forced swim stress, and predator stress) in both mice and rats, suggesting an adaptive role of these epigenetic changes in memory formation and stress response (McGowan et al., 2011; Sun et al., 2013). Intracerebral or systemic administration of histone deacetylase inhibitors (HDACi), alone or combined with antidepressants, resulted in antidepressant-like responses in several animal models (Sun et al., 2013). Histone methyltransferases (e.g., GLP, SUV39H1, G9a) are down-regulated in the nucleus accumbens of susceptible mice exposed to chronic social defeat stress, while these molecules were up-regulated in resilient mice exhibiting low depression-like responses, suggesting that histone methylation may be adaptive in the face of stress and protect against development of depression (Covington et al., 2011). Maternal care was found to influence stress response through epigenetic alterations, with offspring of high maternal care showing increased hippocampal GR expression and enhanced glucocorticoid negative feedback sensitivity, and hence more modest HPA response to stress, through hypomethylation at the NGFI-A nerve growth factor-inducible protein A (NGFI-A) binding site of a GR promoter (Weaver et al., 2004).

Human studies have begun to identify the effects of epigenetic changes on the regulation of the stress response. Suicide victims with childhood abuse had increased methylation of a GR (*NR3C1*) promoter in the hippocampus, and thereby decreased hippocampal GR expression, compared to suicide victims without childhood abuse and to control subjects (victims of sudden, accidental death without childhood abuse) (McGowan et al., 2009). This finding is consistent with those from animal studies showing that history of early adversity is associated with GR expression and stress response in adulthood. Another study showed that DNA methyltransferase (DNMT) expression was altered in a region-specific manner in the brains of suicide victims compared to controls who died of causes other than suicide (Poulter et al., 2008). This study found increased DNMT-3B expression in the prefrontal cortex (PFC), and an associated increase in DNA methylation of the promoter region of the γ-aminobutyric acid (GABA) A receptor subunit alpha-1 gene (*GABRA1*), the product of which was previously demonstrated to be down-regulated in the brains of suicide victims (Merali et al., 2004). Higher methylation of *MAN2C1*, a gene that encodes α-mannosidase, was shown to interact with greater exposure to potentially traumatic events to predict an increased risk of lifetime PTSD (Uddin et al., 2011). A number of epigenetic studies in animal models and humans investigating the association between epigenetic changes and risk for maladaptive stress responses and mental illnesses have recently been published (Radley et al., 2011; Schmidt et al., 2011; Murgatroyd and Spengler, 2012; Rusiecki et al., 2012).

## DEVELOPMENTAL FACTORS IN RESILIENCE

Developmental environment is another crucial contributor to resilience (Rende, 2012). Severe adverse events in childhood can negatively affect the development of stress response systems, in some cases causing long-lasting damage. Numerous rodent and primate studies suggest that animals abused by their mothers in the first few weeks of life show both delayed independence and decreased stress management skills in adulthood (Feder et al., 2011). These changes are reflected in abnormally high anxiety levels, increased HPA axis activity, and increased basal CRH levels in the cerebrospinal fluid (CSF) (Strome et al., 2002; Claes, 2004; McCormack et al., 2006). It is important to note that non-human

primates, who have suffered childhood abuse, resulting in damaged stress response systems, may be more likely to abuse their own children (Maestripieri et al., 2007). In this way, the cycle of abuse is continued through generations.

Similar long-lasting alterations, including changes in the central nervous system (CNS) circuits, have been found in studies of human survivors of childhood trauma (Heim et al., 2010). Prenatal stress and childhood trauma have been linked to a hyperactive HPA axis with attendant risk of negative effects of chronic hypercortisolemia later in life (Frodl and O'Keane, 2012). Furthermore, severe early life stress leads to hyperfunctioning of the locus coeruleus-norepinephrine (LC-NE) system in adulthood (Feder et al., 2011). One study of police recruits with a history of childhood trauma found that in contrast to controls, the police subjects had significantly higher levels of a salivary metabolite of norepinephrine when watching aversive videos (Otte et al., 2005). Childhood abuse can lead to a reduction of hippocampal volume, which is frequently seen in patients with mood disorders (Janssen et al., 2007; Davidson and McEwen, 2012). As the hippocampus is one of the most plastic regions of the brain, there is hope that pharmacological treatments, such as antidepressants, may be able to reverse this decrease in volume by increasing neural progenitor cells (Boldrini et al., 2012). PET studies have also revealed decreased activation in the hippocampus during memory tests in patients with a history of childhood abuse (Heim et al., 2010). Other brain areas seem to be affected by childhood abuse as well. For instance, a recent study suggests that childhood maltreatment has a pronounced effect on two separate neuroimaging markers—reduced hippocampal volume and amygdala responsiveness to negative facial expressions (Dannlowski et al., 2012). Chronic, unmanageable social and psychological stress, and maltreatment, especially early in life, are also linked to shorter telomeres, which have been associated with increased risk of developing somatic diseases such as cancer, diabetes and heart diseases, and psychiatric disorders, particularly depression (Blackburn and Epel, 2012; Price et al., 2013).

Certain factors play major roles in determining whether a childhood traumatic event will lead to vulnerability or instead, to resilience. One of these factors is the degree of control that the person has over the stressor (Feder et al., 2011). Episodes of early uncontrollable stress can lead to "learned helplessness," where a person is conditioned to believe that they are unable to change the circumstances of their situation (Overmier and Seligman, 1967). Learned helplessness is also used as a model for depression in animals. When administered inescapable and erratic shocks, animals tend to develop heightened anxiety states and fear responses (Overmier and Seligman, 1967). Furthermore, their active coping is reduced when faced with later stressors. Learned helplessness in animals is also believed to lead to dysregulation of serotonergic neurons in the dorsal raphe nuclei (Greenwood et al., 2003), as well as a reduction of cell proliferation in the hippocampus (Ho and Wang, 2010). These dysregulations are likely to have severe negative repercussions on both cognition and mood.

On the other hand, when animals are administered shocks that are avoidable by behavioral modification, learned helplessness does not seem to develop (Seligman and Maier, 1967). In this same way, humans that have been able to successfully master a mild or moderate stressor (for example, the end of a friendship or illness of a parent) appear to be resilient to a variety of other later stressors (Feder et al., 2009; Russo et al., 2012). This phenomenon is called "stress inoculation," and occurs when the person develops an adaptive stress response and a higher-than-average resilience to negative effects of subsequent, uncontrollable stressors (Southwick and Charney, 2012). Stress inoculation is a form of immunity against later stressors, much in the same way that vaccines induce immunity against disease (Rutter, 1993). Research in rodents supports the stress inoculation hypothesis and has suggested that this protection against some of the later negative effects may be due to neuroplasticity in the PFC induced by stress inoculation (Southwick and Charney, 2012). In one study, young monkeys were presented with a controllable stressor (periodic short maternal separations) over a course of 10 weeks (Parker et al., 2004). These monkeys experienced acute stress during the separation periods, illustrated by agitation as well as temporary increased levels of cortisol. Yet, at 9 months of age, they experienced less anxiety and lower basal stress hormone levels than monkeys who did not undergo the separations. Additionally, at later time points, the group of stress-inoculated monkeys showed higher cognitive control, higher curiosity in a stress-free situation and larger ventromedial PFC volume (Parker et al., 2005; Lyons et al., 2009).

It is important to note that although research has outlined numerous ways in which developmental environment can negatively impact a person, resilience is in fact a common trait, following even the most severe adversities. Between 50 and 60% of the general population experience a severe trauma during their lifetime, yet the prevalence of PTSD is estimated at 7.8% (Russo et al., 2012). Other studies have found that neural circuits involved in resilience can be modified for many years after adversity. For instance, the majority of adolescents whose development was stunted in childhood due to trauma were able to developmentally "catch-up" when relocated to a supportive, loving environment (Masten, 2001; Rutter, 2012a). The fact that not all animals or humans exposed to uncontrollable traumatic experiences develop stress-related disorders clearly implies that environmental factors interact with genetic endowment and together, affect resilience. In fact, resilient genes may be sufficient to help a person overcome the most traumatic developmental events in some cases (Feder et al., 2011).

## IMPLICATIONS FOR PROMOTING RESILIENCE IN CHILD REARING

The findings that the developmental environment has significant effects on building and enhancing resilience from a young age impart clear messages for child rearing. Several large-scale longitudinal studies have investigated resilience in participants from childhood or adolescence through the transition to adulthood. Results from these studies strongly indicated that key factors including positive family functioning and peer relationships, connections to supportive adults and prosocial romantic partners, planfulness, self-discipline, and cognitive ability, all contribute to a more successful transition to adulthood and more resilient functioning (Burt and Paysnick, 2012). Interventional paradigms in the form of foster care, adoption, and parent training can improve

the quality of parenting, family function, and attachment relationship, and in turn promote adaptive functioning and resilience in children and youth (Sapienza and Masten, 2011).

Children with a history of maltreatment showed lower resilient functioning than those without maltreatment (Cicchetti and Rogosch, 2012). Children with exposure to war and related traumatic experiences (e.g., child soldiers, rape, bombing, forced displacement) showed increased risks for PTSD as well as other medical conditions such as cardiovascular diseases in adulthood (Werner, 2012). Protective factors against deleterious impact of war-related adversities in children include a strong, positive bond between the primary caregiver and the child, the social support from teachers and peers, a shared sense of values, religious beliefs that find meaning in suffering, and humor and altruism as defense mechanisms (Werner, 2012). Besides children from an abusive and life-threatening environment, a newly identified group at risk is youth from affluent families, who may face higher risk of adjustment problems (e.g., substance use, depression, and anxiety) (Luthar and Barkin, 2012). Parents' lax repercussions on discovering substance use was shown to be a major vulnerability factor. Moreover, the levels of teens' symptoms (rule breaking, anxious-depressed, and somatic symptoms) were found to correlate more strongly with the teens' relationships with mothers than with fathers, which may in part reflect greater amount of time spent with mothers, who are generally the primary caregivers of their children. Therefore, positive changes in parenting for affluent youth are of critical importance, including adopting a strict zero-tolerance policy regarding students' law breaking, remaining vigilant about their children's activities outside school, and engaging in talks and workshops for families in distress and holding support groups particularly for mothers (Luthar and Barkin, 2012).

A review of efficacy of different interventions for children and adolescents with a history of trauma exposure indicates that cognitive-behavioral treatment, in both individual and group formats, is effective in reducing psychological harm such as anxiety and depressive disorders and symptoms (Wethington et al., 2008). Stress inoculation training (SIT), a preventive and interventional cognitive-behavioral paradigm, has been shown to be helpful in reducing anxiety and stress-related symptoms in adolescents (Maag and Kotlash, 1994). School-based interventions, including SIT, can improve adaptive coping skills and decrease the likelihood of developing PTSD symptoms in children exposed to war (Werner, 2012).

In summary, it is critical to provide children with a loving, healthy and supportive environment as they grow up, to avoid exposing them to repeated unmanageable stress, and to offer them chances to embrace and conquer life challenges so as to develop mastery of critical life stressors and acquire "stress inoculation" (Southwick and Charney, 2012). Education on successful parenting should be able to help to foster children in a resilience-promoting environment and to minimize occurrence of impaired stress response through generations. Moreover, training programs for children that focus on constructing and maintaining supportive social networks, enhancing prosocial behavior and cognitive reappraisal, and promoting coping self-efficacy and self-esteem, can all contribute to resilience building from an early age (**Figure 1**).

## PSYCHOLOGICAL FACTORS IN RESILIENCE

Significant research has been done on the psychosocial factors of stress tolerance and resilience building (Duryea et al., 1990; Chemtob et al., 1997; Pietrzak et al., 2010). Cognitive processes, personality traits, and active coping mechanisms, among others,

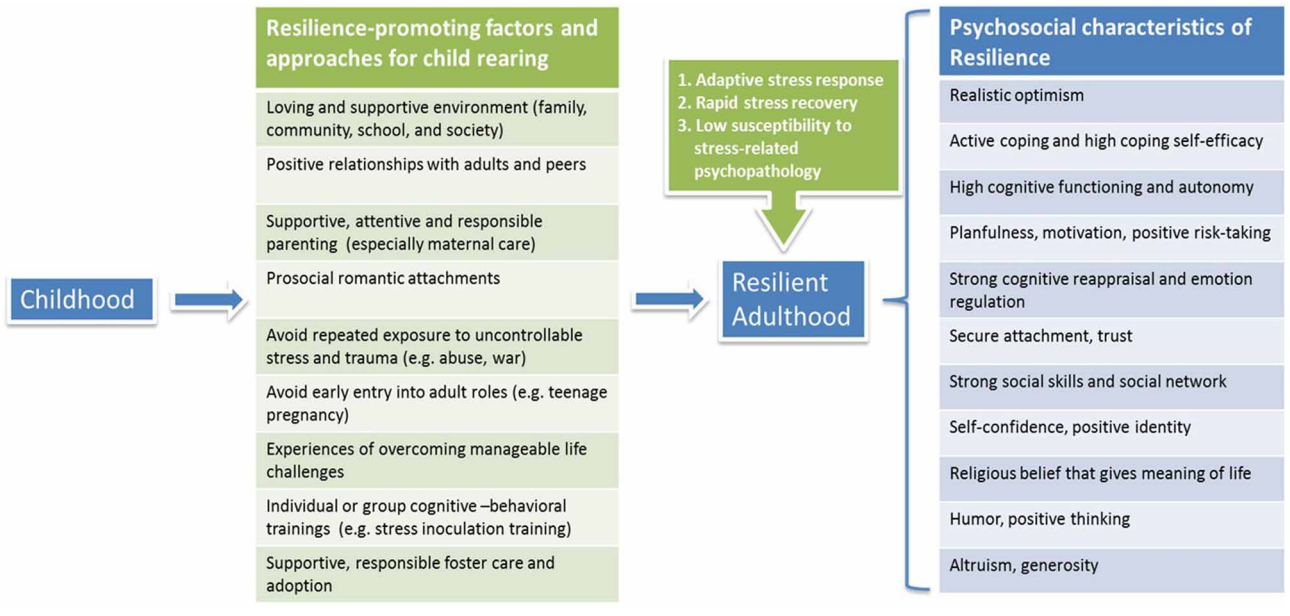


**FIGURE 1 | Promoting resilience in child rearing.**

contribute to resilience. These qualities also interact with biological factors to enhance adaptation in the face and aftermath of traumatic events, and confer resilience (Charney, 2004).

## INDIVIDUAL CHARACTERISTICS AND BEHAVIORS

Characteristics such as high level of intellectual functioning, efficient self-regulation, active coping styles, optimism, and secure attachment were observed in youth who had faced adverse situations and settings, yet did not succumb to the adverse impact of extreme stress (Richardson, 2002).

### *Optimism*

Positive affect has been found to be protective in the face of stress in numerous studies. In addition to decreasing autonomic arousal upon stress exposure (Folkman and Moskowitz, 2000), positive affect is also associated with quicker recovery times and better overall physical health (Scheier et al., 1989; Warner et al., 2012). Similarly, optimism, herein defined as the expectation for good outcomes, has been consistently associated with the employment of active coping strategies, subjective well-being, physical health, and larger and more fulfilling social networks and connections (Stewart and Yuen, 2011; Galatzer-Levy and Bonanno, 2012; Gonzalez-Herero and Garcia-Martin, 2012; Colby and Shifren, 2013). Unlike pessimists, optimists reported less hopelessness and helplessness and are less likely to use avoidance as a coping mechanism when under duress (e.g., among breast cancer patients) (Carver et al., 2010).

### *Cognitive reappraisal*

Strongly associated with resilience is the ability to monitor and assess negative thoughts and replace them with more positive ones, or cognitive reappraisal (McRae et al., 2012). Known as cognitive flexibility or cognitive reframing, this emotion regulation strategy involves changing the way one views events or situations. Consciously reassessing adverse or traumatic events to find the silver lining is associated with resilience (Gross, 2002). Viktor Frankl, the author of *Man's Search for Meaning* and the founder of logotherapy, attributed his psychological endurance and survival of concentration camps mainly to "meaning finding," the belief that the striving to find a meaning in one's life is the most important, powerful motivating and driving force to continue living (Frankl, 2006). In a study examining cognitive protective factors in the face of stress, women with high cognitive reappraisal ability exhibited less depressive symptoms than their cohorts with low cognitive reappraisal ability (Troy et al., 2010). Attachment style may also play a role in reappraisal ability and resilience. In a study of 632 men and women, researchers found that secure attachment was associated with higher cognitive reappraisal and resilience and that these two factors partially mediated individuals' well-being (Karreman and Vingerhoets, 2012). Securely attached participants were more likely to reframe situations as less emotional and less likely to suppress emotional expression. As expected, preoccupied attachment was inversely related to well-being due to less utilization of cognitive reappraisal.

A possible gender difference in emotional regulation/cognitive reappraisal is of note. Neural data suggest that women might employ positive emotions to help them regulate their emotions to a larger extent than men; it is possible that in men, use of emotion regulation is more automatic (McRae et al., 2008). Utilizing a randomized control design, an intervention study in Israeli citizens under ongoing war stress found that gender might act as a moderator in the development of resilience and reduction of helplessness (Farchi and Gidron, 2010). While the "psychological inoculation" intervention was expected to increase coping self-efficacy and to improve mental resilience more so than ventilation, the intervention's efficacy differed by sex. Psychological inoculation, possibly augmenting self-efficacy and hope, appeared to decrease helplessness in men, while the ventilation intervention appeared to decrease helplessness in women. The ventilation intervention may have had calming effects and lent a sense of connectedness that was helpful to women.

### *Active coping*

Coping, using behavioral or psychological techniques utilized to reduce or overcome stress, has been linked to resilience in individuals (Feder et al., 2009) and is coming to be recognized for its intervention potential (Taylor and Stanton, 2007). The literature distinguishes between active coping, involving behavioral and/or psychological strategies to change qualities of the stressor, the stressor itself, or how the stressor is perceived, and avoidant coping, involving activities and mental processes that are employed in lieu of dealing directly with the stressful trigger (Chesney et al., 2006). Emotional or behavioral withdrawal, alcohol use, and other substance use are classic examples of avoidant coping behavior (Lawler et al., 2005). While individuals who primarily exercise avoidant coping are at risk of psychological distress and subsequent negative responses, active coping has consistently been associated with adaptability and psychological resilience (Holahan and Moos, 1987; Moos and Schaefer, 1993). Among chronic pain patients, passive coping strategies were correlated with psychological distress and depression, while active coping strategies were inversely correlated with psychological distress (Snow-Turek et al., 1996). In a study examining two groups of Israeli veterans and former POWs, Solomon and colleagues found that high sensation seeking and low sensation seeking POWs significantly differed in their subjective assessments of suffering, use of coping methods, and emotional states while in prison (Solomon et al., 1995). Low sensation seeking former POWs reported more symptoms of PTSD and other psychiatric symptoms. Further distinguishing coping styles, task-oriented coping was positively correlated with resilience while emotion-oriented coping was related to low resilience among undergraduate students (Campbell-Sills et al., 2006). Drawing a relationship with personality, resilience among these young adults was inversely related with neuroticism but positively so with extraversion and conscientiousness. Even among sport performers, individuals with high hardiness or resilience tend to employ active coping strategies during stressful (competitive) situations compared with low hardiness groups (Hanton et al., 2013).

### *Social support*

Both the presence of social support and the behavior of seeking social support have been associated with psychological hardiness

and flourishing in the face of major adverse life events (Ozbay et al., 2008). The inverse also appears to be true; poorer social support has been linked to psychiatric disorders including PTSD (Tsai et al., 2012). Research with cancer patients found depression to be correlated with poor social support and higher external locus of control (Grassi et al., 1997). Depressed patients consistently reported weak or a lack of support from family, friends, and other social contacts (such as neighbors, colleagues, and less intimate relatives). Such patients were also often characterized by early maladjustment to their diagnosis of cancer (Grassi et al., 1997).

#### *Humor*

Humor has been identified as a form of active coping contributing to resilience not only for its capability for alleviating tension and but also for its ability to attract social support (Vaillant, 1992). Humor is widely used by veterans, repatriates, terminally ill patients, and youth alike and has been shown to be protective against stress (Southwick and Charney, 2012). Cameron and colleagues employed an ecological research method to examine the type and role of humor in resilient adolescents' daily social functioning and found that humor served various socioemotional functions and was a buffer in risky situations (Cameron et al., 2010). In a study of 215 sojourn students from Mainland China studying at a Hong Kong university, humor was seen as imperative to students' ability to adjust to the new culture and thrive in the face of acculturative stress (Cheung and Yue, 2012). In fact, humor increased with an increase in frequency of acculturative hassles.

#### *Physical exercise*

Physical exercise has positive effects on psychological well-being as well as mood, clinical depression, and self-esteem. Physical exercise has been shown to affect neurobiological factors of resilience in animal (Fleshner et al., 2011) and human studies (Wittert et al., 1996; Winter et al., 2007). In a 10-year study of 424 depressed adult patients, Harris and colleagues examined the relationship between physical activity, exercise coping and depression at 1-year, 4-year, and 10-year follow-up points (Harris et al., 2006). While no significant relationship between physical activity and subsequent depression was found, physical activity was negatively correlated with concurrent depression. In other words, physical activity may be beneficial to those currently depressed or facing major stressors. Moreover, in a rat model of depression, voluntary running had antidepressant-like effects in behavioral tests and in parallel enhanced NPY expression and neurogenesis (Bjornebekk et al., 2005, 2006).

#### *Prosocial behavior*

Altruism has also been associated with resilience in both adults and children (Southwick et al., 2005; Leontopoulou, 2010). Staub and Vollhardt examined case studies and qualitative studies where individuals' victimization and suffering bred prosocial behavior, ultimately promoting recovery from trauma, post-traumatic growth, and resilience, and suggested that post-traumatic interventions may promote "altruism born of suffering" (Staub and Vollhardt, 2008). A study of 232 elementary school children in Greece showed that higher altruism resulted in lower classroom competitiveness and was associated with higher empathy and resilience (Leontopoulou, 2010). Studies also show the birth of prosocial behavior and action from trauma enduring during times of civil conflict and unrest as a byproduct of personal healing (Hernández-Wolfe, 2010).

#### *Trait mindfulness*

Trait mindfulness is another psychological factor associated with resilience. Originated as a Buddhist meditation practice, mindfulness concentrates on moment-to-moment awareness of bodily activities, feelings, emotions, or sensations, while purposely perceiving and discarding any distracting thoughts that come into awareness (Thompson et al., 2011). Studies on trait mindfulness suggest that strong pre-trauma mindfulness skills may help prevent ruminative, depressogenic thinking, thereby counteracting the development of depression and PTSD symptoms following trauma (Thompson et al., 2011). A study of 124 firefighters showed that trait mindfulness was negatively related to depressive and PTSD symptoms, physical symptoms, and alcohol problems, suggesting that trait mindfulness may reduce avoidant coping in response to stress and contribute to resilience (Smith et al., 2011).

### MORAL COMPASS

The existence of a moral compass or an internal belief system guiding values and ethics is commonly shared among resilient individuals (Southwick et al., 2005). Though religion or spirituality is often a facet in one's moral compass, the concept of a moral compass is grounded in a more innately human belief in morality. A study of 121 outpatients diagnosed with depression and/or an anxiety disorder showed that a low or lack of purpose in life and less frequent physical exercise were correlated with low resilience, but low spirituality prevailed as a leading predictor of low resilience (Min et al., 2012). Similarly, purpose in life was a key factor linked to resilience in a study of 259 primary care patients with a history of exposure to a range of severe traumatic events (Alim et al., 2008).

## NEUROCHEMICAL FACTORS IN RESILIENCE

A number of neurochemicals have been found to be involved in resilience. These neurochemicals have been shown to interact with and to balance each other to produce regulatory effects on acute and long-lasting adaptations to stress.

### NPY

NPY is widely distributed in the brain (Wu et al., 2011; Sah and Geracioti, 2012). It counteracts anxiogenic effects of CRH in several brain regions that regulate stress and anxiety, including the hypothalamus, hippocampus, amygdala, and locus coeruleus (Sajdyk et al., 2004). Many studies on animal models and humans have confirmed the beneficial role of NPY in mediating resilience and vulnerability to stress and anxiety. Animals with PTSD-like behaviors showed a significant down-regulation of NPY in several brain regions including the amygdala and hippocampus, and centrally administered NPY reversed the negative behavioral effects of predator-scent stress (Cohen et al., 2012). Human

studies found that, under uncontrollable stress induced by harsh military training, plasma NPY levels were markedly increased, and higher NPY levels were associated with better behavioral performance and stress response (Morgan et al., 2000, 2002). Higher plasma NPY levels were also found in combat-exposed veterans without PTSD than in those with PTSD (Yehuda et al., 2006). Significantly lower NPY levels in CSF were found in men with combat-related PTSD compared to healthy controls without PTSD (Sah et al., 2009). Thus, a wealth of studies indicate a positive correlation between NPY levels and resilience to deleterious effects of stress, and suggest a potential pharmacotherapeutic target for effectively reducing anxiety and enhancing resilience to adversity and stress. Studies are currently being conducted in this regard, using possibly effective delivery routes such as intranasal administration.

#### HPA AXIS

Upon stress exposure, CRH is released from the hypothalamus and acts on the pituitary gland, causing it to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenal cortex to release cortisol and dehydroepiandrosterone (DHEA). Cortisol exerts negative feedback effects on the hypothalamus and pituitary, suppressing CRH and ACTH production, while DHEA is thought to have anti-glucocorticoid effects by inhibiting or blocking the effects of cortisol (Jones and Moller, 2011). This complex set of feedback interactions constitutes the HPA axis, which is a key neuroendocrine player modulating behavioral responses to stress (Russo et al., 2012).

Cortisol levels are linked to risk and resilience to stress-related psychiatric disorders, with higher levels associated with depression (Nemeroff and Vale, 2005), and lower levels with PTSD either as a possible trait that predisposes to the development of PTSD or as a consequence of trauma (Radley et al., 2011; Binder and Holsboer, 2012). DHEA together with DHEA sulfate (DHEA-S), have also been implicated in stress response and psychiatric disorders, with lower levels of DHEA(S) associated with depression, and elevated levels of DHEA(S) associated with PTSD (Maninger et al., 2009; Rasmusson et al., 2010). Of note, some studies have generated mixed findings (Hoge et al., 2007; Maninger et al., 2009). Because cortisol and DHEA(S) are released synchronously and function together through their antagonistic, dualistic homeostasis, the DHEA(S)/cortisol ratio has been found to be a crucial parameter that indicates differential stress vulnerability (Morgan et al., 2004; Markopoulou et al., 2009; Jones and Moller, 2011; ó Hartaigh et al., 2012).

CRH and its two receptors, CRHR-1 and CRHR-2, are important mediators of stress response (Southwick et al., 2005). In depression and PTSD, increased CRH levels in CSF have been found, which may relate to the dysregulation of signal transduction via the two receptors (Charney, 2004). CRHR-1 and CRHR-2 are differentially distributed in the brain, with CRHR-1 primarily found in the neocortex, basolateral amygdala, and hippocampus, and CRHR-2 in the lateral septum, medial and cortical nuclei of the amygdala, and dorsal raphe (Holsboer and Ising, 2010). CRHR-1 signaling plays a crucial role in anxiogenic circuits and contributes to anxiety-like response to stress. Consequently, preclinical and clinical studies have examined the antagonism of CRHR1 as a potential therapeutic intervention targeting aberrant CRH levels in mood and anxiety disorders and have generated some encouraging results (Paez-Pereda et al., 2011). CRHR-2 mainly modulates the effects of CRHR-1 signaling and can be either anxiolytic or anxiogenic depending on the circumstances (Hauger et al., 2009; Binder and Nemeroff, 2010).

#### NORADRENERGIC AND DOPAMINERGIC SYSTEMS

The noradrenergic system is activated upon stress, resulting in increased release of norepinephrine primarily from the locus coeruleus to its many projection sites that modulate stress responses and emotional behaviors, including the amygdala, hippocampus, hypothalamus and PFC, all of which constitute the LC-NE system (Aston-Jones and Cohen, 2005; Strawn and Geracioti, 2008). The activation of the LC-NE system under acute stress leads to generation and transmission of negative emotional memories starting from the amygdala, a process that can be inhibited by blocking norepinephrine activity (Charney, 2004). Hyperresponsiveness of the LC-NE system may result in chronic anxiety and fear (Feder et al., 2009). An imaging study in humans showed that disinhibited norepinephrine signaling may contribute to the etiology of PTSD by enhancing basolateral amygdala responses to fear stimuli (Onur et al., 2009). The norepinephrine transporter (NET) and receptors (α- and β-adrenoreceptors) involved in norepinephrine signaling have been implicated as biological mediators of stress-related psychiatric disorders and resilience (Krystal and Neumeister, 2009; Jhaveri et al., 2010). Dopamine release upon stress is increased in the PFC and inhibited in the nucleus accumbens, an area mainly associated with the reward pathway (Charney, 2004). Some studies have found decreased levels of circulating dopamine in depression and elevated urinary and plasma dopamine concentrations in PTSD (Charney, 2004; Dunlop and Nemeroff, 2007). A recent imaging study in humans showed that striatal dopamine transporter (DAT) density was higher in PTSD patients than in traumatized controls, suggesting a possible higher dopamine turnover in PTSD that can contribute to potentiation of exaggerated fear response to a stressful stimulus (Hoexter et al., 2012). Dopamine $D_1$ and $D_2$ receptors can form heterodimers by binding directly to each other, and these heterodimers were markedly elevated in the striatum in postmortem brains from patients with depression (Pei et al., 2010). Disrupting the coupling of $D_1$ and $D_2$ receptors has been shown to produce antidepressant-like effects, providing a possible novel target for antidepressant treatment (Pei et al., 2010; Wong and Liu, 2012).

#### SEROTONERGIC SYSTEM

Serotonin is one of the most studied neurotransmitters in relevance to mood and anxiety. Acute stress leads to increased serotonin turnover in multiple brain areas, including the amygdala, hypothalamus, PFC and nucleus accumbens (Feder et al., 2009). Serotonin affects the regulation of stress response and emotional behaviors through 5-$HT_{1-7}$ receptors in separate brain regions. The 5-$HT_{1A}$ receptor is anxiolytic and may play an important role in the etiology of anxiety disorders. Animal studies have found anxiety-like behaviors after knocking out 5-$HT_{1A}$ (Akimova et al., 2009). A few human imaging studies have also

showed decreased 5-$HT_{1A}$ binding and functioning in the amygdala, anterior cingulate cortex and raphe nuclei in patients with anxiety disorders compared to healthy controls (Akimova et al., 2009). The 5-$HT_{2A}$ receptor, on the other hand, is thought to be anxiogenic, and 5-$HT_{2A}$ antagonists prevent anxious behavior and dysregulated stress responses following early life stress (Benekareddy et al., 2011). Other serotonin receptors (such as 5-$HT_{1B}$ and 5-$HT_{2C}$) have also been implicated in adaptive responses to stress (Krystal and Neumeister, 2009). For example, overexpressing 5-$HT_{1B}$ in the caudal dorsal raphe nucleus led to reduced conditioned fear and helplessness in animal stress models (McDevitt et al., 2011).

#### BDNF

BDNF, a neurotrophic factor expressed in various brain regions including the amygdala, hippocampus, PFC and basal forebrain, is implicated in mood and anxiety disorders (Yamada and Nabeshima, 2003; Angelucci et al., 2005; Duman, 2009). BDNF supports neuronal proliferation, differentiation and growth during development, and promotes neuronal survival and functioning in adulthood (McAllister, 2002). Several studies have shown down-regulation of BDNF in the hippocampus after exposure of animals to various types of stress, and in postmortem studies of suicide-depression patients (Duman and Monteggia, 2006; Duman, 2009). Hippocampal BDNF expression contributed critically to resilient adaptations to chronic stress (Taliaz et al., 2011). BDNF acts through its two main receptors, TrkB and p75 (Castren and Rantamaki, 2010). The BDNF-TrkB pathway has been associated with both PTSD in humans and in animal models of fear conditioning, extinction and inhibitory learning (Mahan and Ressler, 2012). Central administration of BDNF has antidepressant-like effects and can enhance hippocampal neurogenesis (Li et al., 2008; Autry and Monteggia, 2012). Evidence from animal and human studies shows that administration of antidepressants can lead to increase of BDNF and TrkB expression in the hippocampus and PFC, suggesting a role of BDNF-TrkB signaling in the behavioral effects of antidepressants (Masi and Brovedani, 2011). Nevertheless, there is also evidence for antidepressant effects without changes in BDNF or neurogenesis (David et al., 2009; Petersen et al., 2009; Hansson et al., 2011). Much less work has been done regarding the exact role of the BDNF-p75 signaling pathway in resilience, probably due to the low affinity of p75 (Numakawa et al., 2010).

#### GLUTAMATE, GABA, AND ENDOCANNABINOIDS

Glutamate, GABA, and endocannabinoids have also been widely studied and implicated in the stress response, resilience, and pathophysiology of mood and anxiety disorders (Harvey and Shahid, 2012; Hill, 2012; Sanacora et al., 2012). The dysregulation of these systems can lead to profound deficits in successful adaptation to acute and chronic stress. Pharmacological studies targeting these systems in psychiatric disorders have begun to show promising results in achieving therapeutic effects (Hill and Gorzalka, 2009; Murrough and Charney, 2010; Kirilly et al., 2012; Mathew et al., 2012; Mathews et al., 2012).

## NEURAL CIRCUITRY OF RESILIENCE

Animal and human studies have investigated the brain circuits implicated in mood and anxiety and have shown that dysregulated functions and interactions among these circuits can result in low resilience phenotypes (Feder et al., 2009; Franklin et al., 2012). The reward and fear circuits play critical roles in the development of resilient character traits and adaptive social responses to stress.

#### NEURAL CIRCUITRY OF REWARD

Enhanced functioning of the reward circuitry contributes to resilience to stress and trauma (Charney, 2004). A key reward circuit is the mesolimbic dopamine pathway, which carries dopamine signaling from the ventral tegmental area of the midbrain to the nucleus accumbens in the limbic system, and also to other brain regions such as the amygdala, hippocampus, and medial PFC. The mesolimbic dopamine pathway is linked to behavioral responses to rewards (e.g., food, sex, and drugs of abuse), and functional abnormalities in this pathway can contribute notably to key depressive symptomatology such as anhedonia, decreased energy, and reduced motivation seen in individuals with depression (Nestler and Carlezon, 2006). Studies have shown that the onset of depression is likely to happen during adolescence, when reward functioning is generally higher than during childhood and adulthood, and that increased reactivity in the medial PFC and decreased reactivity in the striatum are implicated in adolescent depression (Forbes and Dahl, 2012). Children of depressed parents, therefore at high risk for depression, showed altered amygdala and nucleus accumbens activation to affective stimuli compared to those of non-depressed parents, therefore at low risk for depression (Monk et al., 2008). Depressed and PTSD patients showed weakened responses to rewards in the striatal areas including the nucleus accumbens (Sailer et al., 2008; Pizzagalli et al., 2009). Deep brain stimulation in the nucleus accumbens has antidepressant, anti-anhedonic and anxiolytic effects in patients with treatment-resistant depression, suggesting that modulating a dysfunctional reward system can lead to improvement of the core symptoms in depression (Schlaepfer et al., 2008; Bewernick et al., 2010). Although compelling evidence has shown that an enhanced, highly functional reward system may be beneficial for positive, adaptive response to stress, one study found that Special Forces soldiers of high resilience showed less activation in the subgenual PFC and nucleus accumbens under a high-reward condition compared to healthy civilian controls, suggesting that a potentially "sturdy" reward system may contribute to resilience (Vythilingam et al., 2009). The exact role of the reward system and the associated neurotransmitters in the development of resilience and pathophysiology and even etiology of stress-related psychiatric disorders needs further elucidation.

#### NEURAL CIRCUITRY OF FEAR

Resilience to extreme stress entails the ability to avoid excessive overgeneralized fear responses and to enhance favorable reconsolidation and extinction processes related to fear memories (Charney, 2004). Several studies have identified the components of the neural circuitry of fear response, which includes

the amygdala, hippocampus, medial PFC, nucleus accumbens, ventromedial hypothalamus, and a number of brain stem nuclei (Davis, 2006; Maren, 2008; Quirk and Mueller, 2008). These regions play key roles in fear processing including the fear learning/conditioning, perception of threat, execution of efferent components of fear response, and modulation of fear memories through potentiation, consolidation, reconsolidation, and extinction (Shin and Liberzon, 2010). Patients with PTSD showed hyperactivation in the amygdala and hypoactivation in the ventromedial PFC and anterior hippocampus, which may indicate reduced top-down inhibition of the amygdala and account for exaggerated fear responses (Etkin and Wager, 2007). Other brain regions such as the dorsal anterior cingulate cortex and insular cortex have also been implicated in the maladaptive regulation of fear responses in PTSD, with some studies showing hyperresponsiveness and some showing hyporesponsiveness of these regions (Shin and Liberzon, 2010). Compared to trauma victims without PTSD, individuals with PTSD demonstrated behavioral sensitization to stress, overgeneralization of the conditioned stimulus (CS)-unconditioned stimulus (US) response, impaired CS-US pairings and impaired fear inhibitory learning, all of which are thought to be characteristic of dysregulated fear responses and can result in the core symptoms seen in PTSD, such as intrusive memories and flashbacks, enhanced avoidance of reminders, and autonomic hyperarousal (Mahan and Ressler, 2012). One study found higher potentiation of the startle response to safety cues in patients with PTSD compared to traumatized controls, and that this impaired fear inhibition may be associated with altered HPA-axis functioning in PTSD (Jovanovic et al., 2010).

Animal studies have shown that proper fear conditioning and extinction learning require synaptic plasticity, and thus impaired synaptic plasticity may underlie impaired fear and extinction processes in PTSD (Mahan and Ressler, 2012). The BDNF-TrkB signaling pathway, a ligand-receptor system involved in synaptic plasticity, has been shown to be necessary for sustaining normal functioning of fear conditioning, extinction, and inhibitory learning in three brain regions, the amygdala, hippocampus, and medial PFC, all of which are associated with PTSD (Mahan and Ressler, 2012). Consolidation of fear conditioning and extinction was impaired when BDNF signaling was inhibited in the amygdala (Rattiner et al., 2004; Chhatwal et al., 2006). Heterogeneous *BDNF* knockout mice (*BDNF*±) demonstrated malfunctioning contextual fear conditioning, which can be partially reversed with recombinant BDNF infusion into the hippocampus (Liu et al., 2004). Altered BDNF expression in the prelimbic and infralimbic areas of the medial PFC can also lead to functional changes in fear consolidation and expression, suggesting a role of BDNF as a key mediator of neural plasticity in these regions (Choi et al., 2010; Peters et al., 2010). Glutamatergic and GABAergic signaling pathways have also been implicated in the regulation of fear consolidation, expression and extinction (Mahan and Ressler, 2012). For instance, disrupting NMDA and AMPA receptor functioning impaired the extinction of fear conditioning (Dalton et al., 2008; Liu et al., 2009; Zimmerman and Maren, 2010). Other ligand-receptor signaling systems such as those involving norepinephrine, nitric oxide, endocannabinoids, dopamine and acetylcholine have also been shown to play a modulatory role in the consolidation and extinction of fear conditioning, primarily by modulating glutamatergic and GABAergic signaling (Mahan and Ressler, 2012). These neurochemical systems involved in the fear circuitry provide potential pharmacological targets for reducing dysregulated fear response in PTSD and enhancing resilience to inappropriate fear associations in individuals susceptible to stress-related psychiatric disorders.

### ADDITIONAL NEURAL CIRCUITRY OF RESILIENCE

Neural circuits underlying psychological characteristics that render adaptive social behavior and promote resilience in individuals have been examined. Psychobiological qualities important in prosocial behavior include emotion regulation, empathy, and altruism, among others (Charney, 2004; Feder et al., 2009). Animal and human studies have identified functional neural circuits and interactions among multiple brain regions, such as the amygdala, PFC and nucleus accumbens, that are involved in the regulation of adaptive psychobiological responses to stress and adversities (Charney, 2004; Feder et al., 2009; Kim et al., 2011b; Cusi et al., 2012; Morishima et al., 2012). Reduced Insular activation under stress has been linked to greater non-reactivity to inner experience, a key component of trait mindfulness which may protect against negative bias and reduce depression vulnerability (Paul et al., 2013). By potentially targeting the top-down and bottom-up regulation of these neural circuits, psychotherapeutic interventions including cognitive behavioral therapy with cognitive reappraisal, positive emotion exercises, coping skill training, well-being therapy, and mindfulness meditation, can be efficacious approaches to build and enhance resilient psychosocial responses to stress (Southwick and Charney, 2012).

## SUMMARY

Resilience is a complex multidimensional construct and the study of its neurobiology is a relatively young area of scientific investigation (Southwick and Charney, 2012). Multiple interacting factors including genetics, epigenetics, developmental environment, psychosocial factors, neurochemicals, and functional neural circuitry, play critical roles in developing and modulating resilience in an integrated way. For instance, genetic and epigenetic factors interact with each other and determine the biological characteristics and regulation of neurochemicals and receptors. Environmental factors influence these characteristics and regulation processes through gene and environment interactions throughout development, contributing to adaptive changes in gene regulation, plasticity in the growth and modulation of neurocircuits, and the shaping of psychological factors and behavioral endpoints that underlie the manifestation of resilience.

Our growing understanding of the neurobiology of resilience has significant implications for the prevention and treatment of stress-related psychiatric disorders. Pharmacological interventions targeting the neurochemical systems involving NPY, BDNF, CRH, and HPA axis, among others, are being investigated as potential treatments for depression and PTSD. For instance, pharmacological agents targeting the hyperactivity and malfunction of HPA axis and CRH can possibly reduce the likelihood of pathological response to stress. Also, for individuals with altered NPYergic system, enhancing NPY levels and function may help

to improve stress and anxiety regulation and to minimize the anxiogenic effects of CRH (Southwick and Charney, 2012).

Behavioral training targeting psychosocial risk factors and related neural pathways is also likely to increase resilience to stress (Karatsoreos and McEwen, 2011). Practice and training on enhancing stress-protective factors can lead to augmented plasticity and regulation of neural circuits that modulate reward and motivation, fear response, learning memory, emotion regulation, attention, cognitive executive function, adaptive social behavior, and cognitive reappraisal, thereby result in improved adaptation to stress and trauma, increased speed of recovery from adversities, and decreased susceptibility to stress-related psychopathology throughout life (Southwick and Charney, 2012). Furthermore, maintaining a supportive environment and providing resilience-building classes for child rearing can be particularly beneficial, in that children can learn how to master life challenges and acquire "stress inoculation" while growing up, enabling them to adaptively react to and master future challenges and stressors, thereby reducing susceptibility to stress-related psychopathology.

How to apply what we currently know about resilience to further the promotion of resilience and the prevention and treatment of stress-related psychopathology is one of the most critical questions for future studies. In addition, multidisciplinary research on the neurobiology of resilience should help to further identify risk and protective factors as well as their complex interactions and thereby facilitate the development of evidence-based interventions for enhancing resilience and mitigating risk for stress-related psychiatric disorders.

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# Neuroimaging resilience to stress: a review

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There is a high degree of intra-individual variation in how individuals respond to stress. This becomes evident when exploring the development of posttraumatic symptoms or stress-related disorders after exposure to trauma. Whether or not an individual develops posttraumatic symptoms after experiencing a traumatic event is partly dependent on a person's resilience. Resilience can be broadly defined as the dynamic process encompassing positive adaptation within the context of significant adversity. Even though research into the neurobiological basis of resilience is still in its early stages, these insights can have important implications for the prevention and treatment of stress-related disorders. Neuroimaging studies contribute to our knowledge of intra-individual variability in resilience and the development of posttraumatic symptoms or other stress-related disorders. This review provides an overview of neuroimaging findings related to resilience. Structural, resting-state, and task-related neuroimaging results associated with resilience are discussed. There are a limited number of studies available and neuroimaging research of resilience is still in its infancy. The available studies point at brain circuitries involved in stress and emotion regulation, with more efficient processing and regulation associated with resilience.

**Keywords: resilience, stress, neuroimaging, PTSD, MRI, trauma**

## INTRODUCTION

The lifetime prevalence of exposure to severe traumatic events in the United States ranges between 51.2 and 60.7% (Kessler et al., 1995). Individuals who have been exposed to a trauma can develop stress-related psychopathologies, such as depression, anxiety disorders, or posttraumatic stress disorder (PTSD) (Egeland, 1993; Schnyder et al., 2001; Kilpatrick et al., 2003; Bryant et al., 2011). These disorders are a major cause of long-term disability. The United States National Comorbidity Survey found that ∼7.8% of the population in the US develops a PTSD at least once in their lives, with women having a higher chance than men (Kessler et al., 1995; Mota et al., 2012). In patients with an anxiety disorder, a higher number of episodes of major depressive disorder (MDD) have been reported in patients with a history of trauma compared to those without the experience of a traumatic event (Zlotnick et al., 1997). Notably, not every individual develops posttraumatic symptoms after experiencing a traumatic event. Many people will experience symptoms of an acute stress disorder, but only a minority will develop PTSD or other affective psychopathologies. Major life-events are also known to be precipitating factors for other psychiatric disorders, and again many individuals will show symptoms of psychological distress, but only a relative minority will develop a disorder like MDD or an anxiety disorder. Whether or not an individual develops posttraumatic symptoms or another stress-related disorder after experiencing a traumatic event can be considered from a summative view, i.e., as being accounted for as a balance between positive and negative influences, affecting most people in the same way or to the same degree (Rutter, 2006), but also from a more dynamic and interactive view. According to this view, both vulnerability and resilience in the context of a specific stressor are higher-order, multidimensional phenomena spanning an individual's biological and psychological profile, developmental history, previous (traumatic) experiences, active choices, social context, current environment, social support, and timing of the traumatic event (Charney, 2004; Feder et al., 2009; Cicchetti, 2010; Holman et al., 2011). Importantly, there is not one universally accepted definition of resilience. Resilience is often more broadly defined as a dynamic process encompassing positive adaptation within the context of significant adversity, and also, from a more psychobiological standpoint, as short- and long-term responses that reduce allostatic load (Charney, 2004; Curtis and Cicchetti, 2007). This definition would differentiate resilience from the concept of resistance. Stress resistance prevents the experience of negative consequences of stressor exposure, whereas stress resilience requires one to experience the negative consequences of stressor exposure in order to demonstrate facilitated recovery from that experience (Fleshner et al., 2011). Moreover, in some individuals the experience of negative effects in response to stressors or adversity may also lead to a decreased vulnerability later in life through a "steeling" or inoculation effect (Rutter, 2012).

Research into psychological factors contributing to resilience is longstanding, and has identified factors such as emotional flexibility, locus of control, social problem solving, and cognitive

skills, and several others [for a review see Curtis and Cicchetti (2003)]. More recently, studies have begun to examine biological factors in resilience and their interplay with psychological and environmental factors. In humans, cross-sectional studies have focussed on neuroendocrine and neural markers, while animal models are providing complementing experimental data on behavioral, neural, molecular, and hormonal basis of resilience. Animal data show that in resilient animals there is an absence of the key molecular abnormalities found in susceptible individuals, but also distinct epigenetic and cellular adaptations in response to stressors in various neurotransmitter systems and brain areas (Fleshner et al., 2011; Russo et al., 2012). Insight into biological factors underpinning resilience to stress may open new avenues for prevention and treatment of stress-related disorders (Charney, 2004). In addition, these insights could prove useful in the selection and training of professions known to have a higher risk of trauma exposure (i.e., military personnel, police officers, first responders). Over the past decades neuroimaging has become an increasingly important tool to study neural correlates of adaptive and non-adaptive behavior. Furthermore, neuroimaging allows studying the associations of these neural correlates with other biological factors as well as their interaction with environmental and psychological factors. In addition, neuroimaging facilitates examining the psychobiological mechanisms that underlie these interactions (Meyer-Lindenberg and Tost, 2012).

To investigate neurobiological correlates of resilience to psychological trauma in humans, one would ideally study a group of individuals at baseline, before exposure to trauma, and then assess these individuals repeatedly after exposure to trauma. Those who would have developed sustained symptomatology would then be compared to those who remained symptom-free or only had transient symptoms.

For the sake of this neuroimaging review, we will consider trauma-exposed, non-PTSD (TENP) individuals as resilient subjects. We are aware that defining resilience as the absence of PTSD symptoms after the experience of a traumatic event does not fully cover the multi-dimensional, dynamic nature of the construct of resilience. The term resilience as used throughout our review will therefore be more reflective of the capacity of an individual to avoid negative social, psychological and biological consequences, and cognitive impacts of extreme stress that would otherwise compromise their psychological or physical well-being (Russo et al., 2012). In the present review we will focus on the neuroimaging of resilience to especially severe stressful experiences, such as combat-related trauma, sexual abuse, and sustaining severe injuries through accidents. We will first briefly introduce the most widely used neuroimaging approaches to date. Subsequently, we discuss the brain circuitry of stress and present a review of the available neuroimaging literature on resilience in humans up until 2012.

## NEUROIMAGING METHODS

The rapid growth of modern neuroimaging techniques enables us to study both structure and function of the human brain in great detail. Additionally, it allows us to examine the influence of specific biological or specific environmental factors on brain functioning (Meyer-Lindenberg and Tost, 2012). Nowadays, magnetic resonance imaging (MRI) methods are the most widely used tools to examine brain structure and function in living humans, because of the low risks involved, the non-invasive nature of the technique, and the high quality of the obtained images. MRI techniques can be used to localize neuropathological abnormalities or to determine the size or shape of various structures in the brain (Pitman et al., 2001). The MRI-based diffusion tensor imaging (DTI) method can be used to examine white matter tracts. Functional neuroimaging, i.e., dynamic (indirect) measurements of brain activity during rest or during a cognitive, emotional, or pharmacological challenge, can be assessed using functional MRI (fMRI). With fMRI, changes are measured in regional cerebral blood flow based on changes in the concentration of the blood oxygenation level. A relative estimate of the level of activity within a given region of interest can be derived from this blood oxygenation level-dependent (BOLD) signal (Pitman et al., 2001; Huettel et al., 2009). Brain activity and blood flow can also be measured with functional neuroimaging methods that use radioactive ligands such as positron-emission tomography (PET) or single photon-emission computed tomography (SPECT). PET can use radioactive labeled water, oxygen or glucose (Bremner, 2007a; Townsend, 2008). In addition PET and SPECT methods can use radioactive ligands to visualize biochemical elements such as transporters or receptors for certain neurotransmitters.

## NEUROCIRCUITRY OF STRESS

Converging data from animal studies, lesion studies in humans and neuroimaging research in healthy controls and patient populations, point at the involvement of specific brain structures and circuitries in the generation of emotional, cognitive, and behavioral responses to stressors and the subsequent regulation of these responses. Key structures involved in this neurocircuitry are the amygdala, insula, hypothalamus, hippocampus, and cortical structures like the medial prefrontal cortex (mPFC) and the anterior cingulate cortex (ACC) (Dedovic et al., 2009).

The amygdala is located in the medial temporal lobes of the brain. It is involved in the encoding and consolidation of emotional memory of events (Bremner, 2007b), and regulates part of the fear response by activating the hypothalamic-pituitary-adrenal axis that releases hormones involved in the stress response. The amygdala can also increase the startle response via connections with the pons in the midbrain and is involved in the modulation of the autonomic nervous system via the hypothalamus (Davis, 1992; Jovanovic and Ressler, 2010). Amygdala activation has been reported in response to positive stimuli as well, suggesting a broad involvement of this structure in emotional arousal (Shin and Liberzon, 2010). Lesions in the amygdala of both humans and rodents have shown that the amygdala is also involved in the elimination of the fear response and emotional behavior (Zola-Morgan et al., 1991; Funayama et al., 2001). The insula is involved in high-level cognitive control and attentional processes (Menon and Uddin, 2010). Additionally, together with the hippocampus, the insula plays a role in processing the context of a potential threat (Gilbertson et al., 2002; Feder et al., 2009). The hippocampus not only has an important role in declarative memory, but is probably also a key-regulator

of the hypothalamic-pituitary-adrenal axis activation (Bremner, 2007b). Furthermore, having a small hippocampus could diminish the neuroendocrine regulation, leading to a stronger emotional or hormonal stress response (Gilbertson et al., 2002; Lyons et al., 2007). Animal studies have shown that hippocampal lesions or genetically smaller hippocampi lead to a stronger conditioned fear response and alterations in fear-mediated responses (Phillips and Ledoux, 1992).

The prefrontal cortex (PFC) receives somatosensory, visual and auditory inputs and underlies many cognitive skills. Areas in the PFC have an inhibitory effect on the amygdala (Phelps et al., 2004; Baumann and Turpin, 2010), and the modulation of emotional responsiveness by the PFC through inhibition of the amygdala is supported by lesion studies (Morgan and Ledoux, 1999; Milad and Quirk, 2002). The PFC encompasses many structures, among which the ACC. The ACC is divided into subregions including the dorsal ACC (dACC), the rostral ACC (rACC), and the subgenual ACC (sgACC). Hypofunction of the PFC, the rACC and the sgACC, and hyperactivity of the amygdala and dACC was found to be related to dysregulation of emotion in anxiety or mood disorders (Phan et al., 2005; Shin and Liberzon, 2010). Animal studies have shown that enhanced activation of the infralimbic PFC, the rodent analog of the rACC and sgACC, inhibits the fear response. The ventral-rostral area of the ACC is involved in the processing of emotionally relevant stimuli, while the dorsal-caudal region is more relevant for non-emotional cognitive tasks (Shin et al., 2005, 2007; Mohanty et al., 2007). However, recent studies seem to indicate that both subregions contribute to emotional processing, with ventral-rostral portions of the ACC and the mPFC involved in regulation (Etkin et al., 2011).

With respect to studying stress and resilience to stress, animal studies allow more freedom in manipulating stress responsiveness compared to human research. Interestingly, interventions designed to decrease stress responsiveness by forcing repeated application and selection of the most successful coping strategies have been found to increase the volume of the ventral mPFC (Lyons et al., 2002; Katz et al., 2009), and increase neurogenesis of the hippocampus (Lyons et al., 2010a). In addition, Delgado et al. showed that an inward displacement of the ventral part of the right hippocampus was specific for resilience to stress in rats (Delgado Y Palacios et al., 2011). These findings suggest a firm relationship between successful application of coping strategies and these key structures in the neurocircuitry of stress in animals. For more animal literature on resilience to stress see (Lupien et al., 2009; Lyons et al., 2010b; Franklin et al., 2012) (**Figure 1**).

## REVIEW

For the current review we conducted a Pubmed search up till 2012, using key terms including: "resilience," "vulnerability," "neuroimaging," "PET," "SPECT," "MRI," "posttraumatic," "anxiety" "depression" "affective" "stress," "trauma." Bibliographies were also reviewed for further citations. We limited our search to studies in humans and in English. Papers were selected based on relevance with a focus on studies in PTSD, but studies on MDD were also included. We also included electronic publications ahead of print.

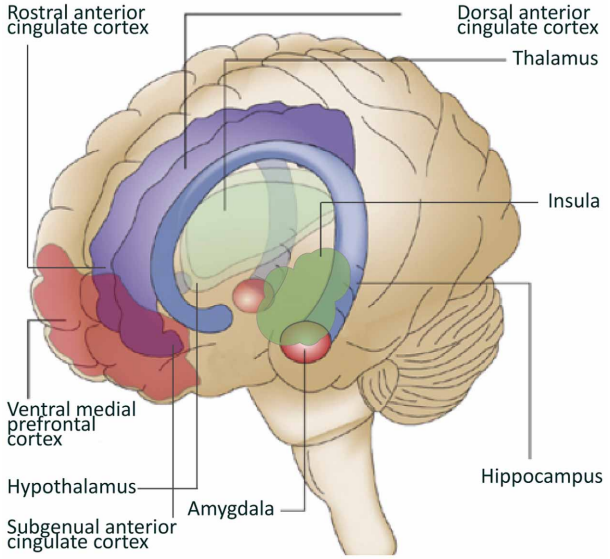


**FIGURE 1 | Brain regions involved in resilience to stress.** Depicted in this figure are brain regions often linked to resilience to stress. Adapted from Schloesser et al. (2008).

We will first discuss studies examining structural neural correlates of resilience to stress, followed by studies examining functional neural correlates of resilience to stress, and studies in which the neural correlates of personality factors known to be involved in resilience, i.e., trait-resilience, were taken into account. **Table 1** presents the studies that allowed examining resilience, i.e., examined stress-resilient subjects, subjects with psychopathology after stress, and healthy controls without both trauma exposure and PTSD. Because studies in PTSD have informed hypotheses on the neural circuitry involved in resilience, findings from studies in PTSD (comparing PTSD individuals with TENP individuals) will be briefly presented in each section before discussing the results of studies focussing on resilience.

## STRUCTURAL NEUROIMAGING OF RESILIENCE

We could only identify a very limited number of MRI studies explicitly designed to explore resilience by focussing on its structural correlates. Many studies did not include a healthy, non-exposed control group, which is needed in a cross-sectional design to establish whether differences between exposed groups are related to vulnerability or resilience. Remarkably, there appear to be no studies on resilience using methods to assess white matter integrity or connectivity, such as DTI, or methods to examine structural aspects other than volume or gray matter density, i.e., shape analysis or cortical thickness. Studies usually report on structural aspects of the hippocampus, amygdala, and ACC.

### HIPPOCAMPUS

One of the core symptoms of PTSD is reliving the traumatic event. With the hippocampus being central in declarative memory, it could be hypothesized that variations in the structure of the hippocampus could contribute to resilience (Bremner,

**Table 1 | An overview of neuroimaging studies specifically examining resilience by using comparisons between three groups: (1) a PTSD group, (2) a TENP group, and (3) a healthy control group without both trauma exposure and PTSD.**

| References | *n* | Protocol | Findings specific to resilience |
|---|---|---|---|
| | PTSD/TENP/HC | | |
| **THREE-GROUP STUDIES** | | | |
| Gurvits et al., 1996 | 7/7/8 | Structural | No resilient specific findings |
| Liberzon et al., 1999 | 14/11/14 | Trauma-related sounds | No resilient specific findings |
| Fennema-Notestine et al., 2002 | 11/11/17 | Structural | Smaller frontal and occipital gray matter volumes |
| Britton et al., 2005 | 16/15/14 | Trauma-script | Decrease in amygdala activation |
| Falconer et al., 2008 | 23/17/23 | Go/No-Go inhibition task | No resilient specific findings |
| New et al., 2009 | 14/14/14 | Emotion regulation | Increased activation in medial prefrontal regions during top-down control |
| Woon and Hedges, 2009 | 121/77/116 | Structural | No resilient specific findings in amygdala volume |
| Blair et al., 2013 | 14/15/19 | Affective stroop task | Increased activation in medial prefrontal regions during top-down control |

| References | *n* | | Protocol | Findings specific to resilience |
|---|---|---|---|---|
| | PTSD Exposed/non-exposed twins | Non-PTSD Exposed/non-exposed twins | | |
| **TWIN STUDIES** | | | | |
| Gilbertson et al., 2002 | 17/17 | 23/23 | Structural | Increased hippocampus volume |
| May et al., 2004 | 20/23 | 23/24 | Structural | Decreased cavum septum pellucidum size |
| Kasai et al., 2008 | 18/18 | 23/23 | Structural | Decreased density in right hippocampus, pregenual ACC, bilateral insulae |
| Shin et al., 2009 | 14/14 | 19/19 | Resting-state | Decreased dorsal anterior cingulate activation |
| Shin et al., 2011 | 12/12 | 14/14 | Multi-source interference task | Decreased dorsal anterior cingulate activation |

*HC, Healthy Controls; PTSD, posttraumatic stress disorder; TENP, Trauma-exposed non-PTSD.*

2007b). Importantly, the hippocampus is also a key-regulator of the hypothalamic-pituitary-adrenal axis activation in response to stressors (Bremner, 2007b). The hippocampus has frequently been studied in PTSD and several studies examining TENP and PTSD subjects found larger volumes of the hippocampus in the TENP subjects (Gurvits et al., 1996; Bremner et al., 2003; Lindauer et al., 2004b; Kitayama et al., 2005; Kasai et al., 2008; Felmingham et al., 2009; Morey et al., 2012), but others did not report this finding (Lindauer et al., 2005; Freeman et al., 2006; Rogers et al., 2009). Smaller hippocampal volumes have also been described in MDD and in healthy "at risk" subjects with a history of depression (Campbell et al., 2004; Videbech and Ravnkilde, 2004).

Because a non-exposed healthy control group was not included in these studies, it is not possible to distinguish whether the differences in hippocampal volume should be attributed to resilience in the TENP group or to the presence of psychopathology or vulnerability in the PTSD group. Controversy exists on the origin of structural differences of the hippocampus in relation to exposure to severe stress. Based on animal studies it has been hypothesized that exposure to traumatic events may damage neurons and inhibit neurogenesis in the hippocampus, showing that the hippocampus is sensitive to the effects of stress (Sapolsky et al., 1990; Bremner, 1999; Golub et al., 2011). This suggests that a smaller hippocampus is a consequence of neurobiological changes associated with extreme or chronic stress. In line with this, smaller left hippocampal volumes were found in women with MDD who have experienced chronic maltreatment in their childhood compared with women with MDD and without childhood maltreatment (Vythilingam et al., 2002). In addition, a decrease in hippocampal volume in MDD has been found to be associated with the duration of depressive episodes (Sheline et al., 1999; Macqueen et al., 2003). In PTSD, psychopharmacological treatment of symptoms has been associated with increases in hippocampal volumes (Vermetten et al., 2003). However, a study in which hippocampal volumes were larger in TENP subjects compared to PTSD subjects, showed that this difference did not change after the PTSD was effectively treated with psychotherapy, suggesting smaller hippocampal volumes to be either a residue or scar, caused by the experience of the trauma, or a factor related to vulnerability, pre-existing the traumatic event (Lindauer et al., 2005). Apart from considerations on how psychotherapy and psychopharmacological treatment influence the brain, an alternative explanation could

be that larger hippocampal volume in the TENP is linked to resilience.

To directly address the important controversy, Gilbertson et al. (2002) in an elegant design examined hippocampal volume in a group of PTSD patients and their non-exposed twins, and in combat-related TENP subjects and their non-exposed twins (Gilbertson et al., 2002). It was possible to correct for childhood abuse and alcohol use, factors known to influence hippocampal volume. The authors found smaller hippocampal volumes in both PTSD patients ($n = 17$) and their non-traumatized co-twins ($n = 17$) compared to TENP subjects ($n = 23$) and their non-traumatized co-twins ($n = 23$). In addition, severity of PTSD symptomatology in patients was negatively correlated with the hippocampal volume of both the patients and their trauma-unexposed identical co-twins, suggesting smaller hippocampal volume to be a familial risk factor for developing stress-related psychopathologies. The TENP subjects showed no differences in hippocampal volumes compared to their non-exposed twins. As there were no non-exposed twin pairs in this study, it could not be examined whether the hippocampal volume of the resilient subjects and their co-twins was perhaps larger than average and a potential familial resilience factor. Another, smaller study examined morphometry of the mesial temporal lobe area in adult female victims of intimate partner violence with ($n = 11$) and without ($n = 11$) PTSD, and in non-victimized controls ($n = 17$) (Fennema-Notestine et al., 2002). There were no differences in hippocampal volumes among the three groups. Interestingly, the authors found smaller overall frontal and occipital gray matter volumes in the resilient (violence exposed non-PTSD) subjects, but the interpretation of the findings is difficult because of the small sample and the presence of childhood emotional abuse.

In conclusion, the available data suggest that smaller hippocampal volumes might be the result of exposure to severe stress and perhaps also a vulnerability factor, but it is not clear whether an increased volume is associated with resilience.

### AMYGDALA

Structural MRI studies in PTSD have also examined structural changes of the amygdala, with some studies reporting a larger amygdala volume in TENP subjects compared to PTSD subjects (Rogers et al., 2009; Morey et al., 2012), while others did not (Gurvits et al., 1996; Bonne et al., 2001; Lindauer et al., 2004b; Kuo et al., 2012).

A recent meta-analytic study, using data from nine different studies in adults, compared the amygdala volumes in PTSD patients ($n = 121$), TENP individuals ($n = 77$), and non-trauma exposed healthy controls ($n = 116$) (Woon and Hedges, 2009). The authors found a larger right amygdala vs. left amygdala in all three groups, which is consistent with a previously conducted study (Pedraza et al., 2004). This suggests that in both PTSD and in resilience to trauma the asymmetry in volume of the amygdala is preserved. In addition, this meta-analysis found no significant differences between amygdala volume in PTSD patients relative to TENP and trauma-unexposed healthy controls (Woon and Hedges, 2009). These results suggest that although the amygdala has a key role in the neurocircuitry of stress, the volume of the amygdala is not associated with influence on vulnerability or resilience toward developing psychopathology after a traumatic event. However, a single case-control study with comparable group sizes to those of the meta-analysis ($n = 99$ PTSD patients; $n = 101$ TENP individuals) found larger left and right amygdalae in the TENP individuals. Evidence of the association between the structure of the amygdala and resilience as well as PTSD symptomatology is currently inconsistent and inconclusive (Morey et al., 2012).

### ANTERIOR CINGULATE CORTEX/PREFRONTAL CORTEX

Studies comparing PTSD subjects with TENP subjects found smaller volumes and lower gray matter density of the ACC in PTSD subjects (Rauch et al., 2003; Woodward et al., 2006; Kasai et al., 2008). More specifically, a smaller volume of the rACC (Rauch et al., 2003; Kasai et al., 2008) and subcallosal cortex was found (Rauch et al., 2003).

Smaller gray matter volumes of the ACC and mPFC have also been found in MDD patients compared to healthy controls, with one of the few longitudinal studies showing loss of volume in MDD in these areas during depressive episodes (Frodl et al., 2008). Similarly, a twin study in subjects with PTSD showed that the gray matter volume reductions were not present in non-PTSD co-twins, suggesting that the reductions are the consequence of the exposure to stress, rather than a possible familial vulnerability factor (Kasai et al., 2008). Furthermore, May et al. (2004) showed in a twin study design a significant reduction of cavum septum pellucidum size in TENP individuals and their co-twins compared to PTSD individuals and their co-twins (May et al., 2004). Increases in the size of the cavum septum pellucidum is linked to impaired limbic development (Raine et al., 2010).

Interestingly, a recent structural neuroimaging study on resilience to MDD examined volumes of the hippocampus, several prefrontal areas, and the basal ganglia in healthy adults without any family history of MDD ($n = 64$), "resilient" healthy individuals with a family history of MDD ($n = 30$), and participants with a current diagnosis of MDD ($n = 33$). A smaller right hippocampal volume, which in PTSD putatively reflects a genetic risk factor, was found in the resilient healthy subjects with a family history of MDD. However, the resilient individuals also showed increased white matter volumes of the right dorsal mPFC as compared to the two other groups. The authors interpreted this as a potential correlate of resilience to stress, possibly linked to the regulatory functions of this region (Amico et al., 2011). To examine the hypothesized modulatory function of emotional responsiveness by the mPFC, Milad et al. (2005) subjected healthy individuals ($n = 14$) to trials of presented pictures of virtual lights followed by an electric shock. In the extinction phase, participants were presented the virtual light without the electric shock. The next day only the virtual lights were presented again and skin conductance was registered as a measure of fear extinction. They found that greater extinction memory (lower skin conductance) was associated with an increased thickness of the ventral mPFC (Milad et al., 2005). These results led the authors to suggest that the size of the ventral mPFC might explain individual differences in the ability to modulate fear. A potential relationship between the ventral mPFC and modulation of emotion responsiveness is

further supported by animal studies (Lyons et al., 2002; Katz et al., 2009). These studies used the early handling paradigm [subjecting pups to short periods of separation from their mother during the first week(s) of life] or social separation in order to decrease stress responsiveness and increase successful application of various coping strategies. Animals subjected to this paradigm were found to have an increased volume of ventral mPFC. Other data in rodents show that in animals resilient to certain stress paradigms the expression of certain genes in glutamatergic neurons in the mPFC increases, suggesting increased neuronal activation. This also suggests that the complexity of neuronal architecture in the mPFC increases, which may be mechanisms underlying volume changes (Russo et al., 2012).

## STRUCTURAL CONNECTIVITY

Studies examining structural connectivity in PTSD have shown abnormalities of structural integrity of cingulate regions, the cingulum bundle and/or the amygdala, and other frontal regions [for a review see Ayling et al. (2012)]. We did not identify studies in which resilience was or could be explored in humans. In monkeys, the recent study by Katz et al. (2009) not only found increased white matter volumes, but also increased myelination in the mPFC after stress inoculation. This could be the substrate for the decreased stress responsiveness observed in these monkeys, given the role of the ventral mPFC in emotion and arousal regulation (Katz et al., 2009).

Taken into account the limited available structural imaging data from both human and animal studies, the findings most consistently indicate that the (ventral) mPFC volume and structure are associated with resilience, with volumetric and structural alterations reflecting or even underlying increased emotion regulation capacities. For two other key-structures in the stress and emotion circuitry, the hippocampus and the amygdala, the available data suggests associations of structure with vulnerability, but not clearly with resilience.

## FUNCTIONAL NEUROIMAGING

Given the concept of resilience as being a dynamic process, encompassing positive adaptation within the context of significant adversity, studies examining functional correlates of resilience are clearly of importance. One approach is to examine the spontaneous brain activity and its temporal and spatial connectivity in the absence of externally presented tasks or stimuli, so-called resting-state fMRI. Older studies have used PET and SPECT methods; more recently there is an increasing use of resting-state fMRI approaches. With resting-state fMRI several functional networks have been detected, including the default mode network, thought to be involved in autobiographic memory and self-referential processing. With respect to studying resilience, resting-state fMRI can also be applied during anticipation and recovery of stress.

A more widely used functional neuroimaging approach is to study the correlates of brain activity and connectivity while subjects have to engage in a specific emotional or cognitive task, as opposed to the situation in resting-state imaging. Emotional tasks may involve paradigms with specific stimuli associated with a previous stressful or traumatic event, such as trauma scripts to study emotion processing related to the specific event, or with emotional stimuli not related to a previous event to study general emotion processing. In addition, it is also possible to induce psychological and social stress before scanning.

### RESTING-STATE STUDIES

We did not identify PET or SPECT studies in which resilience could be explored, i.e., including a TENP group, a psychopathology and a healthy control group. PET and SPECT studies have found increased amygdala activity at rest in PTSD subjects, with one twin study reporting increased resting metabolic activity as a familial risk factor for PTSD (Chung et al., 2006; Shin et al., 2009).

Only a few resting-state fMRI studies have been performed in PTSD so far, and they seem to point at the importance of resting-state connectivity of different areas and networks involved in self-processing and fear conditioning with an amygdala/ACC circuitry. In a small, but very interesting prospective resting-state fMRI study, Lanius et al. (2010) examined the relationship between connectivity of the default mode network and severity of concurrent and prospective PTSD symptoms in 11 acutely traumatized subjects recruited from emergency departments (Lanius et al., 2010). Participants were assessed at 2, 6, 12, and 36 weeks postaccident and scanning took place at week 6 or 12. A seed-based approach with a seed in the posterior cingulate cortex (PCC)/precuneus region was used. The PCC/precuneus region is implicated in autobiographical memory processes and self-processing operations and a key region in the default mode network (Greicius et al., 2003). Connectivity of this region with the perigenual ACC and the right amygdala was positively correlated with current PTSD symptomatology, whereas the connectivity with the right amygdala predicted symptoms 6 weeks subsequently. The authors interpreted their results as reflecting an increased trauma-related input from amygdala and perigenual ACC circuitry into the default mode network, which could lead to disturbed aspects of self-processing. Less resting-state connectivity between the insula and the right amygdala was shown in a combat-related TENP group compared to PTSD subjects (Rabinak et al., 2011). The insula and amygdala have been shown to be connected during fear conditioning and a reduced resting-state connectivity may underlie less exaggerated fear responses, less persistence of traumatic memories and proneness to affective disorders (Etkin and Wager, 2007).

In another, quite large study comparing TENP ($n = 72$) and PTSD subjects ($n = 54$) recruited from earthquake survivors, the resting-state connectivity of the thalamus was examined. The thalamus is connected to nearly all areas in the cortex and acts as a relay between subcortical areas and the cerebral cortex. The TENP group showed decreased positive connectivity between the thalamus and bilateral inferior and left middle frontal gyri, left inferior parietal lobule, and right precuneus. An increased positive functional connectivity between the thalamus and right medial frontal gyrus and left rACC was also found in this group (Yin et al., 2011).

Of interest for the more dynamic concept of resilience, some resting-state fMRI studies in healthy subjects have aimed to identify patterns of adaptive recovery to laboratory-induced stress.

Clearly, this is relevant for elucidating brain mechanisms underlying resilience and vulnerability, as models for stress-related psychopathology usually postulate a loss of the adaptive recovery. Resting-state fMRI seems particularly suited to examine these recovery processes, because brain activity recovery patterns are not disturbed by tasks demands. In a resting-state study in healthy participants, Van Marle et al. investigated poststress amygdala-centered connectivity patterns in order to characterize the aftermath of acute, experimentally induced stress in healthy humans. The investigators recorded resting-state fMRI in 26 female participants immediately following a period of moderate psychological stress induced by means of aversive (vs. emotionally neutral) movie watching. The authors found a prolonged activation in an amygdala-connectivity network after the moderate stress, thought to reflect an extended state of hypervigilance that promotes sustained salience and mnemonic processing after stress (Van Marle et al., 2010).

In another resting-state fMRI study in healthy subjects Veer et al. (2011) examined resting-state functional connectivity during the recovery period after experimentally induced social stress. Forty participants were randomly assigned to the social stress condition or the non-stressful control condition. Resting-state fMRI scans were acquired 60 min after these conditions. In the stressed subjects resting-state fMRI showed an increase in connectivity between the amygdala and the mPFC and between the amygdala and the (PCC)/precuneus region (Veer et al., 2011). The authors interpreted this as showing the top-down inhibitory control by the mPFC and the stress-induced facilitation of self-evaluative processes, involving the default mode network, after or during salient experiences. Both processes can be considered key-elements of the behavioral homeostasis after stress, and this paradigm might be interesting to study adaptive responses in resilient subjects or prospectively.

#### TASK-RELATED FUNCTIONAL STUDIES

As is the case for most other imaging approaches described in this review, task-related functional neuroimaging studies explicitly studying resilience, i.e., using the previously mentioned design with three groups, are scarce. More task-related data are available from studies comparing PTSD subjects with TENP subjects. With disturbed regulation of (emotions evoked by) traumatic memories being a core symptom of PTSD and thought central to its pathophysiology, task-related functional studies typically have employed traumatic memory retrieval scripts to examine alterations in the regulation of emotions induced by traumatic memories. A growing line of research is studying the regulation of non-traumatic induced emotions, focussing on more general emotion regulation capacities in PTSD and TENP. Finally, some recent studies have taken hypervigilance, another core symptom of PTSD in which attention cannot be diverted, as a starting point and studied top-down attentional control systems.

#### STUDIES IN PTSD vs. NON-PTSD CONTROLS

Several functional task-related neuroimaging studies have examined brain activity in PTSD compared to TENP or healthy control subjects [for an extensive review see: Hughes and Shin (2011)]. The most consistent findings in these studies with regard to findings in TENP subjects are an increased activity of the ventral mPFC and rACC, and a relatively lower activity of the amygdala and the dACC as compared to the PTSD subjects during exposure to emotion evocative stimuli. Studies have shown a negative correlation between the increased mPFC and decreased amygdala activity, in line with the regulatory function of mPFC regions over the amygdala. As mentioned above, the first line of research discussed here has used traumatic memory retrieval paradigms, in which subjects are exposed to trauma-related stimuli, such as pictures, sounds, or individual-specific scripts (Liberzon et al., 1999).

Seminal work was done by the group of Bremner et al. (1999a,b) who exposed both Vietnam combat veterans and sexually abused women with and without the diagnosis of PTSD to memories of their trauma during PET scanning and found differences in activity of several brain areas between TENP subjects and PTSD subjects (Bremner et al., 1999a,b). Differences were found in areas involved in emotion regulation, notably in inhibition of the amygdala. In both groups of TENP subjects (war and sexual abuse), an increase in blood flow in the medial prefrontal area, including the subcallosal gyrus, middle temporal gyrus, and right rACC, compared to PTSD patients was found. However, the TENP subjects also showed decreased activity in areas not typically involved in emotion regulation like the PCC, inferior parietal cortex, lingual gyrus, and left precentral gyrus in the motor cortex. In the sexually abused TENP group, there was also increased blood flow in the right hippocampus, inferior fusiform gyrus, supramarginal gyrus, and visual association cortex relative to women with PTSD. This possibly suggests that there may be specific correlates of resilience or vulnerability for specific types of trauma (Bremner et al., 1999a).

More recent studies have predominantly used fMRI paradigms. A study in police officers using traumatic memory retrieval scripts showed an increased activity of the medial frontal gyrus during exposure to trauma scripts in TENP subjects as compared to PTSD subjects (Lindauer et al., 2004a). Traumatic memory retrieval was also used as an fMRI paradigm to examine a group of PTSD and TENP police officers who had experienced the same trauma, but with a (small) subgroup of the PTSD subjects receiving psychotherapy. After therapy symptom scores of this treatment group were similar to those of the TENP group. Subsequent analysis of the scans showed a pattern of increased activity of the mPFC and reduction of amygdala activity during traumatic memory retrieval in the therapy group, comparable to that in the TENP police officers, suggesting a key role for increased emotion regulation capacities in both resilient individuals as well as by therapy (Peres et al., 2011).

Another script-driven fMRI study showed an association between less re-experiencing and less dissociation on the one hand, and activation of the inferior frontal gyrus on the other hand (Hopper et al., 2007). This area was found to be significantly more activated in TENP subjects. Other traumatic script-driven fMRI studies have shown a greater activity of the thalamus region in TENP compared to PTSD patients (Lanius et al., 2001, 2005), which might be interpreted as more efficient information processing capacities in TENP subjects.

Another line of neuroimaging studies in PTSD and TENP subjects examined whether alterations of more general emotion processing capacities are present in PTSD and used paradigms with stimuli unrelated to the specific trauma. Tasks aimed on more general emotion processing included among others pictures of emotional faces (Rauch et al., 2000; Shin et al., 2005), memory tasks with pictures or words unrelated to trauma (Shin et al., 2004; Phan et al., 2006), the emotional counting Stroop task (Shin et al., 2001), or a multi-source interference task (Shin et al., 2011). These studies on more general emotion processing did indeed reveal a similar brain activity pattern in TENP subjects as the studies using trauma-related stimuli, with increased activity of the rACC and ventral mPFC, decreased activity of the amygdala, increased activity of the hippocampus, and a decrease in PCC activity as compared to PTSD subjects. This suggests that more general emotional information processing capacities are involved in vulnerability or resilience.

### FUNCTIONAL STUDIES INVOLVING A THREE-GROUP DESIGN WITH PTSD SUBJECTS, TENP SUBJECTS, AND HEALTHY NON-EXPOSED CONTROLS

Studies comparing PTSD subjects with TENP point at a central role of emotion regulation brain capacities in the adaptive response to trauma, but the lack of a non-exposed healthy control group does not allow any firm conclusion about whether alterations are specific for resilience or vulnerability. A small number of functional imaging studies have employed task-paradigms and included PTSD, TENP, and a non-exposed healthy control group. Britton et al. performed PET scanning during script driven imagery of emotionally evocative and neutral events in combat-related TENP subjects ($n = 15$), combat-related PTSD subjects ($n = 16$), and healthy controls ($n = 14$) (Britton et al., 2005). Emotionally evocative events included general highly stressful events as well as specific traumatic events. PTSD subjects did not show changes in amygdala activity over conditions, but showed deactivation of the rACC during stressful scripts. Healthy controls showed activation of the amygdala and deactivation of the ventral mPFC during the stressful condition, while the resilient subjects showed the same deactivation of the ventral mPFC. Importantly, they also showed a specific pattern of deactivation of the amygdala during imagery of emotionally evocative events. This can be interpreted as a resilience specific mechanism. However, another study using a similar design with three groups found no patterns of amygdala activity that were specific for the TENP group. Liberzon et al. (1999) used [99mTc]HMPAO SPECT to examine combat-exposed PTSD subjects ($n = 14$), combat-related TENP subjects ($n = 11$), and a group of healthy controls ($n = 14$) and exposed them to white noise and combat noises (Liberzon et al., 1999). Both the TENP group and healthy controls showed less amygdala activation than the PTSD subjects.

Most recent studies with a three-group design have used fMRI paradigms. As the ability to exercise voluntary control over emotional responses was found to be linked to better functioning and emotion regulation in healthy volunteers, and as discussed above, patients with PTSD show less activity in the emotion regulation circuitry when confronted with challenging negative stimuli, several researchers have hypothesized that the capacity to voluntarily or automatically regulate emotions may be a resilience factor (Charney, 2004; Yehuda et al., 2006; New et al., 2009). To directly examine this hypothesis, New et al. (2009) investigated deliberate regulation of emotion in PTSD, TENP, and healthy control groups of 14 women exposed to sexual assault (New et al., 2009). Emotionally neutral and negative pictures were presented, with the negative pictures being not related specifically to sexual assault. The participants had to focus specifically on the deliberate modification (up and down regulating) of emotional responses to the stimuli. Contrary to the general regulation hypothesis, both TENP subjects and PTSD subjects were less capable of downregulation responses to negative stimuli and showed less activity in the lateral PFC compared to healthy controls. However, the TENP subjects were more successful in upregulating their responses to negative stimuli, which was associated with increased activity in the dACC compared to both the PTSD group and the control group. Interestingly, the personality trait "optimism" was significantly correlated with the intensity of ACC activation during voluntary upregulation in TENP subjects compared to both PTSD subjects and healthy controls. This interesting preliminary result suggests that specifically the ability to deliberately engage cognitive-emotional strategies to extinguish negative emotional responses and the functional brain correlates are associated with resilience (New et al., 2009).

A last group of studies has focussed on another core symptom of PTSD, hypervigilance, i.e., the increased attentional bias to environmental threat associated cues and the decreased possibility to focus on other stimuli. Previous work in healthy controls showed that emotional attention involves amygdala priming of representations in the temporal cortex, while the involvement of top-down attentional control systems is needed to divert attention toward task-relevant stimuli and weaken (emotional) responding to (emotional) distracters (Vythilingam et al., 2007).

The few neuroimaging studies that have examined top-down attentional control in PTSD vs. TENP subject or controls did find some alterations, but also for this domain it remains unclear whether it concerns a general deficit or a specific deficit within the context of emotional distracters.

Some studies with a three-group design have tried to address this issue. Falconer et al. (2008) used a Go/No-Go fMRI task to measure inhibitory control of non-emotional stimuli in 23 PTSD patients, 17 TENP individuals, and 23 healthy controls. PTSD subjects showed deficiencies in the recruitment of right inferior frontal cortex and the ventral PFC during inhibitory control. However, there were no activation patterns specific to the resilient TENP individuals (Falconer et al., 2008). A recent twin fMRI study by Shin et al. examined whether functional task-related abnormalities of the ACC, after exposure to severe stress, are acquired characteristics or represent a familial risk. They studied combat-exposed PTSD subjects, their non-exposed co-twins (12 pairs) and combat-related TENP subjects and their co-twins (14 pairs). Subjects performed a cognitive attentional task, the multi-source interference task. Vietnam combat veterans in the TENP group and their identical co-twins showed less task-related dACC activity as compared to Vietnam combat veterans with

PTSD and their identical co-twins during the interference task. The dACC activity in the non-exposed twins predicted the severity of the symptomatology in the PTSD subjects (Shin et al., 2011). The results suggest that hyperresponsivity of dACC is a familial risk factor for PTSD. The relative hyporesponsivity in the TENP subjects and their co-twins could be a familial resilience factor.

Recently, Blair et al. (2013) performed an fMRI study on attentional control in 15 TENP individuals, 14 patients with PTSD, and 19 healthy controls. They also explicitly specified a hypothesis on the pattern in the resilient group, expecting resilient subjects to show superior recruitment of regions involved in top-down emotional attention relative to the other two groups during the task performance. Subjects performed the affective number Stroop task, with positive, negative, and emotional pictures selected from the international affective picture system presented as emotional distractors. The PTSD group showed deficiencies in the recruitment of lateral regions of superior and inferior frontal cortex, corresponding with the findings of Falconer et al. (2008), but also a deficiency of recruitment of the parietal cortex that appeared only in the presence of negative distracters (Blair et al., 2013). As hypothesized, the resilient subjects showed an enhanced ability to recruit regions involved in top-down attentional emotional control when compared to the matched healthy controls and the PTSD subjects. Taken together, these studies suggest that deficiencies in the recruitment of especially inferior frontal regions during top-down attentional control in general are specific to PTSD, but resilience specific activity patterns are only present during top-down control of emotional attention.

## TRAIT RESILIENCE

Research has shown that specific personality characteristics contribute to vulnerability and resilience to stress. The Big Five model of personality traits is a widely used model in which individual differences in personality are described in five overall personality factors: neuroticism (also referred to as absence of emotional stability), extraversion, openness, agreeableness, and conscientiousness (Friborg et al., 2005). Studies have shown that neuroticism is a risk factor for the development of PTSD (Breslau et al., 1991; Nakaya et al., 2006). A resilient personality profile was found to consist of low neuroticism, high extraversion, and conscientiousness, but also high scores on openness and agreeableness (Friborg et al., 2005; Campbell-Sills et al., 2006). High trait resilience also coincides with a construct with high scores on optimism, low neuroticism, and behavioral activation sensitivity (Block and Kremen, 1996).

Sub-facets of the Big Five personality traits that are thought to contribute to resilience are high self-esteem, internal locus of control, flexibility in thinking, sense of meaning, and problem-solving skills (Bryant et al., 2011; Daniels et al., 2012). High levels of these traits, together with a fast physiological recovery have been shown to enhance recovery from experimentally induced stress (Tugade and Fredrickson, 2004). Posttraumatic adjustment could also be enhanced by sub-facets of the personality characteristics that include hardiness, believing in having an influence on one's surroundings and also the ability to learn from both positive and negative experiences (Daniels et al., 2012). Furthermore, individuals with high scores on conscientiousness, extraversion and agreeableness are more likely to have a secure and stable environment with supportive social relationships, which also contributes to successful adaptation to stress (Friborg et al., 2005; Daniels et al., 2012).

We identified one study that has examined neural correlates of resilient personality traits, focussing on arousal regulating capacities in healthy volunteers. Waugh et al. (2008) studied the functional neural correlates of trait resilience during anticipation, but also during recovery from threat (Waugh et al., 2008). They operationalized emotional resilience as the flexible and appropriate use of emotional resources. In their event-related fMRI design, healthy participants viewed "threat" cues signaling the possibility of either viewing an aversive picture or a neutral picture, and "non-threat" cues, signaling the viewing of only a neutral picture. High-trait resilient participants exhibited less early and less prolonged insula activity to the neutral pictures shown after a "threat" cue than low-trait resilient participants, indicating quicker and more appropriate adaptation to the neutral stimulus by the high resilient subjects.

In a very interesting small study Daniels et al. (2012) prospectively investigated the neural correlates mediating the relationship between trait resilience and the recovery from a traumatic event. They used a convenience sub-sample of 12 acutely traumatized subjects, derived from a larger sample of 70 acutely traumatized subjects recruited at an emergency department, and fulfilling the DSM-IV PTSD criterion A. Subjects were followed-up for several months to monitor the development of PTSD symptomatology. Trait resilience was assessed with the Connor-Davidson resilience scale (CD-RISC) (Connor and Davidson, 2003). Trait resilience was found to predict a better outcome throughout the first 3 months of follow-up. A trauma script-driven symptom provocation fMRI paradigm with neutral and trauma scripts was used to investigate neural correlates of trait resilience two to four months posttrauma. For imagery of the traumatic vs. the neutral event, CD-RISC scores showed a positive correlation with activity in the right inferior and middle frontal gyrus and the right thalamus. As these regions are known to be involved in arousal regulation and emotional reappraisal, the findings can be interpreted as pointing toward the broader concept of emotion regulation as the mediator between trait resilience and posttraumatic adjustment (Daniels et al., 2012).

## DISCUSSION

Whereas research into psychological factors contributing to resilience is longstanding, only more recently studies have begun to examine biological factors in resilience in humans and their interplay with psychological and environmental factors. Insight into biological factors underpinning resilience to stress may open new avenues for prevention and treatment of stress-related disorders (Charney, 2004). Neuroimaging has become an increasingly important tool to study neural correlates of behavior and to elucidate the role of neural mechanisms in the interaction between genes and environment (Meyer-Lindenberg and Tost, 2012). In a seminal review paper in 2004, Charney stated that with the recent advances it would be possible to create more comprehensive psychobiological models of

the ordinary magic of resilience (Charney, 2004). Based on our present review we have to conclude that neuroimaging of resilience is still in its early stages, with only a limited number of studies allowing to specifically examine functional or structural brain characteristics that may contribute to resilience.

Based on findings in studies comparing stress-related psychopathologies, especially PTSD, with healthy controls, the neural circuitry of resilience is usually postulated to overlap with the brain circuitry involved in emotion and stress regulation. Data from imaging studies comparing TENP subjects with PTSD subjects do indeed find important differences in structural and functional characteristics of emotion regulating brain circuitries, putatively underlying or reflecting increased emotion regulation capacities in TENP subjects. By and large, this pattern is also found in the few studies in which subjects with PTSD, TENP subjects and healthy, non-exposed control subjects are compared. Structural studies point at increased gray matter volumes in structures such as the hippocampus, the ventral mPFC, and the rACC and sgACC. Subsequently, functional studies show increased activity in these structures during tasks using emotion evocative stimuli, such as the traumatic script-driven paradigm. The ventral mPFC, rACC, and sgACC exert top-down control over the amygdala and the stress system, which is putatively more efficient or increased in resilience. In addition, the hippocampus is known to be involved in the processing of traumatic experiences, but also in regulation of the stress system. The mPFC also processes traumatic memories and is involved in the regulation of extinction learning and the modulation of fear responses.

Various subregions of the ACC have been found to be involved in emotion regulation, among others through inhibition of the amygdala. In line with this a decreased reactivity of the amygdala together with an increased rACC activity was found in several studies in TENP subjects. The association between resilience and increased regulation of amygdala activation is further supported by a study showing that symptoms of PTSD were very low in combat veterans with unilateral damage in the ventral mPFC or amygdala (Koenigs et al., 2008). It is also thought that adaptive processes after trauma exposure may occur through functional interactions between the mPFC, ACC, and amygdala (Osuch et al., 2008). In line with the findings in humans, a non-human primate study demonstrated alterations of volume and myelination of the ventral mPFC in animal showing reduced stress responsiveness after an inoculation paradigm (Katz et al., 2009). It should be noted that although the majority of the available studies seems to point at the neurocircuitry involved in aspects of emotion and arousal regulation, studies examining functional connectivity do suggest that in resilience, the connectivity of an amygdala-prefrontal network with several other functional networks, such as the default mode network or the salience network, also plays a role.

Another area of neuroimaging research from which insight into possible neural mechanisms underlying resilience may be derived, is that of the neural correlates of personality traits known to facilitate the adaptation to severely stressful situations. This is particularly the case for trait-resilience, a meta-construct involving traits like low neuroticism, high extraversion, and conscientiousness, with several of these traits known to be linked to the characteristics of the neurocircuitry involved in emotion and stress regulation. The few existing neuroimaging studies examining high vs. low-trait resilient subjects found that high trait resilient subjects are characterized by a brain pattern reflecting more efficient arousal modulation and emotional reappraisal. These patterns overlap with the areas and circuitries that were identified in TENP subjects, again suggesting that the broader concept of emotional control and its neural substrate may indeed be pivotal in resilience.

As resilience is probably best conceptualized as a dynamic, context-dependent phenomenon it could be hypothesized that some of the elements specific for resilience only develop during or after the experience of a traumatic event. However, as most available research in humans is cross-sectional, no causal conclusions can be drawn on the temporal order of trauma exposure and brain changes observed in resilient individuals. Moreover, as in the studies conducted so far resilience has been most frequently defined as the absence of PTSD, although data on symptomatology of other trauma-related disorders were gathered in the majority of the discussed studies, the strictness of the exclusion criteria varied between studies. Therefore, it cannot be excluded that the TENP individuals did suffer, to some extent, from (subclinical) depressive or anxiety symptomatology or substance abuse. Hence, it remains uncertain whether the presented findings are specific for the absence of PTSD psychopathology, or for the absence of psychopathology after exposure to trauma altogether.

Given the current state of the art of neuroimaging of resilience as laid out by this review several avenues to gain further insight into the neural mechanisms involved in resilience can be chosen. Ideally, the neural correlates of resilience should be studied longitudinally. Neurobiological and other variables potentially related to resilience would be measured before and after an individual has been exposed to a severe stressor, after which key variables are assessed over time among individuals who develop trauma-related psychopathology, individuals who do not develop psychopathology, and a control group that has not been exposed to trauma. This would allow the identification of baseline "predictors" of resilience as well as that of potential mediators on different levels, i.e., psychological, (epi)genetic, biochemical and neural, and examine their interaction. A homogeneous cohort of subjects for such a longitudinal study is probably most easily recruited amongst first responders or military personnel, who usually are already assessed extensively before active duty. A caveat is that these populations may consist of a selection of resilient individuals. Such a design would also allow to further examine patterns of resilience based on trajectories of psychological complaints after exposure. In addition, this design would enable examination of the temporal stability of neural correlates of resilience (as assessed at baseline) and their malleability by exposure to a severe stressor.

A first approach could be to focus on the structural neural correlates of resilience. Based on our present review of the structural neuroimaging research of resilience, we would postulate resilience to be associated with alterations of gray matter volume and

structure of especially the (ventral) mPFC before the exposure to severe trauma. In addition to studying gray matter volume with both region of interest (i.e., mPFC) and whole brain approaches, we suggest to subsequently examine both shape and corticol thickness of the brain. Examining structural connectivity by means of DTI scans would also be an important approach for the structural neuroimaging of resilience. Taking into account the neurocircuitry of stress, the findings in PTSD as well as those in animal studies, the structural connectivity of white matter tracts that connect limbic structures with the ventral PFC and subregions of the ACC would be a clear focus. We would hypothesize increased structural connectivity between these areas, underlying increased emotion regulation capacities, in resilient individuals.

A second approach would be to focus on the functional neural correlates of resilience and their temporal characteristics. Based on our review we believe more general emotion and arousal regulation capacities to underlie resilience, with resilient subjects being especially more capable in upregulating their emotions and having top-down control over emotional attention. However, so far only explicit emotion regulation was examined, and the role of automatic emotion regulation capacities, which may have a stronger neural correlate, has not yet been assessed with functional neuroimaging. In addition to emotion regulation paradigms, we think that a broader design should incorporate novel neuroimaging task paradigms that have not been used to examine functional neural correlates of resilience yet. One would be to visualize the neural activity during acute, non-trauma-related stress, using a paradigm such as the Montreal imaging stress task (Dedovic et al., 2005). We would postulate that during this acute social stress paradigm, resilient individuals exert more control over their limbic system by increasing activity of especially their ventral PFC and rACC. In addition to studying neural characteristics of resilience during stress, neural characteristics of resilience during anticipation, and recovery of stress should be incorporated in the same design. This will allow us to get a complete oversight of neural activity patterns used by resilient individuals in order to process stress over time, from anticipation pre-stress up until recovery during the aftermath of stress. Waugh et al. (2008) already showed patterns of brain activity specific for trait resilient individuals during anticipation and recovery of threat (Waugh et al., 2008). Subsequently, some recent resting-state studies have, as we discussed in the present review, focussed on the immediate adaptive recovery after acute (social) stress. This adaptive recovery, which may be a key element in a more dynamic concept of resilience, was not only associated with involvement of emotion regulation circuitry, but also with that of circuits involved in self-referential processing (Lanius et al., 2010; Veer et al., 2011). Furthermore, based on research from other domains, it can be hypothesized that other brain circuits (e.g., underlying social affiliation, reward dependence, but also higher order cognitive skills) are also involved in resilience and should be investigated in concert (Charney, 2004).

Two other avenues to elucidate neural mechanisms in resilience can be considered as stand-alone cross-sectional designs, but also as baseline assessment in a larger scale, longitudinal pre-post exposure design as described above. One would be to further examine neural correlates of trait resilience, a concept probably directly linked to resilience to psychotrauma. This would theoretically require no special study populations, but so far only a few studies have investigated the structural and functional neural correlates of trait resilience. Another avenue would be to examine the functional and structural effects of stress inoculation or "steeling" paradigms in humans, building on the work in non-human primates. This could not only shed more light on changes in neural mechanisms underlying increased resilience, but may also identify potential neural predictors of response to inoculation. An interesting question would be whether inoculation paradigms in adults result in (inoculation) specific or more general adaptations of especially functional neural mechanisms. We are not aware of any neuroimaging studies that have focussed on the effects of inoculation in humans. Finally, we believe that studies examining the functional and structural correlates of resilience could be enriched by including other neurobiological measures, such as measures for autonomic nervous system reactivity, and by examining the genetic influences on brain structure and function.

In conclusion, several years after the seminal review of Charney (2004), neuroimaging of resilience still seems to be in its infancy, but is expected to benefit from the increasing interest in the "positive" concept of resilience and may be informed by neuroimaging approaches already more widely used in affective disorders and normal behavior, and state-of-the art strategies such as neuroimaging approaches for complex gene-environment interactions (Charney, 2004; Meyer-Lindenberg and Tost, 2012).

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# Restoration of hippocampal growth hormone reverses stress-induced hippocampal impairment

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Though growth hormone (GH) is synthesized by hippocampal neurons, where its expression is influenced by stress exposure, its function is poorly characterized. Here, we show that a regimen of chronic stress that impairs hippocampal function in rats also leads to a profound decrease in hippocampal GH levels. Restoration of hippocampal GH in the dorsal hippocampus via viral-mediated gene transfer completely reversed stress-related impairment of two hippocampus-dependent behavioral tasks, auditory trace fear conditioning, and contextual fear conditioning, without affecting hippocampal function in unstressed control rats. GH overexpression reversed stress-induced decrements in both fear acquisition and long-term fear memory. These results suggest that loss of hippocampal GH contributes to hippocampal dysfunction following prolonged stress and demonstrate that restoring hippocampal GH levels following stress can promote stress resilience.

**Keywords: growth hormone, hippocampus, stress, fear, gene therapy, conditioning**

## INTRODUCTION

Stress is defined by a constellation of responses that occur when the body's ability to cope with physical or psychological demands is exceeded (McEwen and Wingfield, 2007). Stress exposure can vary in duration, and it is clear that stress "load," defined by both the length of exposure as well as the number of stressors present, plays a role in determining the consequences of stress (Juster et al., 2011). Short-term stress is thought to recruit adaptive responses that promote coping and resilience. However, the mechanisms for driving adaptive change may be difficult to maintain in the face of repeated challenge, and maladaptations can occur when stress is prolonged (McEwen, 1998). For example, high stress load is a risk factor for the development of numerous types of affective mental illness, particularly those involving fear and anxiety (Mazure, 1995; Belanoff et al., 2001; Lederbogen et al., 2011). Despite an abundant literature on the effects of stress in the brain, most studies have focused on the effects of acute stress. Thus, the mechanisms that lead to maladaptations following chronic stress exposure remain unclear.

While there are many brain regions that are altered by stress and mediate stress-associated changes in behavior, the hippocampus is the region in which the effects of stress are best characterized. The hippocampus plays a role in many types of memory (Jeneson and Squire, 2012), and is also linked to affective regulation (Bangasser and Shors, 2007; Goosens, 2011). Acute stress can both enhance and impair hippocampal function. For example, acute stress can increase (Shors et al., 2001) or decrease (Chen et al., 2008) hippocampal dendritic spine density. Acute stress can also enhance (Shors, 2001) or impair (de Quervain et al., 1998) hippocampus-dependent cognition, an effect that may depend on the level of arousal attained during the stress (Diamond et al., 2007). In contrast, chronic stress generally produces dendritic retraction in hippocampus (Watanabe et al., 1992; Magarinos and McEwen, 1995; Vyas et al., 2002; Sandi et al., 2003), and impairs performance on hippocampus-dependent memory tasks (Nishimura et al., 1999; Pawlak et al., 2005; Kleen et al., 2006). These changes are thought to be mediated, in part, by stress hormone-induced downregulation of growth factors, such as brain-derived neurotrophic factor, in neurons (Lakshminarasimhan and Chattarji, 2012).

Growth hormone (GH) is released into the circulating blood stream by the pituitary, but it is also synthesized by the hippocampus and other brain regions (Nyberg and Burman, 1996; Sun et al., 2005) where it may act as a local neuromodulator. Within the hippocampus, application of exogenous GH is sufficient to induce synaptic plasticity (Zearfoss et al., 2008). Exogenous GH also facilitates hippocampal synaptic transmission (Mahmoud and Grover, 2006; Molina et al., 2012) and hippocampus-dependent eyeblink conditioning is associated with enhanced GH protein synthesis in hippocampal cells (Donahue et al., 2002). Interestingly, hippocampal GH levels are stress-sensitive: GH gene transcription is regulated by glucocorticoid stress hormones (Treacy et al., 1991) and GH protein levels are increased one day after an acute stress exposure (Donahue et al., 2006). These findings suggest that higher levels of hippocampal GH may promote hippocampal function, but these studies are correlational. Here, we examine hippocampal GH following chronic stress and explore the relationship between GH and stress-related changes in hippocampal function by using viral-mediated gene transfer to manipulate GH levels in stressed and unstressed rats prior to training on one of two hippocampus-dependent behavioral tasks. While the hippocampus is a complex structure, and it plays a role in many aspects of memory, we focused on two well-characterized yet distinct aspects of hippocampal function: the role of the hippocampus in forming contextual representations (Maren et al., 2013), assayed by contextual

fear conditioning, and the role of the hippocampus in maintaining a memory "trace" over a delay interval (Shors, 2004), assayed by auditory trace conditioning.

## MATERIALS AND METHODS

### SUBJECTS

All experiments used adult male Long–Evans rats (225–275 g, Taconic, Germantown, NY), housed individually (20–22.2°C; 12 h light-dark cycle, 0700 lights on). Rodent chow and water was provided *ad libitum*. Stressed and unstressed rats were housed in separate cubicles. All procedures were in accordance with the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the MIT Institutional Animal Care and Use Committee, and the Animal Care and Use Review Office (ACURO) of the Army Research Office.

### IMMOBILIZATION STRESS

Immobilization stress was administered for 4 h per day for 10 (contextual fear conditioning experiment) or 14 (trace fear conditioning experiment) consecutive days. Rats were placed in Decapicone plastic bags (Braintree Scientific; Braintree, MA), which were secured at the tail to keep the bagged rat in an upright position. Stress occurred in an isolated lab room, separate from all behavioral testing space. All stress sessions were performed between 1000 and 1600. Unstressed control rats were handled daily for 30 s.

### GROWTH HORMONE ELISA

Hippocampi were homogenized in homogenization buffer (2% HALT protease cocktail and 0.15% NP-40 in PBS; 6 ul buffer per 1 mg of tissue) using a LabGEN 125 homogenizer (Cole-Parmer; Vernon Hills, IL) for 8–10 s on ice. After 5 min of incubation on ice, tubes were spun at 18,000 g for 20 min at 4°C and the supernatant was transferred to a new tube. The resulting solution was assayed as per manufacturer's protocol (Millipore; Billerica, MA).

### AMPLICON CONSTRUCTION

The rat presomatotropin gene was cloned as an 818 bp HindIII cut fragment from the p-RGH1 plasmid (Seeburg et al., 1977), provided by Dr. Douglas Weigent (University of Alabama at Birmingham), into the HindIII cloning site of the HSV amplicon plasmid pα22GFP (Kaufer et al., 2004), in which a bicistronic HSV-based promoter simultaneously drives expression of a transgene from the α-4 promoter and enhanced green florescent protein (eGFP) from the α-22 promoter. The pα22GFP plasmid was used as a control.

### VIRUS PREPARATION

Virus was generated using standard methods (Lim and Neve, 2000). Briefly, plasmids were amplified to generate endotoxin-free DNA, which was transfected into 2–2 cells. The next day, cells were superinfected with 5*dl*1.2 helper virus. After two days, the cells were frozen and thawed three times, sonicated to release infectious viral particles, and centrifuged to clear the medium of cell debris. The resulting supernatant was twice passaged onto 2–2 cells. After the final sonication and centrifugation, the supernatant was purified on a sucrose gradient, pelleted, and resuspended in 10% sucrose in D-PBS. Aliquots of each amplicon were stored at −80°C until use. Amplicon titers ranged from1 to $4 \times 10^8$ IU/ml. Within each experiment, control and GH-expressing viral titers were similar titers.

### PROTEIN (WESTERN) IMMUNOBLOT

Vero cells were plated in 6 cm dishes using standard methods (Lim and Neve, 2000). Purified HSV virus was used to infect cells at multiplicities of infection ranging from 0 to 0.2. After three days, cells were harvested and homogenized. Protein was loaded on to gels for electrophoretic transfer. Membranes were incubated, in succession, with the following primary antibodies overnight at 4°C: 1:5000 rabbit anti-GH (National Hormone and Peptide Program, NIDDK), 1:500 mouse anti-GFP (Roche, Indianapolis, IN), 1:1000 mouse anti-Actin (Millipore; Billerica, MA). Following incubation in secondary antibody, immunoreactivity was visualized using chemiluminescent detection.

### STEREOTACTIC VIRUS DELIVERY

Surgery was performed 18–24 h following the final handling or immobilization stress session. Rats were anesthetized (with either Nembutal at 65 mg/kg, or a ketamine:xylazine:acepromazine cocktail at 100:100.10 mg/kg, i.p.) and mounted in a stereotaxic frame. Small holes were drilled for intra-cranial placement of the injector within the dorsal hippocampus: A/P −3.3 mm, M/L ±2.0 mm, D/V −3.2 mm, relative to brain surface and bregma (Paxinos and Watson, 2005). Virus was infused with either pulled glass pipettes or 33 g stainless steel bevel needles attached to a 10 μl Hamilton syringe (Hamilton Company, Reno, NV). The pipettes or syringes were mounted in stereotaxic barrel holder, and the rate of virus delivery was controlled by a syringe pump (Harvard Apparatus, Holliston, MA). Virus was infused at 0.1 μl/min for 20 min (2 μl total volume per hemisphere). Injectors remained in the brain for 10 min before being withdrawn. Incisions were closed with wound clips and Ketoprofen (1 mg, s.c.) was administered for pain and inflammation.

### PAVLOVIAN FEAR CONDITIONING

All behavioral testing commenced 72 h after stereotactic viral delivery, a time point corresponding to maximal transgene expression with HSV-based viral vectors (Lim and Neve, 2000). Fear conditioning experiments were conducted in a modified chamber (MED Associates; St. Albans, VT) housed in a sound-attenuating cubicle. For auditory trace fear conditioning, rats were placed in individual chambers in a novel context (house and room lights on, 1% acetic acid, grid floors) for 5 min before receiving tone (20 s, 2 kHz, 85 dB)-footshock (1 s, 0.85 mA) pairings, with the stimuli separated by a 35 s trace interval. A 3 min inter-trial interval (ITI) was used. Long-term contextual fear memory was assessed 24 h later, when the rats were returned to the chambers for a 5 min context extinction test. Long-term auditory fear memory was measured 24 h later; the rats were placed in the chamber configured as a novel context (room and house lights off, 0.3% Pine Sol odor, white Plexiglas floor and wall inserts). Rats were allowed 3 min to habituate to the chamber before 4 tones (85 dB, 2 kHz) were presented with ITIs of 3 min 35 s. For contextual fear conditioning, rats were placed in

a novel context (house and room lights on, 0.3% Pine Sol, grid floors) for 3 min before receiving 3 unsignaled footshocks (2 s, 0.5 mA) separated by a 90 s inter-stimulus interval. Long-term contextual memory was measured 24 h later, when the rats were returned to the context for an 8 min context test. Infrared video was recorded throughout all sessions. Freezing was measured offline using commercial software (VideoFreeze, MedAssociates, St. Albans, VT).

### HISTOLOGY

Following completion of the experiment, animals were anesthetized with an overdose of isoflurane and the brains were removed from the cranium. Brains from animals that experienced behavioral testing were bisected along the midline. The dorsal hippocampus was dissected from one hemisphere, placed in a sterile eppendorf tube, and flash frozen in dimethylbutane on dry ice. The tissue was stored at −80°C until GH levels were quantified by ELISA to compare expression in stressed and unstressed control animals. The other hemisphere was placed in 4% paraformaldehyde for 72 h then transferred to a 30% sucrose/4% paraformaldehyde solution for a minimum of 3 days. Hemispheres extracted for each treatment were counterbalanced. Fixed tissue was cut into coronal sections on a cryostat (40 μm) and mounted on slides. Sections were assessed for GFP florescence. Animals with incorrect placements were excluded from all analyses. Brains used to assay viral expression of GH levels were placed in a brain matrix and sliced into coronal sections (2 mm thick; 1 mm on either side of the injection site, visible from the dorsal brain surface). The dorsal hippocampus was dissected from each section and flash frozen. The tissue was stored at −80°C until GH levels were quantified by ELISA.

## RESULTS

It has been shown that an acute stress exposure leads to elevated hippocampal GH (Donahue et al., 2006) and enhanced performance on delay eyeblink conditioning (Shors et al., 1992), a hippocampus-dependent task. However, it is not known how GH is affected by chronic stress. To address this, we quantified GH levels in the dorsal hippocampus of rats after either 14 consecutive days of immobilization stress (STR) or daily handling (no stress, or NS). Hippocampal GH was dramatically downregulated following chronic stress (**Figure 1**; group: $F_{(1,\ 6)} = 8.29$, $p < 0.05$). This finding reaffirms that acute and chronic stress can produce very different effects on the brain.

To explore whether restoration of hippocampal GH following chronic stress would rescue stress-related impairment on hippocampus-dependent tasks, we constructed an HSV-1 based amplicon in which the full-length gene for rat presomatotropin (rGH), the precursor molecule for GH (Seeburg et al., 1977), was co-expressed with green fluorescent protein (GFP) under the control of bicistronic viral promoters (**Figure 2A**). This amplicon, as well as a control amplicon expressing only GFP, was packaged into replication-defective HSV viral vectors. To confirm that the viral vector was working as designed prior to *in vivo* application, we used Western blot with an antibody against rGH and showed that the GH viral vector produced GH protein in infected, dissociated cell cultures, and higher levels of infection led to higher levels

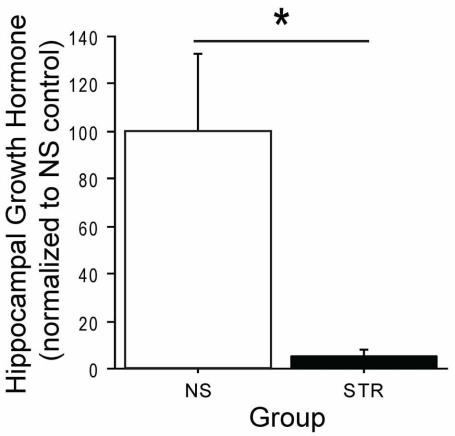


**FIGURE 1 | Chronic stress reduces hippocampal growth hormone (GH).** Hippocampal GH levels were assayed seven days after a two week period of immobilization stress (STR; $n = 4$) or handling (NS; $n = 4$). GH levels were expressed as a percentage, relative to the NS control average. GH was significantly lower in the hippocampi of stressed rats relative to unstressed rats. *Indicates $p < 0.05$.

of expressed protein (**Figure 2B**). We then used these vectors to infect the dorsal hippocampus of rats (**Figure 2C**). The majority of infected cells were pyramidal neurons in areas CA1 and CA2 of the dorsal blade of the hippocampus, with varying levels of infection in the granule cell layer of the underlying dentate gyrus. Rats with infection in the overlying cortex were excluded from the experiment. After four days for post-operative recovery, at a time point that corresponds to peak HSV-mediated gene expression, we quantified the expression of GH protein in unstressed animals that received intra-hippocampal infusions of either the GH or control viral vector (**Figure 2D**). Overexpression of GH led to an approximate doubling of the GH protein in the infected dorsal hippocampus (**Figure 2D**; group: $F_{(1,\ 10)} = 7.84$, $p < 0.05$), suggesting that our infection parameters could approximate physiological GH levels when used in stressed rats where GH levels are nearly depleted (**Figure 1**).

We first examined the role of GH in chronic stress-related changes in auditory trace fear conditioning, a hippocampus-dependent task (Raybuck and Lattal, 2011). Rats were repeatedly exposed to daily immobilization stress (STR) or handling (NS). One day later, rats received intra-hippocampal infusions of either GH or GFP virus. After three days for recovery, rats were subjected to auditory trace fear conditioning. Over the following two days, long-term contextual fear memory and auditory trace fear memory were assessed. Stress did not affect the rapid acquisition of auditory trace fear conditioning (**Figure 3A**; stress: $F_{(1,\ 25)} = 0.02$, $p =$ ns), and this was not differentially impacted by GH expression (Infusion × Stress interaction: $F_{(1,\ 25)} = 0.17$, $p =$ ns). In contrast, the effects of GH expression on long-term contextual and trace auditory fear memory did depend on stress (**Figure 3B**; Infusion × Stress interaction: $F_{(1,\ 25)} = 3.22$, $p = 0.08$; and **Figure 3C**; Infusion × Stress interaction: $F_{(1,\ 25)} = 5.99$, $p < 0.05$). Whereas rats in the STR-GFP group showed lower levels of conditional freezing than rats in the NS-GFP

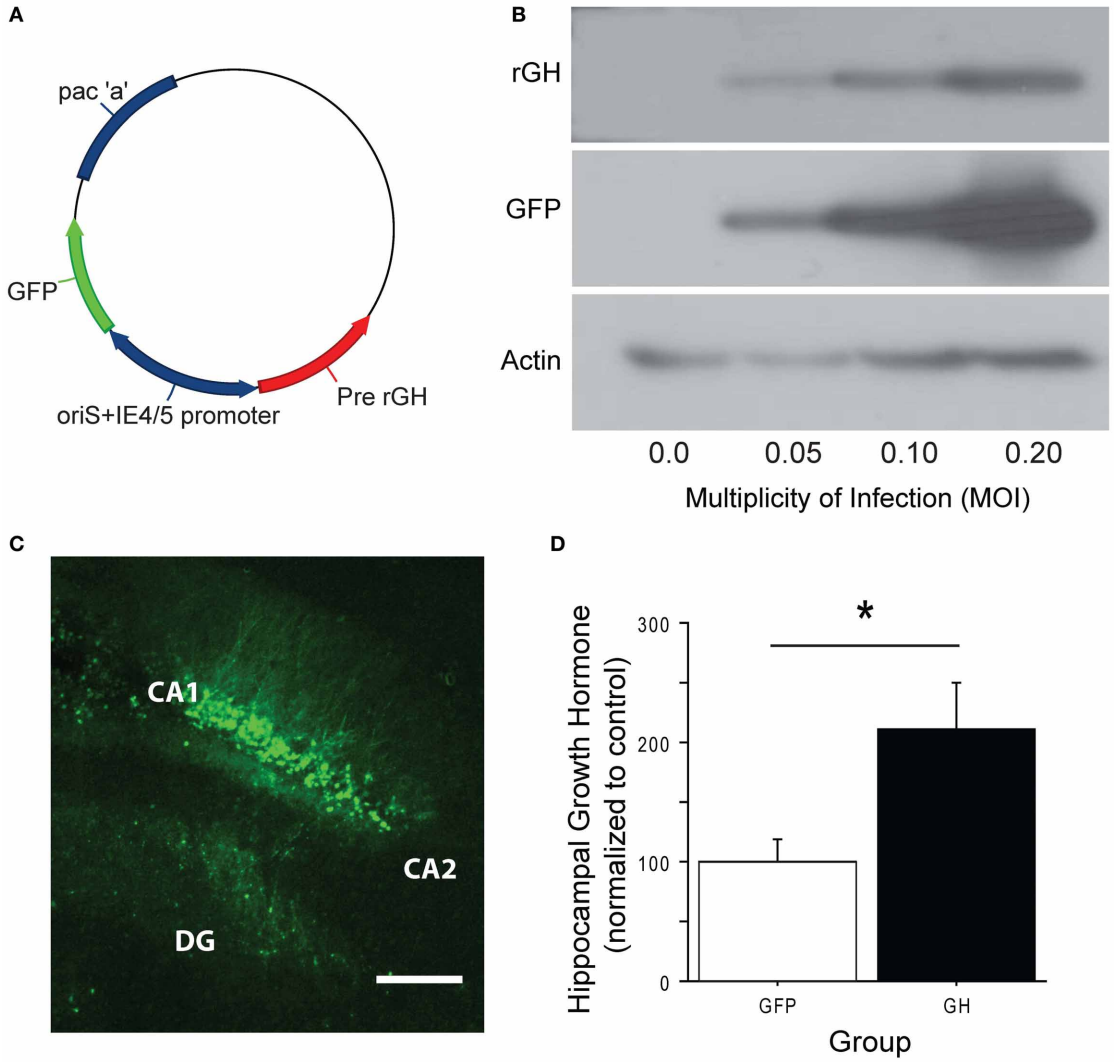


**FIGURE 2 | Construction of an HSV-1 viral vector to overexpress GH.** **(A)** The full-length gene for presomatotropin was cloned into an HSV-1 amplicon under the control of the HSV α-4 promoter. eGFP was co-expressed via the HSV α-22 promoter. **(B)** GH protein expression was confirmed *in vitro*. Vero cells were infected with GH virus at increasing MOIs. As the MOI increased, progressively higher levels of both GH and eGFP were detected. **(C)** The viral vector was infused into the dorsal hippocampus of rats. A representative infection, showing high levels of expression in pyramidal cells of CA1, and sparse infection in the granule cell layer of the dentate gyrus, is shown. Scale bar = 100 microns. **(D)** GH protein expression was quantified in infected dorsal hippocampal slices four days following virus delivery. GH levels were expressed as a percentage, relative to the NS control average. Viral overexpression of GH led to an approximate doubling of GH protein. *Indicates $p < 0.05$.

group, rats in the STR-GH group displayed levels of conditional freezing that were statistically indistinguishable from those displayed by rats in the NS-GFP group (**Figures 3B,C**, *post-hoc* comparisons). These results suggest that the impairments in hippocampal function following chronic stress can be attributed to the loss of hippocampal GH.

To further investigate this, we also examined the role of GH in stress-related changes in foreground contextual fear conditioning, a hippocampus-dependent task (Anagnostaras et al., 2001). Rats were repeatedly exposed to daily immobilization stress (STR) or handling (NS). One day later, rats received intra-hippocampal infusions of either GH or GFP virus. After recovering for three days, rats were subjected to contextual fear conditioning. Long-term contextual memory was measured the next day. The effects of stress on contextual fear acquisition were dependent on the type of virus that had been infused in the hippocampus (**Figure 4A**; Stress × Infusion interaction, $F_{(1,\ 10)} = 1.35$, $p < 0.01$): rats in the STR-GFP group displayed slower acquisition than rats in the STR-GH group (*post-hoc* comparisons). In contrast, rats in the NS-GFP and NS-GH groups acquired fear at virtually identical rates (*post-hoc* comparisons). Similar effects of GH were observed for long-term contextual memory (**Figure 4B**; Stress × Infusion interaction, $F_{(1,\ 10)} = 5.29$, $p < 0.05$): intra-hippocampal GH rescued the memory-impairing effects of stress, leading to conditional freezing levels indistinguishable from NS-GFP controls (*post-hoc* comparisons). However, intra-hippocampal GH in unstressed controls produced a mild impairment in conditional freezing, relative to NS-GFP

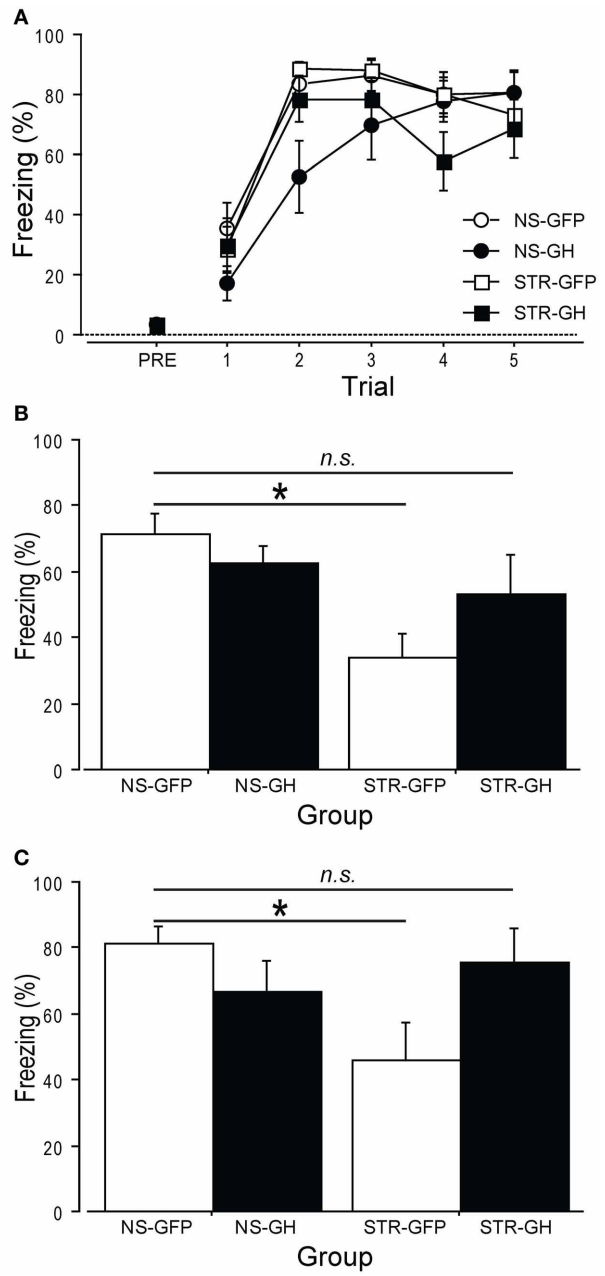


**FIGURE 3 | Overexpression of hippocampal GH rescues stress-related impairments in auditory trace conditioning.** Two weeks of daily immobilization stress (STR) or handling (NS) were administered to rats. Twenty-four hours after the last session, GH or GFP virus was infused bilaterally into the dorsal hippocampus. (NS-GFP, $n = 9$; NS-GH, $n = 7$; STR-GFP, $n = 7$; STR-GH, $n = 6$). **(A)** After three days of recovery, auditory trace conditioning was administered. Neither stress nor GH administration affected conditional freezing during training. **(B)** Long-term contextual fear memory was measured the next day by returning rats to the conditioning context for 5 m. Stress impaired contextual fear memory, and intra-hippocampal GH expression partially reversed this impairment. **(C)** Long-term auditory fear memory was measured the following day by placing the rats in a novel context and presenting four tones in the absence of footshock. Stress impaired auditory fear memory, and intra-hippocampal GH expression fully reversed this effect. *Indicates $p < 0.05$ in a *post-hoc* comparison.

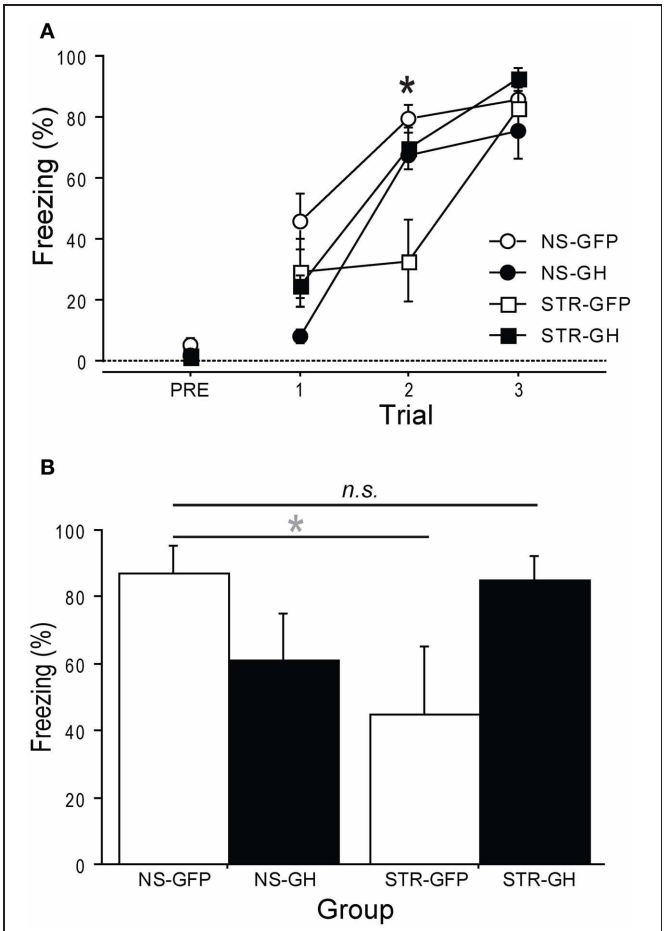


**FIGURE 4 | Overexpression of hippocampal GH rescues stress-related impairments in contextual conditioning.** Ten days of daily immobilization stress (STR) or handling (NS) were administered to rats. Twenty-four hours after the last session, GH or GFP virus was infused bilaterally into the dorsal hippocampus. (NS-GFP, $n = 4$; NS-GH, $n = 3$; STR-GFP, $n = 4$; STR-GH, $n = 3$). **(A)** After three days of recovery, contextual fear conditioning was administered. Stress slowed contextual fear acquisition (*Indicates $p < 0.05$ in *post-hoc* comparisons between STR-GFP and other groups), and this was prevented by intra-hippocampal GH. In contrast, intra-hippocampal GH had no effect in unstressed control rats. **(B)** The next day, the rats were returned to the context for an 8 m context extinction session. Stress impaired long-term contextual fear memory, and this impairment was rescued by expression of GH in the dorsal hippocampus. In contrast, intra-hippocampal GH tended to produce a mild impairment of long-term contextual fear memory in unstressed control rats. *Indicates $p < 0.1$ in a *post-hoc* comparison.

controls ($p = 0.09$; *post-hoc* comparison). These results provide further support for the idea that a loss in hippocampal GH contributes to stress-related impairment in hippocampal function.

## DISCUSSION

Here, we show that chronic stress induces a profound and lasting downregulation of GH in the dorsal hippocampus. Rats that experienced chronic stress also exhibited significant impairment on two hippocampus-dependent tasks. It is tempting to speculate that a stress-induced loss of hippocampal GH may contribute to

stress-related impairment in hippocampal function, though we did not explicitly test whether a loss of GH is sufficient to lead to impairment of hippocampal function. When GH levels were increased in stressed animals using viral-mediated gene transfer, the rats did not exhibit any stress-related decrements in performance. This shows that, regardless of the root cause of stress-related impairment of hippocampal function, restoration of GH after stress termination is sufficient to reverse these changes. While both of the tasks that we used to assay hippocampus-dependent behaviors involve fear, it is highly unlikely that the role of hippocampal GH is specific to tasks involving fear. Indeed, high levels of hippocampal GH are associated with better performance on tasks requiring other facets of hippocampal function, such as working memory (Ramis et al., 2013). Thus, GH likely plays a broad role in hippocampal function.

It is interesting to speculate about the mechanisms engaged by GH signaling following viral expression in the stressed brain. Chronic GH has been shown to upregulate the NR2B subunit of the NMDA receptor (Le Greves et al., 2002), which could lead to enhanced hippocampal function by prolonging neuronal excitation and enhancing long-term plasticity (Tang et al., 1999). While there are no studies to explicitly demonstrate that GH promotes dendritic spine formation, given the tight correlations between stress-related changes in GH levels and stress-related changes dendritic spines (acute stress enhances both, and chronic stress decreases both, in hippocampus), there may also be an unrecognized relationship between the two. Increases in spine density or NR2B expression in hippocampus could promote neuroplastic changes at hippocampal synapses, and learning-related plasticity in hippocampus is thought to underlie context conditioning (Marschner et al., 2008; Kheirbek et al., 2013) and trace conditioning (Thompson et al., 1996; Moyer et al., 2000). GH may also boost hippocampal function by restoring normal levels of neurogenesis (Ransome and Turnley, 2008) following stress (Vollmayr et al., 2003).

Because GH can potentiate hippocampal synaptic plasticity, one might hypothesize that overexpression of GH would lead to enhancement of hippocampal function. Interestingly, overexpression of GH in the hippocampus of unstressed animals had minimal effect on contextual or trace fear conditioning. For animals subjected to contextual fear conditioning, there was a mild trend for unstressed rats to have impaired long-term contextual fear memory when GH was overexpressed in hippocampus. This may be due to an occlusion effect, whereby GH transiently saturates plasticity in the hippocampus such that synapses may not be further potentiated by learning. However, overexpression of GH clearly did not produce a broad occlusion of further hippocampus-dependent learning. An alternative hypothesis to explain the lack of occlusion is that GH may regulate its own expression, and viral expression of recombinant GH could have downregulated expression of endogenous GH, though that does not appear to be the case (**Figure 2D**). Regardless, these results support GH as a novel target for pharmacological intervention following stress, and suggest that interventions that boost GH signaling in hippocampus after stress could promote stress resilience (Fleshner et al., 2011).

## AUTHOR CONTRIBUTIONS

Caitlin M. Vander Weele collected and prepared samples for the GH ELISAs, performed surgeries for the trace conditioning experiment and the ELISA experiments, and collected and analyzed data for the contextual fear conditioning experiment. Christopher Saenz performed surgeries and collected data for the trace fear conditioning experiment. Junmei Yao collected and analyzed data for the GH ELISA. Susana S. Correia planned and executed the Western blot experiment to test viral expression of GH. Ki A. Goosens designed experiments and generated viral constructs, acquired and analyzed data for the trace conditioning experiment, and wrote the manuscript.

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# β-endorphin modulates the effect of stress on novelty-suppressed feeding

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Although stress is implicated in the pathophysiology of mood and anxiety disorders, not all individuals who suffer stressful life events develop psychopathology. Differential susceptibility to stress may be influenced by genetically mediated differences in hypothalamic-pituitary-adrenal (HPA) axis activity and moderation of the stress response by the opioid peptide β-endorphin (β-E). The present study investigated genetic contributions to coping behavior by examining anxious behavior of transgenic mice with varying capacities to synthesize β-E [B6.129S2-*Pomc*$^{tm1Low}$/J; regulated by insertion of a premature stop codon into one or both copies of the proopiomelanocortin (*POMC*) gene], both under normal conditions and following 3 min of forced swim (FS). Ten minutes after this stress exposure or a control manipulation, acutely food-deprived female and male transgenic mice were subjected to a novelty-suppressed feeding (NSF) test, during which their interaction with an almond slice located in the center of an open field box was measured. There was an interaction between genotype and stress for latency to approach the almond and whether or not the almond was approached, such that mice with low or absent β-E displayed a stronger aversion to novelty-feeding after stress exposure than did mice with normal levels. These data provide evidence for a moderating effect of β-E on the behavioral response to stress. Genotypic differences in anxious behavior emerged when mice were stressed prior to behavioral assessment, suggesting that β-E plays a role in coping behavior. These findings indicate that genetic variability in sensitivity of the β-E system to stress may contribute, at least in part, to heritable differences in stress reactivity as well as vulnerability to stress-related psychopathology.

**Keywords: opioids, transgenic, anxiety, depression, mice, hyponeophagia, novelty**

## INTRODUCTION

Over the past few decades, an extensive body of work has emerged linking vulnerability to affective and anxiety disorders with stressful life events. Stressful events often precipitate depressive episodes (Brown et al., 1987; Hammen et al., 1992), and early life stress has been shown to increase the risk for stress-related psychiatric disorders in adulthood (Kendler et al., 1992a; McEwen, 2003). However, not all individuals who suffer stressful life events develop psychopathology; evidence suggests that some individuals are resistant, and others vulnerable, to the adverse effects of stress (de Rijk and de Kloet, 2005; Southwick et al., 2005; Stiller et al., 2011; Castro et al., 2012; see Sandi and Richter-Levin, 2009, for review). Differential vulnerability to stress is regulated by an interaction of genetic and developmental factors with major life stressors (Sullivan et al., 2000; Danese, 2008; Bet et al., 2009). However, the neurobiological mechanisms underlying susceptibility to stress-related disorders remains poorly understood.

One hypothesis is that genetic factors influence coping style to moderate the vulnerability to stress (see Feder et al., 2009, for review). "Coping" describes the behavioral and physiological mechanisms that occur to return an organism to a basal state following stress exposure. Thus, less effective coping, defined as a failure to recover to a baseline state after stress exposure, may render an individual more susceptible to stress-induced psychopathology (McEwen, 2002; Meng et al., 2011). For example, in rats, a behavioral profile characterized by high anxiety is associated with susceptibility to the development of stress-induced depression-like behavior (Sandi et al., 2008; Stedenfeld et al., 2011; Castro et al., 2012). In humans, the neuroticism-anxiety trait, which is associated with disengagement coping (an ineffective strategy; see Carver and Connor-Smith, 2010 for a review of personality and coping) and less flexible coping strategies across situations (Lee-Bagley et al., 2005), strongly reflects liability to major depressive disorder (MDD) and generalized anxiety disorder (GAD; Kendler et al., 2006a, 2007).

Moreover, differences in coping behavior and vulnerability to stress may have a biological basis in hypothalamic-pituitary-adrenal (HPA) axis function (van Santen et al., 2011) and the moderating effects of the endogenous opioid peptide β-endorphin (β-E) on the stress response (Schedlowski et al., 1995; Gianoulakis, 1998; Sarkar et al., 2007; Grisel et al., 2008;

Barfield et al., 2010). Activation of the HPA axis following exposure to stressful stimuli mediates an adaptive response through a hormonal cascade of behavioral and physiological changes aimed at the maintenance of homeostasis in the body (Low, 2004). During stress, the secretion of corticotrophin releasing hormone (CRH) stimulates expression of the proopiomelanocortin (*POMC*) gene in the anterior pituitary, which is subsequently translated into peptides such as adrenocorticotropic hormone (ACTH) and β-E (Charmandari et al., 2005). While ACTH activates the adrenal gland to initiate the peripheral response to stress, β-E attenuates the stress response, at least in part, by inhibiting secretion of CRH (Buckingham, 1986; Plotsky, 1991) and blocking stress-induced nociception (Bodnar et al., 1980; Nakagawasai et al., 1999; Parikh et al., 2011). Reports indicating modulation of the HPA axis by β-E fit well with those evincing a role for β-E in the behavioral response to stress (Amir, 1982; Yamada and Nabeshima, 1995; Ribeiro et al., 2005; Grisel et al., 2008; Barfield et al., 2010). For example, we have shown that transgenic mice with low β-E exhibit increased anxious behavior and show deficits in coping ability during an inescapable aversive situation (Grisel et al., 2008; Barfield et al., 2010). Thus, because stress-induced release of β-E mediates endocrine and behavioral responses that contribute to allostasis of the stress response, insufficient attenuation of the HPA axis arising from low β-E may contribute to maladaptive coping behavior under stressful conditions.

Here, we examined the role of β-E in anxious behavior of mice, both under basal conditions and following exposure to an acute stressor. Anxious behavior was assessed using the novelty-suppressed feeding (NSF) test, an ethologically relevant paradigm that measures the suppression of food intake (in a food-deprived animal) caused by exposure to a potentially anxiogenic novel environment (typically an open field; Merali et al., 2003; see Cryan and Sweeney, 2011 for summary of hyponeophagia paradigms). Because anxiolytics and chronic but not acute antidepressants reduce hyponeophagia (Britton and Britton, 1981; Shephard et al., 1985; Bodnoff et al., 1988; Bessa et al., 2009), the NSF test provides a sensitive and reliable measure of anxiety-related states in animals that resemble those in humans (Merali et al., 2003). Thus, we assessed the effect of genotype (β-E level) and previous stress exposure, as well as their interaction, on anxious behavior in the NSF test. We hypothesized that studying the behavioral response to stress in mice with varying levels of β-E would reveal an interaction of genetic predisposition and environmental stress, such that differences in coping behavior between genotypes would emerge following stress exposure.

## MATERIALS AND METHODS

### SUBJECTS AND DESIGN

Subjects were adult naïve male and female wild-type (C57BL/6J; B6), heterozygous (HT), and β-E-deficient (B6.129S2-*Pomc*$^{tm1Low}$/J; KO) mice. Transgenic mice were developed over a decade ago in the laboratory of Malcolm Low (Rubinstein et al., 1996) by insertion of a premature stop codon into the *Pomc* gene. Homozygotes (KO) are entirely unable to synthesize β-E, though all other *Pomc* products show normal expression. Opioid receptor expression also remains unchanged (Rubinstein et al., 1996). Mice for these studies were bred in-house from stock purchased from Jackson Laboratories (Bar Harbor, ME, USA). The gene mutation has been fully backcrossed to the C57BL/6J strain (>20 generations). HT mice were bred from KO males and B6 females; others were bred under identical conditions from genotype-matched pairs. Mice were weaned at 21 days of age and were group-housed by sex with 3–4 per Plexiglas cage, measuring 20 × 35 × 14.5 cm. Mice were maintained in a colony room at 21 ± 2°C, on a reverse 12:12 light:dark cycle with lights on at 7 p.m. Water and food were available *ad libitum*. All procedures were carried out in accordance with the National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Furman University.

### BEHAVIORAL TESTING

On testing day, food was removed at ∼8 a.m., 1 h after lights-out, in order to facilitate feeding (LeSauter et al., 2009). Behavioral testing occurred during the animals' active phase, between 10 a.m. and 4 p.m., in a dimly lit testing room, so as to enable behavioral assessment of genotypic differences (Branchi and Ricceri, 2002; Hossain et al., 2004; Roedel et al., 2006). Mice were brought into the testing area, weighed, tail marked, and randomly assigned to the control or the forced swim (FS) condition.

Mice in the control condition were individually placed in a Plexiglas cage in the testing room for a 10 min habituation period. Mice in the FS condition were subject to a modified version of Porsolt et al.'s (1977) FS Test for 3 min in a white plastic 5 gallon bucket measuring 30 cm in diameter by 40 cm in height containing 20 cm of water maintained at 23°C. To minimize the possibility of confounding effects (e.g., fatigue) from sex and genotypic differences in behavior (previously reported for 15 min of FS exposure, Barfield et al., 2010), pilot testing was conducted to determine a FS duration that would induce subthreshold amounts of stress. Duration of 3 min was chosen because no sex or genotypic differences in immobile behavior emerged following this brief length of time. Mice were judged immobile when making no movements other than those required to stay afloat, for at least 5 s. Two independent observers recorded latency to immobility, total time spent immobile, and number of immobile segments. Following the FS, mice were individually placed in a Plexiglas cage in the testing room for a 7 min habituation period.

After habituation in the testing room, mice from both the control and the FS conditions were subject to the NSF test (Britton and Britton, 1981; Bodnoff et al., 1988). Mice were individually placed in an open field box (100 × 100 × 4.5 cm) that contained a pre-weighed almond slice in the center, for 5 min. Two independent observers recorded whether the almond was approached, the latency to approach the almond, and the number of times that the mouse sniffed the almond. Following the NSF test, the almond slice was weighed, and the amount of almond eaten was recorded.

### STATISTICAL ANALYSIS

Main effects of and interactions between genotype (B6, HT, KO), sex, and stress condition (control, FST) were analyzed using between-subjects analysis of variance (ANOVA). Significant main effects and interactions were further examined using Fisher's

least significant difference (LSD) test. Three separate Two-Way chi-square tests of independence were performed to determine whether correlations existed between (1) approach behavior (whether or not the almond was approached) and stress condition (2 × 2 design), (2) approach behavior and genotype (2 × 3 design), and (3) approach behavior and genotype with condition (2 × 6 design). Statistical analyses were performed using SPSS Statistics 17.0 (SPSS, Inc., Chicago, IL). In all cases, the criterion for significance (α level) was set at $p \leq 0.05$.

## RESULTS

There were no main effects of or interactions with sex, so male and female data were collapsed for all analyses. As expected, there were no main effects of genotype on any measure of immobility in the 3 min FS exposure.

In terms of latency to approach the almond, there was a main effect of condition [$F_{(1,\ 81)} = 31.261$, $p < 0.001$] and a main effect of genotype [$F_{(2,\ 80)} = 9.696$, $p < 0.001$]. *Post-hoc* analysis (Fisher's LSD) indicated that KOs took the longest to approach the almond ($p < 0.01$) and differed from both B6s and HTs, which did not differ from each other. There was also a significant interaction between genotype and condition for latency to approach the almond [$F_{(2,\ 80)} = 4.899$, $p \leq 0.01$], such that the effect of stress on hyponeophagia increased as β-E levels decreased (**Figure 1**). There were no genotypic differences in the control condition, but in the stressed condition, KOs took the longest to approach the almond (as confirmed by Fisher's LSD, $p < 0.05$). Thus, the main effect of genotype on latency to approach the almond was driven primarily by differences between genotypes in the stressed condition.

In terms of whether or not the almond was approached, FS exposure decreased the likelihood that mice would approach the almond at least once during the 5-min NSF test [$X^2$ $(1, N = 83) = 17.250$, $p < 0.01$]. When data were collapsed across condition, whether the almond was approached depended on genotype [$X^2$ $(2,\ N = 83) = 15.235$, $p < 0.01$] such that as β-E levels decreased, likelihood of approaching the almond also decreased. To determine if genotype and condition were correlated with approach behavior, we further separated genotypes into groups based on condition (i.e., B6 stress, B6 control, HT stress, etc.). Whether or not the almond was approached depended on both genotype and condition [$X^2$ $(5,\ N = 83) = 49.427$, $p < 0.01$]. **Figure 2** depicts the percentages of mice in each genotype and condition that approached the almond. All control mice approached the almond, but whether or not stressed mice approached depended on genotype. Thus, the significant correlation between genotype (collapsed across condition) and whether the almond was approached was driven by genotypic differences that emerged only in the stressed condition.

For number of sniffs, there was a main effect of condition [$F_{(1,\ 81)} = 31.261$, $p < 0.001$] such that stressed mice sniffed the almond less frequently, and a main effect of genotype [$F_{(2,\ 80)} = 8.681$, $p < 0.001$] such that β-E levels were indirectly correlated with degree of hyponeophagia (**Figure 3**). *Post-hoc* analysis indicated that B6s sniffed the almond more than either of the other two lines ($p \leq 0.001$), but HTs and KOs did not differ from each other. There were no significant interaction effects of genotype and condition on number of sniffs [$F_{(2,\ 80)} = 2.043$, $p > 0.05$].

There was a main effect of condition on amount of almond eaten [$F_{(1,\ 81)} = 17.470$, $p < 0.001$] such that stressed mice ate less (**Figure 4**). However, there was no main effect of genotype [$F_{(2,\ 80)} = 0.107$, $p > 0.05$] nor an interaction between genotype and condition for this measure [$F_{(2,\ 80)} = 0.727$, $p > 0.05$].

## DISCUSSION

Employing the NSF test to assess anxious behavior in transgenic mice expressing varying levels of β-E, our findings suggest that β-E modulates the effect of stress on behavior. The ability of exposure to a novel environment to suppress interaction with and ingestion of a highly palatable food was magnified when

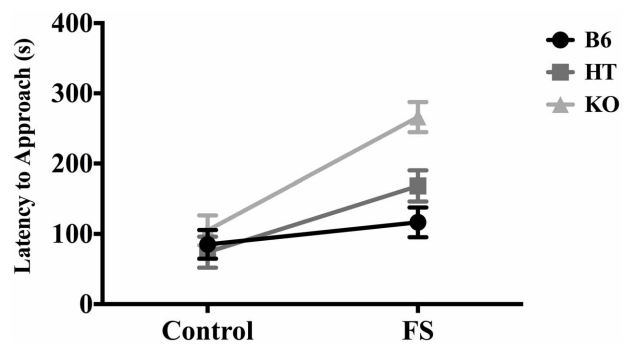


**FIGURE 1 | Latency to approach almond slice during the 5-min novelty-suppressed feeding (NSF) test in wild-type C57BL/6J (B6), heterozygous (HT), and β-E knock-out (KO) mice, following either 10 min single housing (Control) or 3 min of forced swim, followed by 7 min single housing (FS).** Data show mean ± SE. Significant differences (*p*-values ≤ 0.05) between groups were determined following ANOVA by *post-hoc* analysis (Fisher's LSD test). There were main effects of the FS stressor, genotype, and an interaction between stress condition and genotype.

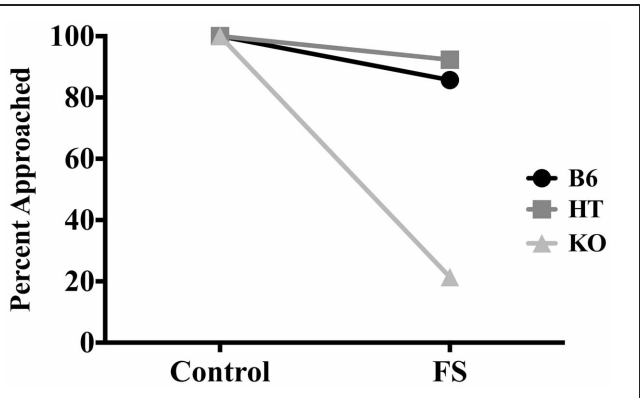


**FIGURE 2 | Percentage of wild-type C57BL/6J (B6), heterozygous (HT), and β-E knock-out (KO) mice in each group that approached the almond slice at least once during the 5-min NSF test.** Significant correlations between experimental variables were determined by Two-Way chi-square tests of independence. Whether or not the almond was approached depended on stress condition, genotype, and interaction of genotype and stress condition (all *p*-values ≤ 0.05).

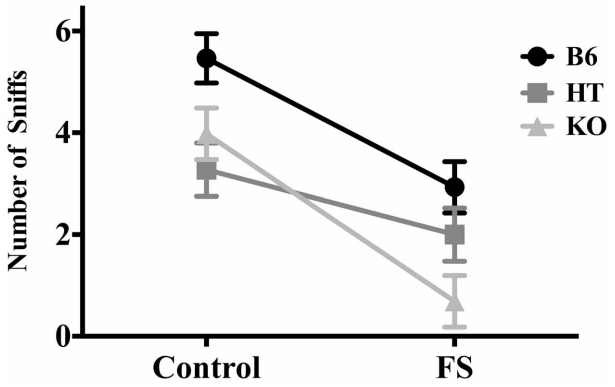


**FIGURE 3 | Number of sniffs to the almond slice made by wild-type C57BL/6J (B6), heterozygous (HT), and β-E knock-out (KO) mice during the 5-min NSF test.** Data show mean ± SE. ANOVA and *post-hoc* analysis (Fisher's LSD test) indicated that forced swim exposure decreased almond sniffing, and a main effect of genotype reflected the fact that B6s sniffed the almond more than either of the other two lines, which did not differ from each other ($p$-values $\leq 0.05$). There were no significant interaction effects of genotype and condition on number of sniffs.

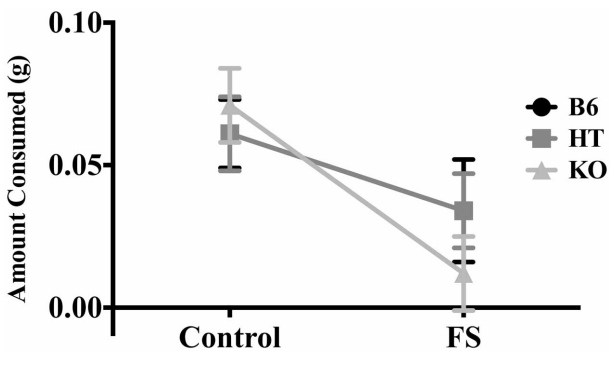


**FIGURE 4 | Average amount of almond eaten during the 5-min NSF test by wild-type C57BL/6J (B6), heterozygous (HT), and β-E knock-out (KO) mice.** Data show mean ± SE. ANOVA indicated that prior forced swim decreased consumption ($p \leq 0.05$), but there were no differences between genotypes, nor was this effect dependent upon genotype.

mice were first exposed to FS in a genotype-dependent manner. (**Figures 1**, **2**). These data are in line with previous reports showing increased hyponeophagia in rodents exposed to unpredictable chronic mild stress (Bessa et al., 2009) or social isolation (Voikar et al., 2005), and extend these findings by suggesting a critical role for β-E.

The main effect of genotype on number of sniffs (**Figure 3**) suggests a direct relationship between peptide levels and interaction with the novel food stimulus. However, there was also an interaction between genotype and stress for latency to approach the almond (**Figure 1**) and whether the almond was approached (**Figure 2**); mice with lower levels of β-E displayed a stronger aversion to novelty-feeding after exposure to stress than did mice with higher levels. Moreover, there were no differences between wild-type (B6), HT, or, β-E knock-out (KO) mice under control conditions. Because the effects of stress on hyponeophagia are magnified with lower levels of β-E, these data suggest that β-E plays an active role in coping behavior by mitigating the behavioral response to stress.

As expected based on pilot testing, there were no effects of or interactions between genotype and sex on immobile behavior of mice during the 3 min FS. In a previous study in our lab, using the same three strains of mice, we found effects of sex and genotype on immobility during a 15 min FS Test (Barfield et al., 2010). However, for the present study, we aimed to induce a subthreshold amount of stress that would not produce genotypic or sex differences in behavior during the FS so as to minimize the possibility that behavior in the NSF test would be confounded by factors such as fatigue from the FS. We found that 3 min of exposure to the FST was just stressful enough for genotypic differences in novelty-feeding to emerge. Furthermore, although we found an interaction between genotype and stress for latency to approach the almond and whether or not the almond was approached, there was no such interaction for number of sniffs and amount of almond eaten. It is possible that interactions between genotype and stress for the latter two measures may emerge with FS times longer than 3 min.

Likewise, although we found no effects of sex on behavior during the NSF test, it is possible that this design did not induce sufficient stressor intensity to allow for detection of sex differences. Thus, the present findings do not preclude the possibility of sex differences in coping behavior. Given that sex differences in the risk for and prevalence of stress-related disorders in humans are well-documented (Kessler et al., 1993; Zilberman et al., 2003; Marcus et al., 2005; Hasin et al., 2007), future research should aim to develop animal models that reflect such differences.

The findings presented here support our earlier findings using the plus maze, light-dark box (Grisel et al., 2008), FS Test, and tail suspension test (TST; Barfield et al., 2010), suggesting that β-E contributes to the ability to behaviorally manage stressful stimuli. For example, we have shown an inverse relationship between β-E levels and anxious behavior (as measured by percent of open arm entries and time spent in the open arms in the plus maze, and time spent in the light compartment of the light-dark box; Grisel et al., 2008). We have also shown a direct relationship between β-E levels and immobility in the FST and TST (Barfield et al., 2010). Because these tests subject mice to inescapable aversive situations, whereby failure to exhibit actions aimed at escape may represent an effective coping strategy, these results suggest that β-E facilitates coping behavior. The present study provides additional evidence to support this role of β-E by showing that under conditions of acute stress, mice alter their behavior in an anxiogenic situation to mitigate a deficiency in β-E. Moreover, these data extend our earlier findings to suggest that behavior becomes increasingly influenced by underlying neurobiology when an organism is exposed to stressors.

An interaction of both genetic predisposition and environmental stressors contributes to increased risk for developing stress-induced psychopathology (Danese, 2008; Bet et al., 2009). In line with this view, it is possible that an individual who produces lower than normal amounts of β-E may suffer from an overactive HPA axis and an impaired ability to effectively manage

stressful stimuli (behaviorally and physiologically). These factors may render an individual particularly susceptible to the aversive effects of stress and to developing anxiety and depression. Indeed, evidence from studies utilizing selectively bred rodent lines suggests that individual differences in HPA activity and anxiety traits may contribute to differential susceptibility to stress. Rats with a behavioral profile characterized by high anxiety and low exploration are particularly vulnerable to developing depression-like behaviors and HPA axis hyper-reactivity when exposed to subchronic stress, while low anxiety rats are more resistant to the development of stress-induced depression-like behavior (Castro et al., 2012). Additionally, rats classified as low-responders to novelty (high anxiety) who are exposed to chronic mild stress exhibit increased latencies to approach and consume food in the NSF test, while the behavior of rats classified as high-responders to novelty (low anxiety) is unaffected by chronic stress (Stedenfeld et al., 2011). Chronically stressed low-responder rats also become anhedonic more rapidly and to a greater degree than chronically stressed high-responder rats (Stedenfeld et al., 2011).

The role of genetic factors in the etiology of MDD and anxiety disorders is well recognized (Unschuld et al., 2009; see Sullivan et al., 2000, for review), as heritability is estimated to be around 40% for MDD (Kendler et al., 2006b), and 32% for GAD; Hettema et al., 2001. At least in part because of the complexity of these disorders, candidate gene studies have not been able to unambiguously identify susceptibility genes (Levinson, 2006). Moreover, the high comorbidity of MDD and anxiety disorders (Gorman, 1996; Kessler et al., 1996, 2008; Kaufman and Charney, 2000; Hettema et al., 2003) suggests that risk factors for these disorders are not mutually exclusive (Krueger, 1999; Ohara et al., 1999; Vollebergh et al., 2001; Gorwood, 2004). Indeed, twin studies indicate significant overlap of genetic risk factors for depression and anxiety (Hettema et al., 2003; Kendler et al., 2007). In particular, it has been suggested that the genes influencing liability to MDD are the same as those influencing liability to GAD (Kendler et al., 1992b). Nevertheless, genome-wide association studies suggest that a large number of genes, each with a small effect, influence susceptibility to MDD, and there is overlap in genetic risk factors with GAD (Demirkan et al., 2011).

Because the ability to cope with stress is an important factor influencing susceptibility to stress-related disorders (Meng et al., 2011; Mahmoud et al., 2012), it is possible that shared liability genes for anxiety and depression influence stress reactivity (Kendler et al., 1991; Gorwood, 2004; Yu et al., 2012). Indeed, stress-induced activation of the HPA axis is moderately to highly heritable (Federenko et al., 2004). Healthy individuals with depressed first-degree relatives show a moderately elevated cortisol response following challenge with dexamethasone (DEX-CRH test), though not as elevated as that of patients with MDD (Holsboer et al., 1995), and healthy individuals with diagnosed parental history of anxiety or depression show higher cortisol awakening levels than individuals without parental history (Vreeburg et al., 2010). Moreover, the response of the β-E system to acute stress exposure is also highly heritable (Dai et al., 2002, 2005), and genetic variation in the μ-opioid receptor contributes to the differential response of the HPA axis to stress (Chong et al., 2006; Schwandt et al., 2011).

Although a compelling number of studies report evidence for dysregulation of the HPA axis in patients suffering from depression and anxiety (Young et al., 1991; Carroll et al., 2007; Lloyd and Nemeroff, 2011), the above findings suggest that a hyperactive HPA axis in normal individuals may represent a vulnerability marker for stress-related psychopathology. Because β-E plays a role in moderating the effects of stress (Amir, 1982; Yamada and Nabeshima, 1995) as well as termination of the stress response (Buckingham, 1986), individual differences in HPA axis activation and subsequent release of β-E may influence differential vulnerability to stress-induced changes in the coordination and dynamics of the stress response. Indeed, depressed patients show hypertrophy of the adrenal gland (Rubin et al., 1995), indicative of HPA hyperactivity, and mice with low or absent β-E have enlarged adrenal glands, suggesting chronic upregulation of the HPA axis with decreased β-E levels (Grisel et al., 2008).

The present study, along with earlier studies in our lab, provides evidence of a moderating effect of β-E on the behavioral response to stress (Grisel et al., 2008; Barfield et al., 2010), implicating a role for this peptide in coping behavior. In particular, we found an effect of interaction between genetic predisposition and environmental stressors on anxious behavior in mice. Behavioral differences between "genetically vulnerable" (low or absent β-E) and "genetically resistant" mice emerged when mice were exposed to a stressor before the NSF test. These data suggest that low β-E levels impair the ability to return to a basal state following stress exposure, and thus compromise coping ability. Considering the evidence for heritability of stress-induced HPA axis activity together with the findings presented here, it is possible that genetically determined differences in sensitivity of the β-E system to stress contribute, at least in part, to heritable differences in vulnerability to developing anxiety and depression (Charmandari et al., 2005; Hegadoren et al., 2009; Merenlender-Wagner et al., 2009).

MDD and anxiety disorders affect a significant portion of the nation, with a lifetime prevalence of ∼20% for MDD and 28% for anxiety disorders (Kessler et al., 2005). Although the neural mechanisms involved are poorly understood, evidence from clinical and pre-clinical studies implicates the role of HPA axis abnormalities in the pathophysiology of mood and anxiety disorders (Carroll et al., 2007; see Arborelius et al., 1999, for review). Thus, genetically mediated interindividual differences in HPA axis activity may help explain why some individuals are particularly vulnerable, and others resilient, to anxiety and depression (Holsboer et al., 1995; Wüst et al., 2000; McEwen, 2002; Vreeburg et al., 2010). Altogether, our findings suggest that β-E facilitates coping behavior. Low levels of this peptide may impair the coordination and dynamics of the stress response, thereby enhancing vulnerability to stress-related psychopathology. Further investigation of the role of β-E in allostasis of the stress response may yield insight into the etiology of anxiety and depression.

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# On the incongruity between developmental plasticity and methodological rigidity

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## LIVING ORGANISMS ADJUST THEIR PHENOTYPES ACCORDING TO ENVIRONMENTAL INFLUENCES

One thousand nine hundred thirty six: this is the number of citations retrieved in "pubmed" using the search terms: "developmental plasticity rodent." The "results by year" graphical trend, automatically plotted, indicates that this number has been linearly increasing over the last 30 years. Therefore, developmental plasticity, the range of different phenotypes potentially branching from an identical genotype (West-Eberard, 2003), is not a novel concept (Weininger et al., 1954). The notion that environmental factors modulate individual maturation, specifically during highly plastic developmental stages, is also not novel. Disciplines like psychology and evolutionary ecology devised theoretical and practical tools to understand the link between experiential factors and phenotypic adjustments. Whilst Freud proposed that adult neuroses build upon infantile experiences (Freud, 1918), contemporary authors demonstrated that several psychiatric disorders often root in early childhood, when abuse and/or neglect may increase individual vulnerability to depression (Heim and Nemeroff, 1999, 2001). These studies attempted to define the potentially disruptive nature of severe developmental stress. Complementary to them, other studies demonstrated that stress during development may represent a constructive force: specifically, moderate precocious environmental challenges have been proposed to favor resilience (Lyons and Macrì, 2011), i.e., program the organism to handle repeated stressors in a more efficient way. Studies conducted in rodents (Macrì et al., 2011), birds (Henriksen et al., 2011), primates (Parker et al., 2006; Parker and Maestripieri, 2011), and humans (DiCorcia and Tronick, 2011; Flinn et al., 2011; Seery, 2011), demonstrate that precocious exposure to mild stress (being briefly separated from dams during lactation, exposed to low doses of stress hormones, or reared to mothers requested to seek for food instead of being allowed unlimited foraging) promotes resilience.

At the same time that behavioral neuroscientists started disclosing the inextricable link between developing organisms and their environments, ethologists and evolutionary ecologists attempted to understand the functional meaning of these processes. Bateson and colleagues discussed the representative example of the freshwater crustacean *Daphnia* (Bateson et al., 2004). Offspring of this species develop a protective "helmet," reducing the odds of being predated, if their mothers were exposed to a predator odor. Yet, the energetic costs associated with helmet patterning reduce individual competitive success in a predator-free environment. Thus, the success of each phenotype is dictated by the presence or absence of predators and, ultimately, by the correspondence (match) between neonatal forecasting and adult life conditions. A directional phenotypic adjustment in conformity with developmental cues has also been observed in rodents (Sachser, 1993; Sachser et al., 1994; Liu et al., 1997) and humans (Hales and Barker, 2001; Wells, 2007, 2011). The concept of resilience should be integrated within this theoretical framework, whereby precocious challenges may forecast an adult environment characterized by the presence of multiple stressors, to which the individual phenotype is accordingly tuned. Maladaptive or pathological outcomes may occur under several circumstances, among which the following two are of particular interest: (1) external challenges are too elevated to permit adaptive processes thereby exceeding individual adaptive capacities (Sultan, 2003); (2) developmental experiences do not provide a reliable indication of the challenges to be encountered later in life (phenotypic mismatch).

Along with the observation that experiential factors adjust individual development, so also the fundamental underlying mechanisms started being detailed. To investigate these mechanisms, laboratory rodents have often constituted the methodology of choice. For example, several studies demonstrated that being reared to a careful rat mother favored adult resilience through a non-genomic mother-offspring transfer mechanism (Francis et al., 1999). Specifically, increased adult resilience was shown to depend on maternally mediated epigenetic regulations at the level of DNA methylation (Weaver et al., 2004). Ultimately, laboratory animals constitute the cornerstone against which developmental plasticity is demonstrated and dissected.

I therefore find it quite ironic that such plasticity tends to be contrasted when it comes to using laboratory rodents as experimental subjects. Such contrast becomes particularly evident when current housing and breeding standards are considered. Thus, either in the case of conventional or enriched housing there exists a strong strive to equate living conditions across different facilities. Such strive is theoretically justified by the need to minimize and equalize environmental sources

of variation to isolate the biological factors contributing to the individual phenotype, and to obtain reproducible results across different laboratories. I believe that these considerations entail several research questions: (1) does a unique laboratory standard produce identical individuals? (2) To what extent do laboratory rodents suit their environment? (3) If standardization were inefficient, what would the alternative be?

## WOULD A UNIQUE LABORATORY STANDARD GUARANTEE FULL REPRODUCIBILITY OF EXPERIMENTAL FINDINGS?

Under the assumption that different environments beget different phenotypes, it may be tenable to propose that a unique standard housing/breeding system should produce similar results. Before attempting to design a comprehensive digest listing how a rodent should be kept and tested (a hard duty), and select the committee devoted to this task (an even harder one), experimental support to the assumption that identical environments beget identical data should be obtained. This would require a set of identical experiments to be performed in independent facilities. Crabbe and colleagues performed such nobody-would-dare-to-do-experiment (Crabbe et al., 1999). The authors attempted to decompose the genetic and environmental influences on behavior through performing several tests in eight different mouse strains brought, kept, reared, and tested under the same conditions in three laboratories. Notwithstanding a spectacular level of across-lab standardization, the authors observed that mouse behavior was influenced by the test site, and concluded that "experiments characterizing mutants may yield results that are idiosyncratic to a particular laboratory." The fact that this study involved the use of mutant mice is even more daunting as mutant mice derive from strains that have been mated with siblings for so many generations to become virtual genetic copies (for a discussion see Sapolsky, 2006). These results have also been replicated in an independent study (Wolfer et al., 2004).

Ultimately, identical subjects kept under "allegedly" identical environmental conditions may behave differently.

## TO WHAT EXTENT DO LABORATORY RODENTS SUIT THEIR ENVIRONMENT? CAN WE IMAGINE REARING CONDITIONS CAPABLE OF PROMOTING RESILIENCE?

Whilst evaluating whether behavioral data are reproducible across laboratories is attainable, determining whether laboratory rodents suit their environment is much more complex. Specifically, it is necessary to devise strategies aimed at evaluating whether rodents are adapted to their living conditions. Reproductive fitness may not constitute a biologically meaningful parameter in captivity, as hardly ever are laboratory rodents faced with contextual features capable of altering their life history strategies (the trade-offs between reproductive efforts and other fitness-relevant activities, like foraging and morphological growth, Del Giudice et al., 2011). Alternatively, we may address whether laboratory rodents exhibit behavioral abnormalities (reflecting brain dysfunctions) that are generally not displayed in natural conditions. Apparently functionless repeated behaviors (stereotypies) may constitute an informative parameter: up to 98% of ICR (Wuerbel et al., 1996) and 80% of C57BL/6 mice perform them under standard housing conditions (Garner, 2005). An elevated rate of abnormal spontaneous behavior has been proposed to constitute an index of poor welfare (Laviola et al., 1994; Gross et al., 2011). Additionally, to evaluate whether laboratory rodents are "normal," it may be worth looking at the statistical distribution of the data collected upon them. Several studies show that experimental data are extremely variable and may greatly diverge from a "normal" distribution (Macrì et al., 2007). This has been proposed to stem from maladaptive adjustments to the laboratory environment (Garner, 2005) which, in turn, may relate to the fact that the neonatal living conditions do not constitute good predictors of the challenges to be encountered in adulthood (Wuerbel, 2001). Specifically, neonate laboratory rodents are exposed to extremely quiet, stable and safe conditions (the maximal source of stress being cage cleaning once/twice a week), including effortless *ad libitum* feeding conditions (unlikely to impose the foraging demands regularly met by a rodent dam in the wild). These conditions may not be good predictors of the continuous challenges to which rodents are often exposed in adulthood (e.g., injections, modified housing and re-grouping, food restrictions, etc.). In other words, laboratory rodents are not prepared to cope with the challenges imposed by laboratory routines. Thus, moderately challenging rearing conditions, aimed at promoting resilience, might prepare experimental subjects to efficiently handle the stressors associated with laboratory procedures, ultimately increasing experimental validity (reproducibility) and normalizing the statistical distribution of experimental data. To test these predictions, we exposed neonate mice to a supplementation of corticosterone (mimicking neonatal stress) and evaluated, in adulthood, the inter-individual variation and frequency distribution of data obtained in these individuals compared to standard laboratory controls. As predicted, adult mice exposed to challenging neonatal conditions exhibited reduced inter-individual variation across the following variables: anxiety-related behavior, pain perception, corticosterone response to restraint stress, and immune response to bacterial infection (Macrì et al., 2007). Thus, matching the stressful nature of the neonatal environment with actual adult test conditions may benefit the quality of laboratory data; by the same token, I believe that devising stress-free testing strategies (e.g., home-cage automated tasks: Galsworthy et al., 2005; Branchi et al., 2010; Voikar et al., 2010; Zoratto et al., 2012a,b) may benefit the quality of experimental data without requiring perinatal challenges to be administered to laboratory rodents.

## CAN EXPERIMENTAL REQUIREMENTS MEET ANIMAL NEEDS?

I previously discussed experimental studies in which current laboratory standards failed to yield reproducible results (Crabbe et al., 1999; Wolfer et al., 2004); I also proposed that breeding strategies aimed at "preparing" developing rodents to their future experimental habitat, may increase the reproducibility of experimental findings. Yet, this proposition is still clearly incomplete as it implies the use of animals kept and tested under univocal conditions.

Animal models generally attempt to test experimental hypotheses that pertain to a wide population, likely composed of variable individuals derived from different environments: a population in which developmental plasticity regularly occurs. Proposing a univocal standard would, by definition, neglect such plasticity. In analogy with phases 2-3-4 of clinical testing (in which the treatment is given to incrementally larger groups of people), I believe that pre-clinical experimental hypotheses should be tested in heterogeneous populations rather than in subsets of genetically and environmentally identical individuals. Therefore, future strategies shall include individual diversity in the experimental design across housing conditions, genetic predispositions and test paradigms. Theoretically, the potential influences of a given gene on a certain phenotype should be tested in mice derived from different strains rather than in a single background. Likewise, the effects of experimental variables should be tested in mice housed in systematically variable environmental conditions. This effort should be statistically supported by the adoption of random block experimental designs, allowing the analysis of the independent contribution (percentage of explained variance) exerted by the different factors involved. Several studies started investigating this possibility. Richter and collaborators demonstrated that data obtained in heterogeneous experimental populations, characterized by individuals derived from different stocks and breeding systems, yield more consistent results than studies involving the use of homogeneous groups (Richter et al., 2009, 2010, 2011).

Ultimately, I propose that future experimental approaches may implement the concepts discussed herein. Specifically, I foresee large-scale endeavors in which data are collected in systematically variable experimental populations (hetherogeneization); at the same time, experimental subjects constituting each statistical unit should be adapted to their specific test conditions (phenotypic match). Since an entirely stress-free laboratory environment cannot be applied on a large scale, the possibility to prepare rats and mice to multiple challenges should become a needed objective. It is thus necessary to devise diverse rearing systems capable of promoting laboratory-specific resilience. As researchers we are aware that, in order to dissect adaptive processes, we regularly expose experimental subjects to external sources of stressors. By the same token, we should acknowledge that analogous adaptive processes take place also when we are not specifically observing them, i.e., throughout the entire course of development. Rather than being neglected, individual plasticity should be incorporated in rearing and testing systems through the provision of consistent developmental information, matching precocious and adult environment.

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# Microarray analyses reveal novel targets of exercise-induced stress resistance in the dorsal raphe nucleus

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Serotonin (5-HT) is implicated in the development of stress-related mood disorders in humans. Physical activity reduces the risk of developing stress-related mood disorders, such as depression and anxiety. In rats, 6 weeks of wheel running protects against stress-induced behaviors thought to resemble symptoms of human anxiety and depression. The mechanisms by which exercise confers protection against stress-induced behaviors, however, remain unknown. One way by which exercise could generate stress resistance is by producing plastic changes in gene expression in the dorsal raphe nucleus (DRN). The DRN has a high concentration of 5-HT neurons and is implicated in stress-related mood disorders. The goal of the current experiment was to identify changes in the expression of genes that could be novel targets of exercise-induced stress resistance in the DRN. Adult, male F344 rats were allowed voluntary access to running wheels for 6 weeks; exposed to inescapable stress or no stress; and sacrificed immediately and 2 h after stressor termination. Laser capture micro dissection selectively sampled the DRN. mRNA expression was measured using the whole genome Affymetrix microarray. Comprehensive data analyses of gene expression included differential gene expression, log fold change (LFC) contrast analyses with False Discovery Rate correction, KEGG and Wiki Web Gestalt pathway enrichment analyses, and Weighted Gene Correlational Network Analysis (WGCNA). Our results suggest that physically active rats exposed to stress modulate expression of twice the number of genes, and display a more rapid and strongly coordinated response, than sedentary rats. Bioinformatics analyses revealed several potential targets of stress resistance including genes that are related to immune processes, tryptophan metabolism, and circadian/diurnal rhythms.

**Keywords: Affymetrix gene microarray, Weighted Gene Correlational Network Analysis, bioinformatics, laser capture microdissection, stress resistance, dorsal raphe nucleus**

## INTRODUCTION

Depression and anxiety frequently coexist and are the most common mood disorders affecting society. The World Health Organization estimates that 121 million people currently suffer from depression. Individuals suffering from depression have significant impairment in quality of life (Rapaport et al., 2005), are at increased risk for developing coronary heart disease (Wulsin and Singal, 2003) and type 2 diabetes (Knol et al., 2006), and have higher mortality due to suicide. By 2030, depression is expected to be a leading cause in the global burden of disease (Mathers and Loncar, 2006).

Stressful life events often precede the onset of depression (Kendler et al., 1999; van Praag, 2005) and anxiety. Despite the high occurrence and significant disability associated with stress-related mood disorders, the pathophysiology of these conditions is not fully understood. Important to note is that not every individual who experiences a stressful life event develops a serious mood disorder, and these individuals may possess resistance to the negative affective consequences of stress. Pinpointing the factors by which stress resistance occurs could provide a better understanding of the neurobiological mechanisms underlying stress-related mood disruptions.

To investigate the neural circuitry underlying stress-related mood disorders, researchers use animal models (Krishnan and Nestler, 2008). Rats exposed to an acute inescapable stressor, such as tail shock, later exhibit behaviors argued to resemble symptoms of human anxiety and depression (Maier and Watkins, 1998), and these behaviors are responsive to pharmaceutical treatment with anxiolytics (Drugan et al., 1984) and antidepressants (Sherman et al., 1982). Inescapable stressor exposure also produces various physiological perturbations. Long-term increases in basal levels of plasma corticosterone and decreases in corticosteroid-binding

globulin occur in rats following tail shock (Fleshner et al., 1995). Additionally, acute stress increases interleukin-1β (IL-1β), leading to immune modulation (Moraska et al., 2002), and centrally, contributes to behavioral consequences of stress (Maier and Watkins, 1995). Circadian-regulated processes are also susceptible to acute stress. Thompson et al. (2013) observed a decrease in amplitude and disruption in diurnal pattern of core body temperature and heart rate in rats exposed to tail shock. Moreover, inescapable stress produces alterations in brain serotonergic circuits. The serotonergic system has long been implicated in underlying the behavioral consequences of inescapable stress exposure in rats (Maier and Watkins, 2005) and has been heavily implicated in human affective disorders (Sharp and Cowen, 2011).

Numerous components of the serotonergic system such as serotonin (5-HT) receptors, the 5-HT transporter, and extracellular 5-HT levels are sensitive to stress. Serotonergic nerve terminals and receptors also occupy regions of the brain involved in neuroendocrine and behavioral responses to stress (Chaouloff, 1993). One region of particular interest is the dorsal raphe nucleus (DRN), a small midbrain structure containing a high concentration of stress-responsive 5-HT cell bodies (Grahn et al., 1999). Hyper activation and sensitization of DRN 5-HT neurons is thought to underlie the depression- and anxiety-like behaviors induced by inescapable stress exposure (Maier et al., 1995; Christianson et al., 2008).

The DRN receives afferent, and provides efferent, projections to brain regions involved in fear, anxiety, and depression. These regions include the prefrontal cortex, striatum, bed nucleus of the stria terminalis (BNST), amygdala, and locus coeruleus (LC). Efferent DRN projections render these regions susceptible to stress-induced 5-HT activity in the DRN. Furthermore, these regions are themselves sensitive to stress (Cullinan et al., 1995), provide afferent input to the DRN, and may modulate DRN 5-HT activity. Nerve terminals containing corticotropin-releasing factor (CRF), a neuropeptide produced in response to elevated cortisol levels, for example, are present in the DRN (Swanson et al., 1983). Given that the BNST projects to the DRN and contains many CRF neurons (Day et al., 1999), the BNST is believed to be a primary source of CRF to the DRN. Interestingly, CRF injected into the DRN increases 5-HT activity in a subpopulation of cells (Lowry et al., 2000), and injection of CRF into the caudal DRN produces behaviors resembling those produced by inescapable stress exposure (Hammack et al., 2002). Thus other brain regions influence DRN 5-HT levels, and interactions between those regions and the DRN likely contribute to the DRN's role in stress-related mood disorders.

Also important to consider is that within the DRN, interactions between diverse cell populations may influence stress-induced 5-HT activity. The DRN is not just a homogenous structure of 5-HT neurons. Other populations of neurons containing the neurotransmitters γ-aminobutyric acid (Belin et al., 1979; Day et al., 2004), dopamine (Lindvall and Björklund, 1974; Stratford and Wirtshafter, 1990), and glutamate (Commons et al., 2005) also exist. Cells containing neuropeptides such as substance P (Hökfelt et al., 1978) and neuropeptide Y (de Quidt and Emson, 1986) are also present. These various neuropeptides and neurotransmitters/receptors are capable of modulating 5-HT (Ferré et al., 1994; Song et al., 1996; Tao and Auerbach, 2000; Valentino et al., 2003). Therefore, stress-induced alterations in 5-HT activity within the DRN and at DRN projection sites may be influenced indirectly through non-serotonergic neuronal modulation of serotonergic neurons. Non-serotonergic neurons in the DRN are also sensitive to 5-HT, and can have inhibitory and excitatory responses to 5-HT release (Marinelli et al., 2004). Dynamic interactions between serotonergic and non-serotonergic neurons originating at DRN afferent sites and within the DRN likely contribute to the effect of stress on net DRN 5-HT release within the DRN and at DRN projections sites.

Non-neuronal cell types, such as astrocytes and microglia, may also influence DRN neural activity. Microglia are the resident "immune cells" of the brain and are sensitive to stress-induced elevation of glucocorticoids (Nair and Bonneau, 2006; Sugama et al., 2007). Activated microglia release interleukin-1 (IL-1) (Giulian et al., 1986), tumor necrosis factor-α (TNF-α) (Sawada et al., 1989), and interleukin-6 (IL-6) (Righi et al., 1989). Inescapable stress increases IL-1β in the brain (Nguyen et al., 1998). Stress-induced activation of microglia may occur in the DRN and effect 5-HT neurons. Consistent with this idea, administration of interferon-γ (IFN-γ) and TNF-α reduced the survival of 5-HT neurons in organotypic DRN sections (Hochstrasser et al., 2011).

Overall, the DRN is an important region of investigation in studying the neurobiological mechanisms of stress-related mood disorders. Elucidation of variables influencing the serotonergic response to stress within the DRN may provide a better understanding of the development of these disorders. Furthermore, identification of interventions that prevent or manipulate the serotonergic response to stress and/or influence the various factors capable of modulating 5-HT activity within the DRN, may lead to the identification of novel therapeutic targets.

In humans, physical activity is one factor known to influence an individual's response to stress. Exercise reduces the risk of developing stress-related depression and anxiety (Fox, 1999). Similarly, in rats, 6 weeks of voluntary wheel running protects against the behavioral consequences of inescapable stress exposure (Greenwood et al., 2003). It is believed that wheel running prevents these behaviors by attenuating stress-induced activation of 5-HT neurons within the DRN. Wheel running may do this by producing plasticity at (1) DRN afferent sites (2) DRN efferent sites or (3) within the DRN itself (Greenwood and Fleshner, 2011). Given that hyperactivity of 5-HT neurons in the DRN is necessary for the development of stress-induced behaviors in rats and our lab has previously shown that wheel running attenuates stress-induced c-fos expression in DRN 5-HT neurons (Greenwood et al., 2003), we will focus on exercise-induced plastic changes that may occur within the DRN itself.

In particular, the 5-$HT_{1A}$ inhibitory autoreceptor has been implicated in the mechanism by which wheel running could constrain stress-induced 5-HT activity and protect against the behavioral consequences of inescapable stress. 5-$HT_{1A}$ receptors inhibit the activity of 5-HT neurons (Sprouse and Aghajanian, 1987) and reduce 5-HT release (Casanovas et al., 1997). Six weeks of wheel running increases 5-$HT_{1A}$ mRNA expression in the DRN (Greenwood et al., 2003, 2005) and thus, may increase

5-HT$_{1A}$ receptor-mediated inhibition of DRN 5-HT neurons during inescapable stress.

The protective effect of wheel running could also occur indirectly, through a non-serotonergic route. One possibility is through neuropeptides. Wheel running increases brain-derived neurotropic factor (BDNF) (Neeper et al., 1995), a neuropeptide important for maintaining neuronal health and function, and galanin (Tong et al., 2001) in the hippocampus, and also upregulates gene expression of galanin in the LC (Holmes et al., 2006; Sciolino et al., 2012). Wheel running may also increase levels of BDNF and galanin in the DRN. Both factors are coexpressed in 5-HT neurons in the DRN (Merlio et al., 1992; Xu and Hökfelt, 1997) and are capable of modulating 5-HT activity. Infusion of BDNF into the DRN modifies the neuronal firing of 5-HT by decreasing the regularity of the firing pattern (Celada et al., 1996). Additionally, an *in vitro* study revealed that galanin hyperpolarizes 5-HT neurons within the DRN (Xu et al., 1998). Exercise-induced increases in BDNF and galanin may protect against stress-induced activation of 5-HT neurons through modulating and, in the case of BDNF, inhibiting 5-HT neuronal activity.

Another method by which exercise may confer protection is through an immune-related mechanism. Evidence suggests a role of cytokines in human mood disorders (Maes, 2008) and stress-induced behaviors in rats. Injection of an IL-1 receptor antagonist into the brain blocks stress-induced depression- and anxiety-like behaviors in rats (Maier and Watkins, 1995), suggesting that activity at brain IL-1 receptors is important for the production of these behaviors. Speaker et al. (2011) observed that 6 weeks of wheel running attenuates stress-induced increases in plasma IL-1β, one of two cytokines that bind IL-1 receptors. It is possible that 6 weeks of wheel running also reduces stress-induced increases in brain IL-1β, and through reducing ligand availability, protects against stress-induced alterations in brain IL-1 receptor activity. Given that the DRN contains many IL-1 receptors (Cunningham and De Souza, 1993), it may be particularly sensitive to stress-induced and/or exercise-induced alterations in IL-1 receptor activity.

Though the precise mechanisms are not fully understood, the protective effect of exercise likely involves preventing stress-induced alterations in the serotonergic system, either by directly constraining activity of 5-HT neurons within the DRN or indirectly, through altering other neurotransmitter systems or neuropeptides within the DRN that are capable of modulating 5-HT neurons. Furthermore, DRN 5-HT neurons may be influenced by exercise-induced plastic changes that reduce afferent input to the DRN, activate afferent inhibition of the DRN during stress (Greenwood and Fleshner, 2011), or produce alterations in postsynaptic 5-HT receptor function (Greenwood et al., 2012). Elucidating the mechanism by which exercise produces stress-resistance and protects against the behavioral consequences of stress may lead to the identification of novel therapeutic targets and development of more targeted drugs for the treatment of human stress-related mood disorders.

One approach to reveal novel targets is by employing the use of microarray technology. Microarray technology permits the investigation of the expression of tens of thousands of genes simultaneously, at the level of mRNA transcription. Predesigned chips that contain sequences, known as probes, derived from every gene within a specified genome can be probed with mRNAs obtained from experimental samples in order to gain information about gene expression under the given conditions (Cox et al., 2012). When used in conjunction with laser capture microdissection, microarrays can reveal expression patterns of genes within specific cells. Using microarray and laser capture microdissection, therefore, it is possible to assess the effect of exercise and/or stress on gene expression in cell populations specific to the DRN.

The purpose of this experiment was to investigate the effect of exercise and/or stress on gene expression within the DRN. We hypothesized that wheel running produces changes in mRNA transcription within the DRN, and physically active rats exposed to stress have different gene expression profiles compared to sedentary rats exposed to stress. The differences in gene expression patterns within the DRN between physically active and sedentary rats exposed to stress may underlie the molecular mechanisms by which exercise protects against behaviors produced by inescapable stress exposure. Whole genome Affymetrix microarray analysis was used to assess gene expression. Our goal was to use an exploratory approach to (1) systematically organize the transcriptome (17,170 genes) obtained from the microarray analysis into a more manageable and focused gene set and (2) extrapolate physiological implications from this focused gene set by identifying novel targets of exercise-induced stress resistance within the DRN. To ensure a comprehensive assessment of the data, the organizational process involved two approaches, (1) identification of genes based on changes in differential expression in response to exercise and/or stress (2) identification of genes based on changes in coexpression in response to exercise and/or stress. For the differential expression analysis, two measures of significance were utilized. In a more conservative approach, genes were identified by log fold changes in gene expression. In a second, less stringent approach, genes statistically significantly differentially expressed by $p < 0.05$ were identified. These $p$-values were corrected for multiple comparisons using the False Discovery Rate adjustment method. The coexpression analysis narrowed the transcriptome from 17,170 genes to 11 modules of highly coexpressed networks of genes. These networks of genes were then correlated to the response to exercise and/or stress. Both the differential and coexpression analysis returned sets of genes that were further sorted by their relationship to functional categories derived from bioinformatics databases. Novel targets of exercise-induced stress resistance were identified within these functional categories.

## MATERIALS AND METHODS

### ANIMALS

The University of Colorado Boulder Animal Care and Use Committee approved all protocols for this study. A total of 48 adult, male Fisher 344 rats weighing 170–180 grams at time of arrival (Harlan Laboratories) were used in this experiment. Upon arrival, animals were individually housed in Nalgene Plexiglas cages (45 × 25.2 × 14.7 cm). The housing environment was maintained on a 12:12 h light:dark cycle, controlled for humidity, and held at a constant temperature of 22°C. Rats were

allowed ad libitum access to food and water and were weighed weekly to ensure each animal remained healthy. Following arrival, animals were acclimated to the housing conditions for 1 week before experimental manipulation.

#### WHEEL RUNNING

Animals were randomly assigned to remain sedentary (Sed, $n = 23$) or were housed with a running wheel (Run, $n = 25$), and allowed voluntary access to the wheel for 6 weeks. During the 1-week acclimation period, wheels were rendered immobile with metal stakes. Daily wheel revolutions each animal ran were logged with Vital View software (Mini Mitter). The product of the total number of daily revolutions and the wheel circumference (1.081 m) was calculated to obtain daily running distance. Daily running distance was summed in order to get an average weekly running distance.

#### INESCAPABLE STRESS

Animals were randomly assigned to remain in their home cages (HC) or receive inescapable tail shock (Stress). The stress procedure occurred between 0700 and 1200. Animals subjected to stress were restrained in acrylic cylinders (23.4 × 7 cm diameter). The tail projected from the back of the restraint device. An electrode was positioned 3 cm from the base of the tail and served as the vehicle by which shock was delivered. The shock procedure consisted of 100, 5-s tail shocks administered on a random 60-s inter-trial interval. Rats received 1.0 mA tail shocks for 50 min, at which time the intensity of shock was increased to 1.5 mA tail shocks for the remainder of the session. The entire stress procedure lasted 1 h and 48 min. Rats were sacrificed by rapid decapitation immediately following termination of tail shock (Stress0) or 2 h post termination of tail shock (Stress2). The sacrificing of rats that remained in their home cage was time matched with those animals subjected to tail shock.

#### TISSUE COLLECTION AND CRYOSECTIONING

RNAse free conditions were maintained throughout tissue processing. After rats were sacrificed, brains were extracted and flash frozen at −20°C, in 2-Methylbutane (Fisher Scientific), for 4 min. Brains were stored at −80°C prior to sectioning. Brains were prepared with M-1 embedding matrix before sectioning at −21°C with a cryostat (Leica CM1850). Tissue was sectioned to a thickness of 20 um through the rostral to mid-caudal (approximately −7.3 to −8.2 mm relative to Bregma) portions of the DRN. This specific region of the DRN was targeted because it is involved in modulating stress- and anxiety-like behaviors (Hale et al., 2012) and prior evidence suggests that alterations in gene expression occur in this region following 6 weeks of wheel running (Greenwood et al., 2003, 2005). Sections were freeze-mounted to PEN membrane frame slides (MDS Analytical Technologies) and stored at −80°C until further use.

#### LASER CAPTURE MICRODISSECTION AND RNA ISOLATION

Laser capture microdissection was performed to procure a precise sample of the DRN from each rat. Slides containing sections of DRN were allowed to thaw for 20 s prior to being fixed in 75% ethanol, subjected to a Histogene Stain (for visualization purposes), and dehydrated in graded ethanol concentrations, in accordance with the Arcturus Histogene LCM Frozen Section Staining Kit protocols (Applied Biosystems). Following staining procedures, slides were loaded into the laser capture microdissection system (Arcturus XT, Life Technologies). The regions of DRN targeted for capture were the dorsal and ventral portions of the rostral to mid-caudal DRN. Samples were captured so that each sample contained the entire portion of the dorsal and ventral portion of the DRN at the given rostral-caudal level. DRN samples were obtained by using an infrared laser to adhere the tissue to a cap coated with a thermoplastic film (Capsure Macro LCM Caps, Applied Biosystems). An ultraviolet laser was used to separate the DRN from the rest of the brain section. An average of 23 DRN samples, ranging in size from 300,000 to 800,000 $um^2$ (depending on rostral to caudal level), were successfully dissected and pooled for each rat to ensure maximal total RNA yield. Following laser capture microdissection, caps were incubated in RNA extraction buffer (Applied Biosystems) for 30 min and frozen at −80°C until future use. RNA was isolated using the Arcturus Picopure RNA Isolation Kit (Applied Biosystems) in accordance with kit protocols. Samples were stored in Elution Buffer (Applied Biosystems) at −80°C until microarray analysis.

#### MICROARRAY ANALYSIS

Samples were sent to the Genomics and Microarray Core Facility at the University of Colorado Denver for whole genome analysis using microarray. RNA integrity was evaluated with the Agilent Bioanalyzer 2100 and RNA 6000 Nano/Pico Kit (Agilent Technologies). Concentrations of extracted RNA were assessed with the Nanodrop spectrophotometer (Nanodrop Technologies). One sample was removed from further processing due to poor integrity of RNA ($n = 47$). A total of 100–150 ug RNA per each sample was converted to double stranded cDNA and then transcribed into cRNA using the Ambion WT Expression Kit, in accordance with kit protocols. Following generation of cRNA, second cycle, first strand cDNA synthesis was carried out in order to transform the cRNA into single-strand cDNA. The cDNA was fragmented and the Genechip WT Terminal Labeling Kit (Affymetrix) was used to label the single-stranded DNA with biotin. Samples were hybridized to an Affymetrix Genechip Rat Gene 1.1 ST Array Platform. Hybridization, washing, staining, and scanning were executed using the GeneTitan instrument (Affymetrix).

#### MICROARRAY DATA PRE-PROCESSING

The Bioconductor toolset within the R statistical software program was used to format the raw microarray data. This preprocessing was completed using the 'expresso' option in the 'affy' package of the Bioconductor toolset and included background adjustment, log fold transformation, and normalization. To control for inter-array variability, the dataset was normalized using the Robust Multi Array Average method. Gene chip and RNA quality were assessed by examining total mRNA expression for each animal.

#### MICROARRAY CONTRAST GENERATION

Following pre-processing and normalization, a standardized expression value was obtained for each gene for each rat.

The expression values for each gene were averaged for each experimental group. The LIMMA package was used to generate nine contrasts between experimental groups. These contrasts included [($Sed_{Stress0}$ v. $Sed_{HC}$) v. ($Run_{Stress0}$ v. $Run_{HC}$)], [($Sed_{Stress2}$ v. $Sed_{HC}$) v. ($Run_{Stress2}$ v. $Run_{HC}$)], $Run_{HC}$ v. $Sed_{HC}$, $Sed_{Stress0}$ v. $Sed_{HC}$, $Sed_{Stress2}$ v. $Sed_{HC}$, $Run_{Stress0}$ v. $Run_{HC}$, $Run_{Stress2}$ v. $Run_{HC}$, $Run_{Stress0}$ v. $Sed_{Sress0}$, and $Run_{Stress2}$ v. $Sed_{Sress2}$. For each contrast, the difference in the expression level of each individual gene was calculated by subtracting the expression level of the 2nd group in each contrast from the expression level of the 1st group in each contrast. For example, the contrast $Run_{HC}$ v. $Sed_{HC}$ indicates that the expression level of gene X in the $Sed_{HC}$ group was subtracted from the expression level of gene X in the $Run_{HC}$ group, or ($Run_{HC}$—$Sed_{HC}$). The first two contrasts represent the interaction between exercise and stress at each time point. For each contrast, *p*-values and test statistics were calculated for each gene according to the absolute value of difference in gene expression observed between the groups. The False Discovery Rate multiple-test adjustment method was applied in the calculation of these *p*-values in order to control for the chance of yielding false positive (significant) results. The log fold change (LFC) in gene expression was also calculated for each gene in each contrast. Out of 27,000 possible genes, 17,170 gene transcripts were reliably detected. These genes were considered the transcriptome, or the genes expressed in cells of the DRN as a result of the experimental conditions.

### DIFFERENTIAL GENE EXPRESSION IDENTIFICATION AND BIOINFORMATICS

In an initial approach, genes differentially expressed by a LFC $\geq \pm 1.1$ were identified for each contrast. A second approach was performed utilizing the same contrasts as previously described. However, less stringent requirements for statistical significance were utilized ($p < 0.05$) to identify differentially expressed genes between groups. Genes that were significantly differentially expressed at a $p < 0.05$ were organized into nine sets, one set for each contrast, and imported into the bioinformatics system, Web Gestaldt. Specifically, KEGG (Kanehisa and Goto, 2000) and Wikipathways (Wiki) (Kelder et al., 2012) pathway enrichment analysis were applied to each gene set in order to identify the top functionally enriched pathway categories related to the genes significantly differentially expressed in each contrast. Both KEGG and Wiki databases were used in an effort to generate a more comprehensive analysis. The KEGG system is recognized as one of the major pathway databases (Bauer-Mehren et al., 2009), whereby data is derived from published work. KEGG pathway content includes categories in metabolism, genetic information processing, organismal systems, environmental information processing, cellular processes, and drug development. The Wiki system is curated by the scientific community and serves as a complementary and enhancing source to the KEGG database (Bauer-Mehren et al., 2009). Finally, ANOVAs were performed on select genes of interest that were identified through the pathway analysis.

### WEIGHTED GENE CORRELATIONAL NETWORK ANALYSIS

Given that genes often operate in a coordinated manner to accomplish a physiological function, a more sophisticated approach utilizing Weighted Gene Correlational Network Analysis (WGCNA) was also performed. That is, in the absence of absolute differences in gene expression, the coexpression of genes may differ across experimental conditions. The WGCNA package within the R software program was used to perform this analysis. Following standard preprocessing and normalization of the data, a gene expression profile was available for each rat. Based on this expression profile, rats were clustered hierarchically within a dendogram based on Euclidian distance, or similarity between expression profiles. The dendogram was visualized to see how the physical traits (experimental conditions) related to the various clusters. Next, modules of highly coexpressed genes were identified and related to physical traits. Importantly, the genes within each module are more highly correlated with each other than to the rest of the transcriptome. Physical traits were categorized by experimental group (Sed.HC, Sed.Stress0, Sed.Stress2, Run.HC, Run.Stress0, Run.Stress2) and differences between groups (RunvsSed.HC, Stress0vsHC.Sed, Stress2vsHC.Sed, Stress0vsHC.Run, Stress2vsHC.Run, RunvsSed.Stress0, RunvsSed.Stress2). A correlation value and *p*-value associated with the strength of correlation was calculated for each module. These values are considered a representation of the correlation and correlational strength of the module to each physical trait. Modules with a correlational strength of $p < 0.001$ were targeted for further investigation by ANOVA. Following ANOVA analysis, modules that had statistically significant main effects of exercise, stress and/or an exercise by stress interaction were subjected to KEGG and Wiki analyses.

## RESULTS

### BODY WEIGHT AND RUNNING DISTANCE

A repeated measures ANOVA was used to analyze body weights. Repeated measures ANOVA analysis revealed statistically significant main effects of time [$F_{(6,\ 252)} = 518.415$; $p < 0.0001$] and exercise [$F_{(1,\ 42)} = 7.759$; $p = 0.0080$] and a reliable time by exercise interaction [$F_{(6,\ 252)} = 2.634$; $p = 0.0171$] on body weight. Running distance steadily increased over the course of the experiment from 5959.305 ± 382.081 m during week 1 to 19355.603 ± 2983.808 m during week 6 [$F_{(5,\ 115)} = 18.870$; $p < 0.0001$].

### ASSESSMENT OF RNA AND MICROARRAY GENECHIP QUALITY

To verify microarray chip quality and mRNA integrity, boxplots were constructed that represented total mRNA expression for each rat. Visual inspection of boxplots revealed four outliers. One sample was previously identified with spectrophotometry analysis. The additional three outliers were dropped from further analysis. Final group totals were $Sed_{HC}$ ($n = 6$), $Sed_{Stress0}$ ($n = 7$), $Sed_{Stress2}$ ($n = 7$), $Run_{HC}$ ($n = 8$), $Run_{Stress0}$ ($n = 8$), $Run_{Stress2}$ ($n = 8$), for a total of ($n = 44$) rats.

### DIFFERENTIAL GENE EXPRESSION ANALYSIS RESULTS

#### *The effect of wheel running and/or exposure to stress on log fold changes in gene expression of ±1.1 in the DRN*

In a conservative initial approach, genes differentially expressed by a LFC $\geq \pm 1.1$ in response to exercise and/or stress were identified. Overall, relatively few genes had LFCs in expression $\geq \pm 1.1$. The effect of stress on LFCs in gene expression $\geq \pm 1.1$ is not

different between sedentary and physically active rats immediately following or 2 h after stress exposure. When considering the effect of exercise, only one gene was statistically significantly altered. This gene was transthyretin, which was downregulated in physically active rats. Following stress exposure, transythretin remained downregulated in physically active rats immediately after, but not 2 h post stress. Compared to home cage controls, stress produced alterations in gene expression regardless of physical activity status at both time points. **Figure 1** shows the effect of stress on total number of genes altered (A) and in which direction (B) by a LFC $\geq \pm 1.1$ in sedentary rats and physically active rats immediately following and 2 h post stress relative to home cage non-stressed controls. Exposure to stress produced alterations in gene expression immediately following and 2 h after in both sedentary and physically active rats. A greater number of genes changed in physically active rats (41 and 27) than sedentary rats (18 and 26) for both time points (**Figure 1A**). All 18 genes that were differentially expressed immediately following stress in sedentary rats were also differentially expressed immediately following stress in physically active rats. Twenty-two of the twenty-six genes that were differentially expressed 2 h post stress in sedentary rats were also differentially expressed 2 h post stress in physically active rats. Two genes were differentially expressed exclusively in the physically active rats exposed to stress. These genes were CD180 and fos-like antigen 1, which were different at both time points. There were also two genes that were differentially expressed exclusively in sedentary rats exposed to stress compared to home cage non-stressed controls. These genes were only differentially expressed 2 h post stress and were fibronectin 1 and solute carrier organic anion transporter family, member 1c1.

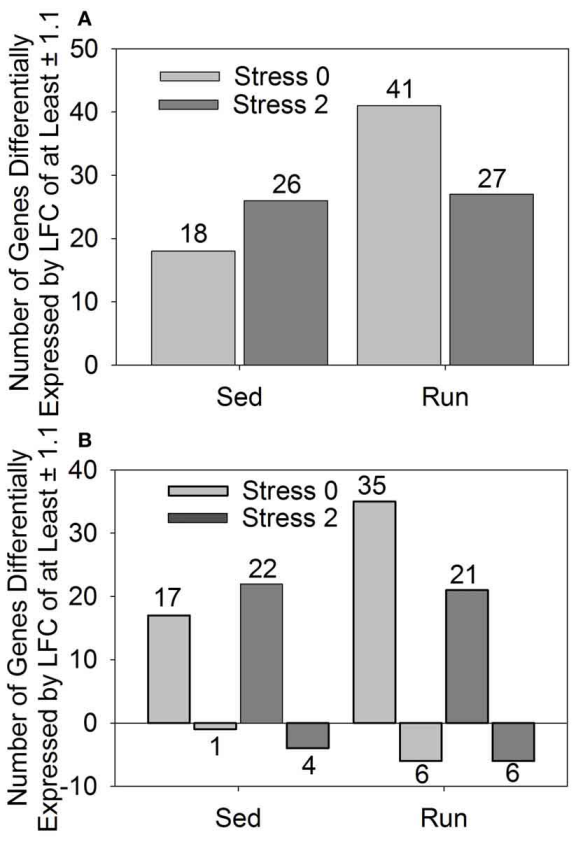


**FIGURE 1 | Number of genes differentially expressed by a log fold change $\geq \pm 1.1$ in rats exposed to stress compared to home cage non-stressed controls. (A)** Total number of genes differentially expressed and **(B)** Number of genes differentially upregulated or downregulated.

**Figure 1B** shows the number of genes that were upregulated and downregulated by a LFC $\geq 1.1$ in sedentary and physically active rats in response to stress. For all groups, a greater number of genes were differentially upregulated than downregulated in stressed rats compared to home cage non-stressed controls.

### *The effect of wheel running and/or exposure to stress on changes in gene expression of $p < 0.05$ in the DRN*

A second less stringent approach utilized *p*-values that were corrected for multiple comparisons to identify genes that were differentially expressed by $p < 0.05$ in response to exercise and/or stress. The effect of stress on differential gene expression is different depending on physical activity status. Differential expression of 1028 genes was observed immediately following stress and 637 genes were differentially expressed 2 h post stress in sedentary rats compared to physically active rats. (These results are detailed in **Figure 3** and **Table 3**). Compared to sedentary rats, physically active rats had differential expression in 2350 genes (1290 upregulated, 1060 downregulated). Immediately following stress, differential expression of 634 genes (413 upregulated, 221 downregulated) was observed in physically active rats compared to sedentary rats. Two hours post stress, 997 genes (610 upregulated, 387 downregulated) were differentially expressed in physically active rats compared to sedentary rats. Compared to home cage non-stressed controls, stress produced changes in gene expression in both sedentary and physically active rats. **Figure 2** shows the effect of stress on total number of genes altered (A) and in which direction (B) by $p < 0.05$ in sedentary rats and physically active rats immediately following and 2 h post stress relative to home cage non-stressed controls. Exposure to stress produced alterations in gene expression immediately following and 2 h after stress in both sedentary and physically active rats. For both time points, physically active rats exposed to stress had a greater number of genes differentially expressed relative to home cage non-stressed controls than sedentary rats exposed to stress (**Figure 2A**). For both sedentary and physically active rats, stress altered the expression of a greater number of genes 2 h after compared to immediately following stress. This difference was greater in sedentary rats.

**Figure 2B** indicates the number of genes that were upregulated and downregulated by $p < 0.05$ in sedentary and physically active rats in response to stress. For both sedentary and physically active rats, a greater number of genes were differentially upregulated 2 h after compared to immediately following stress. In contrast, the number of genes differentially downregulated was greater immediately following stress compared to 2 h after for both sedentary and physically active rats. For both time points, physically active rats had a greater number of genes upregulated than sedentary rats. Similarly, physically active rats had a greater number of genes downregulated than sedentary rats at both time points.

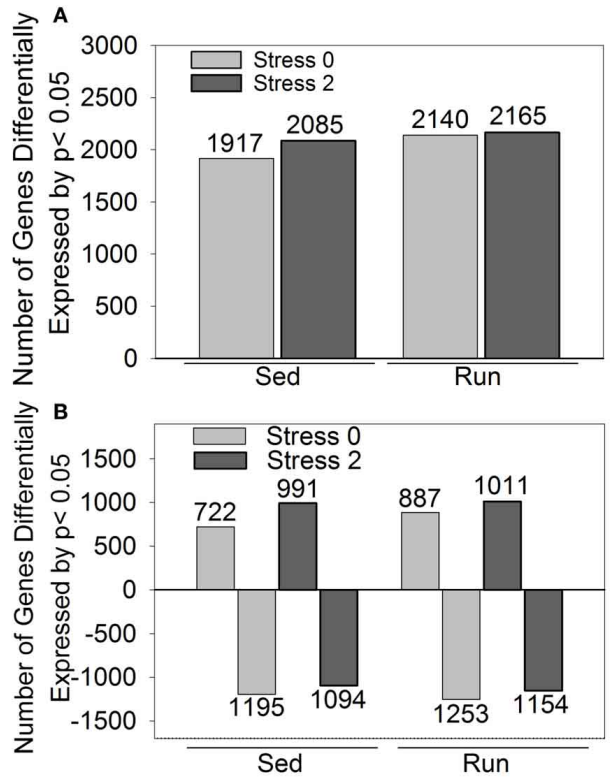


**FIGURE 2 | Number of genes differentially expressed by $p < 0.05$ in rats exposed to stress compared to home cage non-stressed controls.** **(A)** Total number of genes differentially expressed. **(B)** Number of genes differentially upregulated or downregulated.

### *KEGG functionally enriched pathway categories related to genes differentially expressed in the DRN following 6 weeks of wheel running and/or exposure to stress*

Genes that were differentially expressed at $p < 0.05$ across the various contrasts were imported into Web Gestaldt bioinformatics system and subjected to analysis with KEGG functional terms. **Table 1** shows the top ten functionally enriched pathway categories, for each contrast, related to genes differentially expressed following 6 weeks of wheel running and/or exposure to stress. Gene count refers to the number of genes from the data sets that contribute to each functional category. The *p*-value represents the statistical significance of each functionally enriched category identified.

KEGG analysis revealed that genes that were differentially expressed between sedentary and physically active rats in response to stress were related to functional categories including metabolic pathways, mitogen-activated protein kinase (MAPK) signaling, neuroactive ligand receptor interaction, transforming growth factor-β (TGF-β) signaling, epidermal growth factor family of receptor tyrosine kinases (ErbB) signaling, and vascular endothelial growth factor (VEGF) signaling immediately following and or 2 h post stress.

Six weeks of wheel running modulated the expression of genes involved in physiological processes including metabolic activity,

**Table 1 | KEGG functionally enriched pathway categories generated from genes significantly differentially expressed at $p < 0.05$ in the DRN in response to exercise and/or stress.**

| Contrast | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| **($Sed_{Stress0}$ vs. $Sed_{HC}$) vs. ($Run_{Stress0}$ vs. $Run_{HC}$)** | | |
| Olfactory transduction:04740 | 70 | 1.65e–13 |
| Ribosome:03010 | 18 | 9.81e–12 |
| Metabolic pathways:01100 | 69 | 1.48e–10 |
| MAPK signaling pathway:04010 | 23 | 6.84e–07 |
| Neuroactive ligand receptor interaction:04080 | 20 | 1.92e–05 |
| Pathways in cancer:05200 | 23 | 2.34e–05 |
| Endocytosis:04144 | 17 | 5.79e–05 |
| TGF beta signaling pathway:04350 | 10 | 6.16e–05 |
| ErbB signaling pathways:04012 | 10 | 0.0001 |
| Arachidonic acid metabolism:00590 | 9 | 0.0010 |
| **($Sed_{Stress2}$ vs. $Sed_{HC}$) vs. ($Run_{Stress2}$ vs. $Run_{HC}$)** | | |
| Olfactory transduction:04740 | 66 | 1.78e–21 |
| Allograft rejection:05330 | 8 | 7.41e–06 |
| Pathways in cancer:05200 | 18 | 1.16e–05 |
| Autoimmune thyroid disease:05320 | 8 | 2.27e–05 |
| Graft-vs.-host disease:05332 | 7 | 5.39e–05 |
| C21-Steroid hormone metabolism:00140 | 4 | 4.44e–05 |
| Androgen and estrogen metabolism:00150 | 6 | 3.77e–05 |
| Type I diabetes mellitus:04940 | 7 | 0.0001 |
| Non-small cell lung cancer:05223 | 6 | 0.0003 |
| VEGF signaling pathway:04370 | 7 | 0.0003 |
| **$Run_{HC}$ vs. $Sed_{HC}$** | | |
| Metabolic pathways:01100 | 141 | 3.24e–17 |
| Olfactory transduction:04740 | 115 | 4.84e–12 |
| MAPK signaling pathway:04010 | 48 | 3.22e–12 |
| Pathways in cancer:05200 | 53 | 2.28e–11 |
| Ribosome:03010 | 23 | 6.46e–10 |
| FC epsilon RI signaling pathway:04664 | 21 | 2.54e–09 |
| Cell cycle:04110 | 27 | 3.83e–09 |
| Long term depression:04730 | 19 | 5.84e–09 |
| Gap junction:04540 | 21 | 1.65e–08 |
| Vascular smooth muscle contraction:04270 | 24 | 4.09e–08 |
| **$Sed_{Stress0}$ vs. $Sed_{HC}$** | | |
| MAPK signaling pathway:04010 | 58 | 1.10e–21 |
| Metabolic pathways:01100 | 104 | 2.33e–09 |
| VEGF signaling pathway:04370 | 19 | 5.06e–09 |
| Neurotropin signaling pathway:04722 | 25 | 9.26e–09 |
| Pathways in cancer:05200 | 42 | 2.58e–08 |
| Chronic myeloid leukemia:05220 | 17 | 4.77e–07 |
| Toll-like receptor signaling pathway:04620 | 18 | 7.50e–07 |
| GnRH signaling pathway:04912 | 16 | 1.93e–05 |
| Leukocyte transendothelial migration:04670 | 18 | 2.29e–05 |
| FC epsilon RI signaling pathways:04664 | 14 | 2.75e–05 |
| **$Sed_{Stress2}$ vs. $Sed_{HC}$** | | |
| MAPK signaling pathway:04010 | 56 | 9.54e–19 |
| Pathways in cancer:05200 | 53 | 8.51e–13 |
| Adipocytokine signaling pathway:04920 | 22 | 1.95e–12 |

*(Continued)*

**Table 1 | Continued**

| Contrast | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| Metabolic pathways:01100 | 116 | 3.16e–11 |
| VEGF signaling pathway:04370 | 19 | 1.68e–08 |
| Neuroactive ligand receptor interaction:04080 | 36 | 1.80e–07 |
| Jak-STAT signaling pathway:04630 | 25 | 2.22e–07 |
| Leukocyte transendothelial migration:04670 | 22 | 3.05e–07 |
| Regulation of actin cytoskeleton:04810 | 31 | 4.89e–07 |
| Toll-like receptor signaling pathway:04620 | 19 | 4.78e–07 |
| **$Run_{Stress0}$ vs. $Run_{HC}$** | | |
| MAPK signaling pathway:04010 | 63 | 2.05e–23 |
| Metabolic pathways:01100 | 125 | 9.13e–14 |
| Pathways in cancer:05200 | 50 | 5.84e–11 |
| Cytokine-cytokine receptor interaction:04060 | 35 | 5.59e–10 |
| Adipocytokine signaling pathway:04920 | 17 | 6.23e–08 |
| Focal adhesion:04510 | 31 | 8.65e–08 |
| P53 signaling pathway:04115 | 17 | 3.64e–07 |
| Neuroactive ligand receptor interaction:04080 | 35 | 7.63e–07 |
| Calcium signaling pathway:04020 | 28 | 1.25e–06 |
| Neutrotrophin signaling pathway:04722 | 22 | 3.15e–06 |
| **$Run_{Stress2}$ vs. $Run_{HC}$** | | |
| Pathways in cancer:05200 | 74 | 3.25e–25 |
| MAPK signaling pathway:04010 | 62 | 3.30e–22 |
| Metabolic pathways:01100 | 127 | 8.15e–14 |
| Jak-STAT signaling pathway:04630 | 35 | 1.15e–13 |
| Neuroactive ligand receptor interaction:04080 | 44 | 4.00e–11 |
| Chronic myeloid leukemia:05220 | 23 | 6.70e–11 |
| Cytokine-cytokine receptor interaction:04060 | 37 | 6.25e–11 |
| Focal adhesion:04510 | 35 | 8.43e–10 |
| Prostate cancer:05215 | 23 | 1.01e–09 |
| Pancreatic cancer:05212 | 19 | 1.20e–08 |
| **$Run_{Stress0}$ vs. $Sed_{Stress0}$** | | |
| Cytokine-cytokine receptor interaction:04060 | 12 | 7.82e–05 |
| Pathways in cancer:05200 | 15 | 0.0003 |
| Chemokine signaling pathway:04062 | 9 | 0.0018 |
| MAPK signaling pathway:04010 | 11 | 0.0038 |
| Toll-like receptor signaling pathway:04620 | 6 | 0.0038 |
| Olfactory transduction:04740 | 28 | 0.0032 |
| Apoptosis:04210 | 6 | 0.0047 |
| Arachidonic acid metabolism:00590 | 5 | 0.0058 |
| Jak-STAT signaling pathway:04630 | 7 | 0.0086 |
| TGF beta signaling pathway:04350 | 5 | 0.0103 |
| **$Run_{Stress2}$ vs. $Sed_{Stress2}$** | | |
| Neuroactive ligand receptor interaction:04080 | 23 | 2.38e–07 |
| Prostate cancer:05215 | 13 | 6.23e–07 |
| Pathways in cancer:05200 | 24 | 4.38e–06 |
| Ribosome:03010 | 11 | 1.22e–05 |
| Focal adhesion:04510 | 16 | 4.16e–05 |
| Regulation of actin cytoskeleton:04810 | 17 | 3.59e–05 |
| Melanoma:05218 | 9 | 8.38e–05 |
| Olfactory transduction:04740 | 44 | 0.0004 |
| Cell adhesion molecule:04514 | 12 | 0.0006 |
| Wnt signaling pathway:04310 | 11 | 0.0011 |

olfactory transduction, MAPK signaling, cell cycle, and long term depression.

Compared to home cage non-stressed controls, both sedentary and physically active rats exposed to stress had significant enrichment of functional categories related to MAPK signaling, metabolic pathways, adipocytokine signaling, and neuroactive ligand receptor interaction. Significant enrichment of functional categories including VEGF signaling and toll-like receptor signaling was exclusive to sedentary stressed rats compared to home cage non-stressed controls and occurred at both time points. Compared to home cage non-stressed controls, stress modulated the expression of genes involved in cytokine-cytokine receptor interaction exclusively in physically active rats at both time points.

A direct comparison of sedentary and physically active rats exposed to stress, revealed enrichment differences in functional categories related to cytokine-cytokine receptor interaction, chemokine signaling, MAPK signaling, toll-like receptor signaling, apoptosis, janus kinase-signal transducer and activator of transcription (Jak-Stat) signaling, TGF-β signaling, neuroactive ligand receptor interaction, cell adhesion molecules, and wingless-type mouse mammary tumor virus integration site (WNT) signaling either immediately following and/or 2 h post stress.

### *Wiki functionally enriched pathway categories related to genes differentially expressed in the DRN following 6 weeks of wheel running and/or exposure to stress*

Genes that were differentially expressed at $p < 0.05$ across the various contrasts were imported into Web Gestaldt bioinformatics system and subjected to analysis with Wiki functional terms. **Table 2** shows the top ten functionally enriched pathway categories, for each contrast, related to genes differentially expressed following 6 weeks of wheel running and/or exposure to stress. Gene count refers to the number of genes from the data sets that contribute to each functional category. The *p*-value represents the significance of each functionally enriched category identified.

Wiki analysis revealed that genes that were differentially expressed between sedentary and physically active rats in response to stress were related to functional categories including metabolic pathways, MAPK signaling, adipogenesis, biosynthesis of aldosterone and cortisol, and diurnally regulated genes with circadian orthologs. In addition, various immune-associated categories were also identified including those related to the inflammatory and cytokine response as well as signaling pathways for interleukin-5 (IL-5), IL-6, B-cell receptor, TGF-β receptor, and TNF-α-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

Six weeks of wheel running modulated the expression of genes involved in physiological processes related to signaling pathways for MAPK, epidermal growth factor receptor 1 (EGFR1), TNF-α-NF-κB, Insulin, G-protein, IL-5, and B-cell receptor. Compared to home cage non-stressed controls, both sedentary and physically active rats exposed to stress had enrichment of functional categories related to signaling pathways for MAPK, insulin, TGF-β receptor, IL-6, EGFR1, delta notch, and toll-like

**Table 2 | Wiki functionally enriched pathway categories generated from genes significantly differentially expressed at $p < 0.05$ in the DRN in response to exercise and/or stress.**

| Contrast | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| **($Sed_{Stress0}$ vs. $Sed_{HC}$) vs. ($Run_{Stress0}$ vs. $Run_{HC}$)** | | |
| Cytoplasmic ribosomal proteins:WP30 | 17 | 3.08e–09 |
| MAPK signaling pathway:WP358 | 14 | 2.17e–05 |
| IL-5 signaling pathway:WP44 | 9 | 4.29e–05 |
| Insulin signaling:WP439 | 13 | 7.33e–05 |
| B cell receptor signaling pathway:WP285 | 13 | 0.0001 |
| Diurnally regulated genes with circadian orthologs:WP1306 | 6 | 0.0005 |
| TGF beta receptor signaling pathway:WP362 | 11 | 0.0005 |
| Adipogenesis:WP155 | 10 | 0.0008 |
| Fas pathway and stress induction of HSP regulation:WP89 | 6 | 0.0007 |
| IL-6 signaling pathway:WP135 | 9 | 0.0008 |
| **($Sed_{Stress2}$ vs. $Sed_{HC}$) vs. ($Run_{Stress2}$ vs. $Run_{HC}$)** | | |
| Biosynthesis of aldosterone and cortisol:WP508 | 2 | 0.0038 |
| Diurnally regulated genes with circadian orthologs:WP1306 | 4 | 0.0037 |
| Steroid biosynthesis:WP66 | 2 | 0.0070 |
| TNF alpha NF-kB signaling pathway:WP457 | 6 | 0.0579 |
| GPCRs, class A rhodopsin-like:WP473 | 7 | 0.0485 |
| Kit receptor signaling pathway:WP147 | 4 | 0.0206 |
| Inflammatory response pathway:WP40 | 2 | 0.0666 |
| Cytokines and inflammatory response:WP271 | 2 | 0.0494 |
| Ovarian infertility genes:WP263 | 2 | 0.0535 |
| Metapathway biotransformation:WP1286 | 5 | 0.0379 |
| **$Run_{HC}$ vs. $Sed_{HC}$** | | |
| MAPK signaling pathway:WP358 | 29 | 2.69e–09 |
| EGFR1 signaling pathway:WP5 | 29 | 3.80e–08 |
| TNF alpha NF-KB signaling pathway:WP457 | 30 | 3.17e–08 |
| Insulin signaling:WP439 | 25 | 4.32e–07 |
| Renin-angiotensin system:WP376 | 13 | 3.97e–07 |
| Myometrial relaxation and contraction pathways:WP140 | 24 | 5.98e–07 |
| Regulation of actin cytoskeleton:WP351 | 24 | 6.89e–07 |
| G protein signaling pathways:WP73 | 18 | 1.14e–06 |
| IL-5 signaling pathway:WP44 | 15 | 3.31e–06 |
| B cell receptor signaling pathway:WP285 | 24 | 5.24e–06 |
| **$Sed_{Stress0}$ vs. $Sed_{HC}$** | | |
| MAPK signaling pathway:WP358 | 36 | 2.63e–16 |
| Insulin signaling:WP439 | 31 | 1.25e–12 |
| TGF beta receptor signaling pathway:WP362 | 25 | 2.96e–09 |
| GPCRs, class A rhodopsin-like:WP473 | 32 | 4.84e–09 |
| Adipogenesis:WP155 | 23 | 6.55e–09 |
| EGFR1 signaling pathway:WP5 | 26 | 7.67e–08 |
| IL-6 signaling pathway:WP135 | 19 | 1.53e–07 |
| Toll-like receptor signaling pathway:WP1309 | 16 | 5.76e–07 |
| IL-3 signaling pathway:WP319 | 18 | 5.39e–07 |
| B cell receptor signaling pathway:WP285 | 23 | 1.21e–06 |
| **$Sed_{Stress2}$ vs. $Sed_{HC}$** | | |
| MAPK signaling pathway:WP358 | 38 | 6.26e–17 |
| Adipogenesis:WP155 | 28 | 5.99e–12 |
| EGFR1 signaling pathway:WP5 | 33 | 1.42e–11 |
| B cell receptor signaling pathway:WP285 | 28 | 5.19e–09 |
| Insulin signaling:WP439 | 27 | 4.64e–09 |
| IL-3 signaling pathway:WP319 | 21 | 1.29e–08 |
| IL-6 signaling pathway:WP135 | 21 | 1.93e–08 |
| Toll-like receptor signaling pathway:WP1309 | 18 | 5.10e–08 |
| Delta notch signaling pathway:WP199 | 18 | 5.10e–08 |
| TNF alpha NF-KB signaling pathway:WP457 | 28 | 6.69e–08 |
| **$Run_{Stress0}$ vs. $Run_{HC}$** | | |
| MAPK signaling pathway:WP358 | 33 | 8.27e–13 |
| Insulin signaling:WP439 | 31 | 1.27e–11 |
| Adipogenesis:WP155 | 27 | 5.08e–11 |
| EGFR1 signaling pathway:WP5 | 31 | 4.54e–10 |
| Apoptosis mechanisms:WP284 | 21 | 1.50e–09 |
| Apoptosis:WP1290 | 21 | 1.91e–09 |
| Diurnally regulated genes with circadian orthologs:WP1306 | 13 | 6.73e–09 |
| Cardiovascular signaling:WP590 | 14 | 3.15e–08 |
| Toll-like receptor signaling pathway:WP1309 | 24 | 7.80e–08 |
| T cell receptor signaling pathway:WP352 | 23 | 9.49e–08 |
| **$Run_{Stress2}$ vs. $Run_{HC}$** | | |
| MAPK signaling pathway:WP358 | 40 | 4.75e–18 |
| Adipogenesis:WP155 | 33 | 1.25e–15 |
| EGFR1 signaling pathway:WP5 | 39 | 2.01e–15 |
| Insulin signaling:WP439 | 35 | 2.93e–14 |
| B cell receptor signaling pathway:WP285 | 33 | 5.96e–12 |
| IL-6 signaling pathway:WP135 | 26 | 5.32e–12 |
| Delta notch signaling pathway:WP199 | 21 | 3.62e–10 |
| TGF beta signaling pathways:WP505 | 17 | 6.00e–10 |
| GPCRs, class A rhodopsin-like:WP473 | 34 | 5.30e–09 |
| TGF beta receptor signaling pathway:WP362 | 26 | 6.09e–09 |
| **$Run_{Stress0}$ vs. $Sed_{Stress0}$** | | |
| Delta notch signaling pathway:WP199 | 8 | 2.30e–05 |
| Kit receptor signaling pathway:WP147 | 7 | 6.36e–05 |
| IL-5 signaling pathway:WP44 | 7 | 7.03e–05 |
| IL-3 signaling pathway:WP319 | 7 | 0.0007 |
| IL-6 signaling pathway:WP135 | 7 | 0.0007 |
| TGF beta signaling pathways:WP505 | 5 | 0.0011 |
| Toll-like receptor signaling pathway:WP1309 | 6 | 0.0012 |
| Notch signaling pathway:WP517 | 4 | 0.0017 |

*(Continued)*

**Table 2 | Continued**

| Contrast | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| Endochondral ossification:WP1308 | 5 | 0.0017 |
| Hedgehog signaling pathway:WP574 | 3 | 0.0026 |
| **$Run_{Stress2}$ vs. $Sed_{Stress2}$** | | |
| Adipogenesis:WP155 | 11 | 0.0001 |
| GPCRs, class A rhodopsin-like:WP473 | 15 | 0.0002 |
| B cell receptor signaling pathway:WP285 | 12 | 0.0004 |
| Hypothetical network for drug addiction:WP1281 | 5 | 0.0007 |
| IL-6 signaling pathway:WP135 | 9 | 0.0006 |
| Regulation of actin cytoskeleton:WP351 | 11 | 0.0006 |
| Calcium regulation in the cardiac cell:WP326 | 10 | 0.0009 |
| Cytoplasmic ribosomal proteins:WP30 | 9 | 0.0017 |
| Androgen receptor signaling pathway:WP68 | 9 | 0.0019 |
| Myometrial relaxation and contraction pathways:WP140 | 10 | 0.0019 |


**FIGURE 3 | Effect of physical activity status on stress-induced differential gene expression in the DRN by $p < 0.05$.** Number of genes differentially expressed immediately following (red) and 2 hours post (blue) stress, and genes differentially expressed at both time points (purple).

receptor. Other genes affected by stress were related to functional categories including G protein coupled receptors (GPCRs) and adipogenesis. Compared to home cage non-stressed controls, stress modulated the expression of genes involved in interleukin-3 (IL-3) signaling exclusively in sedentary rats at both time points. Significant enrichment of categories related to apoptosis, diurnally regulated genes with circadian orthologs, cardiovascular signaling, and T-cell receptor signaling was exclusive to physically active stressed rats compared to home cage non-stressed controls.

A direct comparison of sedentary and physically active rats exposed to stress, revealed significant differences in functional categories related to signaling pathways including delta notch, kit receptor, IL-3, IL-5, IL-6, TGF-β receptor, toll-like receptor, and B-cell receptor. Significant enrichment of functional categories related to adipogenesis and GPCRs was also observed.

**Table 3 | Functionally enriched KEGG and Wiki pathway categories related to genes differentially expressed in physically active and sedentary rats immediately following and 2 h post stress.**

| Pathway analysis | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| **KEGG ENRICHMENT ANALYSIS** | | |
| Olfactory transduction:04740 | 19 | 2.80en–08 |
| Pathways in cancer:05200 | 8 | 0.0001 |
| Metabolic pathways:01100 | 15 | 0.0001 |
| Allograft rejection:05330 | 4 | 0.0001 |
| MAPK signaling pathway:04010 | 7 | 0.0002 |
| Autoimmune thyroid disease:05320 | 4 | 0.0003 |
| VEGF signaling pathway:04370 | 4 | 0.0004 |
| Intestinal immune network for IgA production:04672 | 3 | 0.0010 |
| C21-Steroid hormone metabolism:00140 | 2 | 0.0014 |
| Graft-vs. host disease:05332 | 3 | 0.0022 |
| **WIKI ENRICHMENT ANALYSIS** | | |
| Diurnally regulated genes with circadian orthologs:WP1306 | 3 | 0.0006 |
| B cell receptor signaling pathway:WP285 | 4 | 0.0040 |
| Cytokine and inflammatory response:WP271 | 2 | 0.0040 |
| Tryptophan metabolism:WP270 | 2 | 0.0128 |
| Regulation of actin cytoskeleton:WP351 | 3 | 0.0201 |
| TGF beta signaling pathway:WP505 | 2 | 0.0195 |
| MAPK signaling pathway:WP358 | 3 | 0.0238 |
| Kit receptor signaling pathway:WP147 | 2 | 0.0309 |
| IL-5 signaling pathway:WP44 | 2 | 0.0318 |
| GPCRs, class A rhodopsin-like:WP473 | 3 | 0.0564 |

### *The effect of stress on differential expression of genes in the DRN is different depending on physical activity status*

The effect of stress on gene expression in the DRN is different depending on physical activity status. **Figure 3** shows the number of genes changed by $p < 0.05$ that were differentially expressed between sedentary rats and physically active rats immediately following or 2 h post stress. Of these genes, 1028 were differentially expressed immediately following stress. Differential expression of 637 genes was observed 2 h after stress. Differential expression of 169 genes was observed in sedentary rats compared to physically active rats immediately following stress and differential expression of these genes was also present 2 h after stress.

Genes that were differentially expressed at both time points ($n = 169$) were subjected to KEGG pathway analysis (**Table 3**) in order to identify functionally enriched pathway categories related to these genes. Stress differentially impacted the expression of genes related to functional categories including olfactory transduction, metabolic pathways, MAPK signaling pathways, and VEGF signaling pathways in physically active compared to sedentary rats.

Genes that were differentially expressed at both time points ($n = 169$) were also subjected to Wiki pathway analysis (**Table 3**). Stress differentially impacted the expression of genes related to functional categories including diurnally regulated genes with circadian orthologs, tryptophan metabolism, and GPCRs in physically active compared to sedentary rats. Various immune-associated pathway categories were also identified such as cytokine and inflammatory response pathways as well as signaling pathways for B-cell receptor, TGF-β, and IL-5.

### *The effect of wheel running and/or exposure to stress on the expression of select genes related to functionally enriched wiki pathway categories*

Select genes from Wiki functional pathway categories were targeted for analysis by ANOVA to reveal the main effect of wheel running, main effect of stress, and exercise x stress interaction on gene expression in the DRN immediately following and 2 h post stress. **Table 4** shows the statistical results of the ANOVAs for each gene. **Figure 4** displays the graphs for each ANOVA. Genes were selected from the following functional categories: diurnally regulated genes with circadian orthologs, tryptophan metabolism, and various immune-related pathways including B-cell receptor signaling, TGF-β signaling, IL-5 signaling, and cytokines and inflammatory response. Within the diurnally regulated genes with circadian orthologs category, genes selected for ANOVA were Kruppel-like factor 9 (*Klf9*), protein phosphate 1 (*Ppp1r3c*), and G0/G1 switch 2 (*G0s2*). Within the tryptophan metabolism category, genes selected for ANOVA were aldehyde dehydrogenase 1 family (*Aldh1a2*) and tryptophan 2,3-dioxygenase (*Tdo2*). Within the immune-related categories, genes selected for ANOVA included hematopoietic cell specific Lyn substrate 1 (*Hcls1*), phospholipase C, gamma 2 (*Plcg2*), Cas-Br-M ecotropic retroviral transforming sequence b (*Cblb*), hemopoietic cell kinase (*Hck*), interleukin 4 (*IL-4*), transforming growth factor, beta 1 (*TGF-β1*), and BMP and activin membrane-bound inhibitor (*Bambi*).

## WEIGHTED GENE CORRELATIONAL NETWORK ANALYSIS

### *Hierarchical clustering of rats based on gene expression profiles*

In order to construct modules of highly correlated genes that were related to exercise and/or stress exposure, a WGCNA was performed. Hierarchical clustering was used to categorize rats based on their individual expression profile of the genes within the transcriptome (17,170). First, a dendogram was constructed to cluster rats based on gene expression profile. Rats that were closer in distance within the dendogram were considered to have a more closely related gene expression profile. After the dendogram was constructed, clusters of the dendogram were related to physical traits whereby experimental condition (exercise and stress) were considered physical traits. With the exception of the outliers ($n = 7$), rats fell into two main categories, home cage non-stressed rats on the left and rats exposed to stress on the right. All groups within the stress cluster contained both sedentary and physically active rats with no visible grouping pattern by physical activity status. Within the home cage non-stressed control cluster, one stressed outlier was identified. Rats with the same physical activity status were clustered together within the non-stressed cluster.

### *Identification of modules of coexpressed genes in the DRN correlated to 6 weeks of wheel running and/or exposure to stress*

Modules of highly coexpressed genes were identified that were also highly correlated (either positively or negatively) to exercise and/or stress. Eleven modules were derived from the transcriptome and were related to the various physical traits. Each module was assigned an arbitrary color. The number of genes contributing to each module was: yellow-199, blue-1077, purple-36, magenta-46, turquoise-3350, red-99, black-67, brown-373, green-153, pink-53, and grey-11,717. A correlation value and *p*-value associated with the correlational strength for each module-trait relationship was calculated. Modules of interest were identified based on statistical significance of the correlational strength ($p < 0.001$). A more stringent cutoff for statistical significance was used

**Table 4 | Effect of exercise on stress-induced alterations in the expression of select genes in the DRN.**

**FUNCTIONAL CATEGORY**

| *Gene* | Exercise | Stress | Exercise x stress |
|---|---|---|---|
| **DIURNALLY REGULATED W/CIRCADIAN ORTHOLOGS** | | | |
| *Klf9* | $F_{(1, 38)} = 0.012$, $p = 0.915$ | $F_{(2, 38)} = 7.842$, $p = 0.001$ | $F_{(2, 38)} = 4.552$, $p = 0.016$ |
| *Ppp1r3c* | $F_{(1, 38)} = 0.504$, $p = 0.482$ | $F_{(2, 38)} = 6.551$, $p = 0.003$ | $F_{(2, 38)} = 4.014$, $p = 0.026$ |
| *G0s2* | $F_{(1, 38)} = 3.239$, $p = 0.079$ | $F_{(2, 38)} = 3.813$, $p = 0.031$ | $F_{(2, 38)} = 4.408$, $p = 0.019$ |
| **TRYPTOPHAN METABOLISM** | | | |
| *Aldh1a2* | $F_{(1, 38)} = 2.713$, $p = 0.107$ | $F_{(2, 38)} = 0.384$, $p = 0.683$ | $F_{(2, 38)} = 2.891$, $p = 0.067$ |
| *Tdo2* | $F_{(1, 38)} = 0.658$, $p = 0.422$ | $F_{(2, 38)} = 1.243$, $p = 0.300$ | $F_{(2, 38)} = 5.022$, $p = 0.011$ |
| **IMMUNE-RELATED** | | | |
| *Hcls1* | $F_{(1, 38)} = 3.417$, $p = 0.072$ | $F_{(2, 38)} = 0.494$, $p = 0.614$ | $F_{(2, 38)} = 2.896$, $p = 0.067$ |
| *Plcg2* | $F_{(1, 38)} = 1.898$, $p = 0.176$ | $F_{(2, 38)} = 0.129$, $p = 0.879$ | $F_{(2, 38)} = 4.352$, $p = 0.019$ |
| *Cblb* | $F_{(1, 38)} = 7.493$, $p = 0.009$ | $F_{(2, 38)} = 0.968$, $p = 0.389$ | $F_{(2, 38)} = 3.179$, $p = 0.052$ |
| *Hck* | $F_{(1, 38)} = 8.404$, $p = 0.006$ | $F_{(2, 38)} = 1.858$, $p = 0.169$ | $F_{(2, 38)} = 4.010$, $p = 0.026$ |
| *IL-4* | $F_{(1, 38)} = 0.284$, $p = 0.597$ | $F_{(2, 38)} = 0.121$, $p = 0.886$ | $F_{(2, 38)} = 2.983$, $p = 0.062$ |
| *TGF-β1* | $F_{(1, 38)} = 0.528$, $p = 0.472$ | $F_{(2, 38)} = 22.91$, $p < 0.0001$ | $F_{(2, 38)} = 6.430$, $p = 0.003$ |
| *Bambi* | $F_{(1, 38)} = 8.404$, $p = 0.006$ | $F_{(2, 38)} = 1.858$, $p = 0.169$ | $F_{(2, 38)} = 4.010$, $p = 0.026$ |

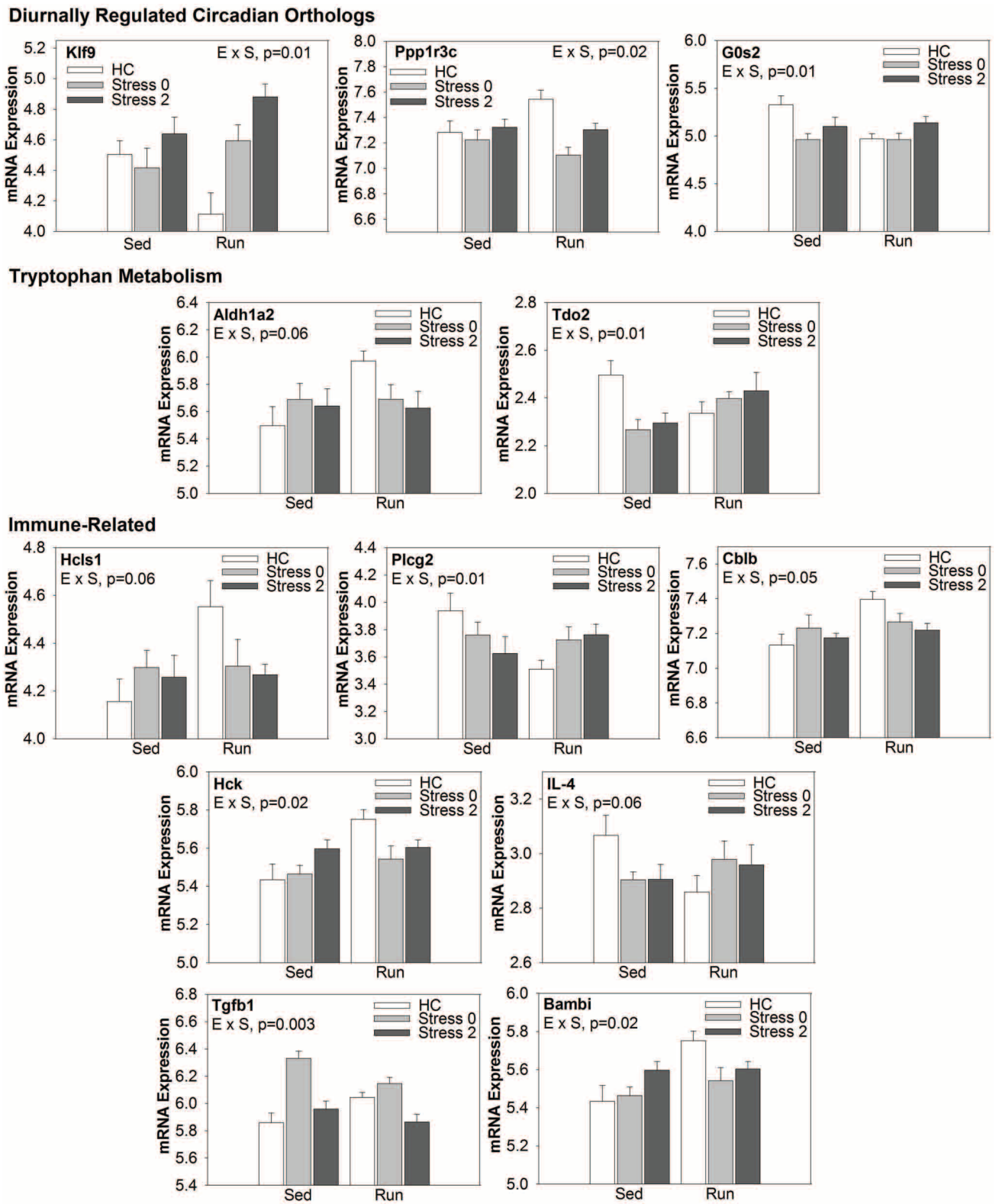


**FIGURE 4 | Effect of exercise on stress-induced alterations in the expression of select genes in the DRN, organized by Wiki functional category.**

given the wide range of $p$-values ($p = 3e - 39$ to $p = 1.0$). The grey module was excluded from analysis due to the large number of genes contributing to the module, and therefore, lack of specificity.

Of the 11 modules, 2 modules, the brown and black, were responsive to stress in both physically active and sedentary rats. The brown module was highly positively correlated to stress, indicating a strong increase in expression of genes within the brown module in response to stress. The physically active rats had a greater correlation value immediately following stress (0.98) compared to sedentary rats (0.83), suggesting that there was a more coordinated response among genes in the brown module in physically active rats. The black module was also highly positively correlated to stress, indicating a strong increase in expression of genes within the black module in response to stress. For both time points, physically active rats had a greater correlation value (0.96 and 0.83) compared to sedentary rats (0.8 and 0.72), suggesting that there was a more coordinated response among genes in the black module in physically active rats in response to stress.

Five additional modules were also identified. Genes within the blue, purple, and green modules were responsive to stress only in the sedentary rats. The blue module was negatively correlated (−0.5) with stress in sedentary rats, indicating a decrease in expression of genes within the blue module in response to stress. The purple module was negatively correlated (−0.5 and −0.65) with stress in sedentary rats immediately following and 2 h post stress, indicating a decrease in expression of genes within the purple module in response to stress. The green module was positively correlated (0.48) immediately following stress in sedentary rats, indicating an increase in expression of genes within the green module in response to stress.

Expression of genes within the purple and turquoise modules was associated with physical activity. The purple module was negatively correlated with physical activity (−0.48) indicating a decrease in expression of genes within the purple module in response to wheel running. The turquoise module was positively correlated with physical activity (0.53) indicating an increase in expression of genes within the turquoise module in response to wheel running.

Finally, the magenta module was positively correlated (0.53) with physically active rats 2 h post stress compared to sedentary rats 2 h post stress. This suggests that relative to sedentary rats, physically active rats have increased expression of genes within the magenta module 2 h following stress exposure.

### *The effect of wheel running and/or exposure to stress on modules of coexpressed genes in the DRN*

Modules of interest (correlational strength $p < 0.001$) were also subjected to analysis by ANOVA in order to determine the effect of exercise and/or stress on alterations in the coexpression of genes in the DRN. **Figure 5** shows the graphs of the ANOVA analysis for each module. There were no statistically significant ($p < 0.05$) effects of exercise, stress, or an exercise by stress interaction in the blue, purple, or green module.

ANOVA analysis of the magenta module revealed a statistically significant main effect of exercise [$F_{(1,\ 38)} = 6.588$; $p = 0.0143$], but no significant main effect of stress or interaction. ANOVA analysis of the turquoise module also revealed a statistically significant main effect of exercise [$F_{(1,\ 38)} = 5.495$; $p = 0.0244$], but no significant main effect of stress or interaction. ANOVA analysis of the black module module revealed a statistically significant main effect of stress [$F_{(2,\ 38)} = 56.872$; $p < 0.0001$] and significant interaction [$F_{(2,\ 28)} = 3.431$; $p = 0.0427$], but no significant main effect of exercise. ANOVA analysis of the brown module revealed a statistically significant main effect of stress [$F_{(2,\ 38)} = 168.838$; $p < 0.001$], but no significant main effect of exercise or interaction.

### *KEGG functionally enriched pathway categories related to genes differentially coexpressed in the DRN following 6 weeks of wheel running and/or exposure to stress*

For modules found to be statistically significant through ANOVA analysis, genes that contributed to each module were imported into Web Gestaldt bioinformatics system and subjected to analysis with KEGG functional terms. **Table 5** shows the top ten functionally enriched pathway categories, for each module, related to the genes differentially coexpressed following 6 weeks of wheel running and/or exposure to stress. Gene count refers to the number of genes from the data sets that contribute to each functional category. The $p$-value represents the statistical significance of each functionally enriched category identified.

KEGG analysis revealed that genes within the black module were related to functional categories including prion diseases, Wnt signaling, chemokine signaling, toll-like receptor signaling, VEGF signaling, gonadotropin-releasing hormone (GnRH) signaling, apoptosis, and small cell lung cancer. Genes within the brown module were related to functional categories including Jak-Stat signaling, adipocytokine signaling, pathways in cancer, hematopoietic cell lineage, p53 signaling, focal adhesion, chronic myeloid leukemia, and ErbB signaling. Both the black and brown modules contained genes that were related to functional categories of pathways involving cytokine-cytokine receptor interaction and MAPK signaling.

Genes within the magenta and turquoise modules were related to functional categories including metabolic pathways and ubiquitin mediated proteolysis. Refer to **Table 5** for other functional categories related to genes within these modules.

### *Wiki functionally enriched pathway categories related to genes differentially coexpressed in the DRN following 6 weeks of wheel running and/or exposure to stress*

For modules found to be statistically significant through ANOVA analysis, genes that contributed to each module were imported into Web Gestaldt bioinformatics system and subjected to analysis with Wiki functional terms. **Table 6** shows the top ten functionally enriched pathway categories, for each module, related to the genes differentially coexpressed following 6 weeks of wheel running and/or exposure to stress. Gene count refers to the number of genes from the data sets that contribute to each functional category. The $p$-value represents the statistical significance of each functionally enriched category identified.

Wiki analysis revealed that genes within the brown module were related to functional categories including adipogenesis, IL-6 signaling, triacylglyceride synthesis, ErbB signaling, p38 MAPK

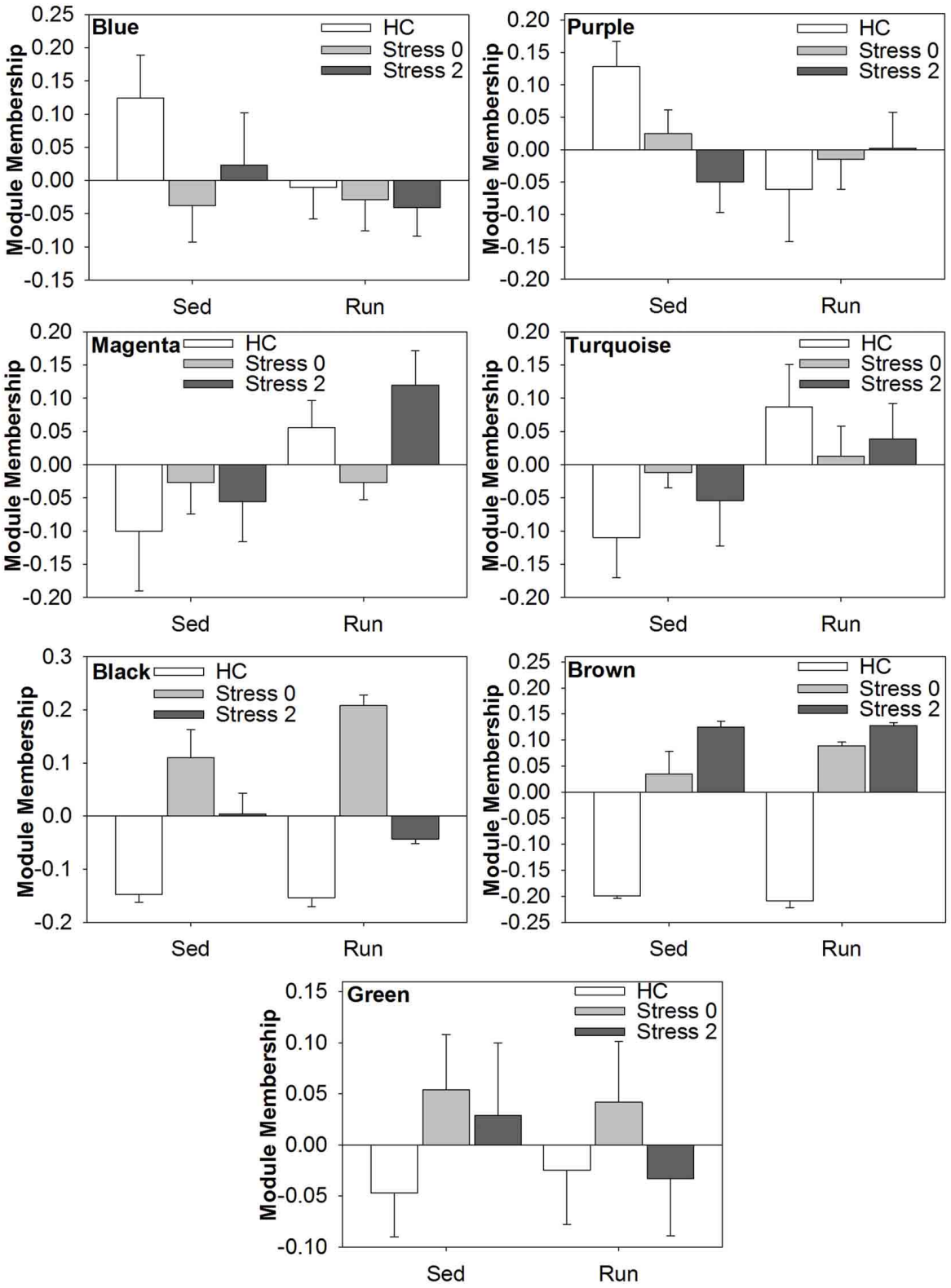


**FIGURE 5 | Effect of exercise and/or stress on modules of coexpressed genes in the DRN.**

**Table 5 | KEGG functionally enriched pathway categories generated from modules of genes correlated to exercise and/or stress.**

| Module | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| **MAGENTA MODULE** | | |
| Focal adhesion:04510 | 3 | 0.0014 |
| Type II diabetes mellitus:04930 | 2 | 0.0015 |
| Pancreatic cancer:05212 | 2 | 0.0031 |
| Colorectal cancer:05210 | 2 | 0.0047 |
| Pathways in cancer:05200 | 3 | 0.0061 |
| Neurotropin signaling pathway:04722 | 2 | 0.0099 |
| Insulin signaling pathway:04910 | 2 | 0.0099 |
| Ubiquitin mediated proteolysis:04120 | 2 | 0.0091 |
| Metabolic Pathways:01100 | 2 | 0.3704 |
| **TURQUOISE MODULE** | | |
| Metabolic Pathways:01100 | 231 | 3.92e–38 |
| Ribosome:03010 | 43 | 7.08e–24 |
| Ubiquitin mediated proteolysis:04120 | 45 | 9.21e–18 |
| Axon guidance:04360 | 42 | 6.30e–15 |
| Huntington's disease:05016 | 56 | 2.63e–13 |
| Alzheimer's disease:05010 | 57 | 7.96e–13 |
| Oxidative phosphorylation:00190 | 43 | 3.01e–12 |
| Regulation of actin cytoskeleton:04810 | 51 | 3.72e–12 |
| Spliceosome:03040 | 37 | 3.89e–12 |
| Cell cycle:04110 | 37 | 5.05e–12 |
| **BLACK MODULE** | | |
| Cytokine-cytokine receptor interaction:04060 | 4 | 0.0005 |
| Prion Diseases:05020 | 2 | 0.0020 |
| Wnt signaling pathway:04310 | 3 | 0.0023 |
| MAPK signaling pathway:04010 | 4 | 0.0014 |
| Chemokine signaling pathway:04062 | 3 | 0.0039 |
| Toll-like receptor signaling pathway:04620 | 2 | 0.0127 |
| VEGF signaling pathway:04370 | 2 | 0.0087 |
| GnRH signaling pathway:04912 | 2 | 0.0132 |
| Apoptosis:04210 | 2 | 0.0137 |
| Small cell lung cancer:05222 | 2 | 0.0127 |
| **BROWN MODULE** | | |
| MAPK signaling pathway:04010 | 23 | 3.94e–15 |
| Cytokine-cytokine receptor interaction:04060 | 13 | 9.53e–08 |
| Jak-Stat signaling pathway:04630 | 10 | 2.02e–06 |
| Adipocytokine signaling pathway:04920 | 7 | 4.19e–06 |
| Pathways in cancer:05200 | 14 | 6.26e–06 |
| Hematopoietic cell lineage:04640 | 7 | 1.07e–05 |
| P53 signaling pathway:04115 | 6 | 9.48e–05 |
| Focal adhesion:04510 | 9 | 0.0002 |
| Chronic myeloid leukemia:05220 | 6 | 0.0002 |
| ErbB signaling pathway:04012 | 6 | 0.0002 |

signaling, and Wnt signaling and pluripotency. Genes within the black module were related to functional categories including hypertrophy model, small ligand GPCRs, prostaglandin synthesis and regulation, myometrial relaxation and contraction, diurnally regulated genes with circadian orthologs, and peptide GPCRs. Both the black and brown modules contained genes that were

**Table 6 | Wiki functionally enriched pathway categories generated from modules of genes correlated to exercise and/or stress.**

| Module | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| **MAGENTA MODULE** | | |
| Insulin signaling:WP439 | 3 | 0.0005 |
| Apoptosis:WP1290 | 2 | 0.0039 |
| IL-3 signaling pathway:WP319 | 2 | 0.0048 |
| Apoptosis mechanisms:WP284 | 2 | 0.0038 |
| **TURQUOISE MODULE** | | |
| mRNA processing:WP529 | 41 | 2.45e–17 |
| Electron transport chain:WP59 | 35 | 6.06e–17 |
| TNF alpha NF-KB signaling pathway:WP457 | 47 | 2.77e–14 |
| EGFR1 signaling pathway:WP5 | 42 | 6.39e–12 |
| Regulation of actin cytoskeleton:WP351 | 37 | 2.25e–11 |
| TGF beta receptor signaling pathway:WP362 | 35 | 1.04e–10 |
| B cell receptor signaling pathway:WP285 | 38 | 1.96e–10 |
| G protein signaling pathway:WP73 | 27 | 2.60e–10 |
| Oxidative phosphorylation:WP1283 | 19 | 3.28e–09 |
| Proteasome degradation:WP302 | 19 | 1.51e–08 |
| **BLACK MODULE** | | |
| Hypertrophy Model:WP442 | 3 | 4.38e–06 |
| Insulin signaling:WP429 | 4 | 0.0001 |
| GPCRs, class A rhodopsin-like:WP473 | 4 | 0.0005 |
| Small ligand GPCRs:WP161 | 2 | 0.0004 |
| Prostaglandin synthesis and regulation:WP303 | 2 | 0.0012 |
| Myometrial relaxation and contraction pathways:WP140 | 3 | 0.0018 |
| MAPK signaling pathway:WP358 | 3 | 0.0022 |
| TGF beta receptor signaling pathway:WP362 | 3 | 0.0016 |
| Diurnally regulated genes with circadian orthologs:WP1306 | 2 | 0.0022 |
| Peptide GPCRs:WP131 | 2 | 0.0043 |
| **BROWN MODULE** | | |
| MAPK signaling pathway:WP358 | 15 | 1.29e–11 |
| Adipogenesis:WP155 | 12 | 1.41e–09 |
| Insulin signaling:WP429 | 9 | 1.04e–05 |
| TGF beta receptor signaling pathway:WP362 | 8 | 3.95e–05 |
| IL-6 signaling pathway:WP135 | 7 | 4.22e–05 |
| Triacylglyercide synthesis:WP356 | 4 | 4.69e–05 |
| ErbB signaling pathway:WP1299 | 5 | 7.64e–05 |
| P38 MAPK signaling pathway:WP294 | 4 | 0.0002 |
| GPCRs, class A rhodopsin-like:WP473 | 9 | 0.0002 |
| Wnt signaling pathway and pluripotency:WP1288 | 6 | 0.0002 |
| **GREEN MODULE** | | |
| TNF alpha NF-KB signaling pathway:WP457 | 5 | 7.31e–05 |
| Electron transport chain:WP59 | 3 | 0.0013 |

*(Continued)*

**Table 6 | Continued**

| Module | Gene count | *P*-value |
|---|---|---|
| **FUNCTIONALLY ENRICHED PATHWAY** | | |
| Androgen receptor signaling pathway:WP68 | 3 | 0.0029 |
| Cytoplasmic ribosomal proteins:WP30 | 3 | 0.0028 |
| Oxidative phosphorylation:WP1283 | 2 | 0.0070 |
| Proteasome degradation:WP302 | 2 | 0.0081 |
| G1 to S cell cycle control:WP348 | 2 | 0.0110 |
| Wnt signaling pathway:WP375 | 2 | 0.0227 |

related to functional categories of pathways in insulin signaling, MAPK signaling, GPCRs of class A rhodopsin-like, and TGF-β receptor signaling.

For the magenta and turquoise modules, functional categories associated with immune pathways such as IL-3 signaling, insulin signaling, TGF-β receptor signaling, B-cell receptor signaling, and TNF-α-NF-κB signaling were identified.

## DISCUSSION

### OVERALL THEMES

The mechanism by which exercise protects against the behavioral consequences of inescapable stress is unknown. The current data suggest that rats with 6 weeks of prior access to a running wheel have a different physiological response to stress, as measured by gene expression in the DRN, than sedentary rats. Here we report that (1) relative to home cage non-stressed controls, physically active rats have a greater number of genes differentially expressed in response to stress both immediately following and 2 h after stress exposure than sedentary rats (2) modules made up of genes that are highly coexpressed and responsive to stress operate in a more strongly coordinated manner in response to stress in physically active rats compared to sedentary rats (3) many of the stress-responsive genes within the DRN are known to be involved in various immune-related pathways, such as cytokine signaling and inflammatory processes.

These data demonstrate that in response to stress, physically active rats mount a more active response, at the level of mRNA transcription in the DRN. Relative to home cage non-stressed controls, physically active rats had a greater number of genes altered by a LFC $\geq \pm 1.1$ and a greater number of genes significantly differentially altered by $p < 0.05$ than sedentary rats in response to stress. This is interesting because it suggests that the protective effect of exercise is not through a dampening of the stress response. Rather, physically active rats may mount a more robust response that functions, in concert, to protect the brain against the negative behavioral consequences of stress. Furthermore, physically active rats may respond to stress in a more efficient manner. This is demonstrated by the observation that modules made up of coexpressed genes that are highly stress-responsive, are more strongly upregulated in physically active rats in response to stress than sedentary rats. Given that these modules are highly upregulated in response to stress despite physical activity status, it is possible that the genes within these modules are expressed in order to protect the DRN from stress-induced damage. Physically active rats have a more strongly coordinated coexpression of these stress-responsive, "protective" genes and this more effective synchronization may be important for exercise-induced stress resistance.

Interestingly, many genes within the DRN that are altered in response to stress are involved in immune-related signaling processes including the signaling of proteins (MAPK) involved in the stimulation of proinflammatory factors, immune cell receptors (toll-like, B cell, T cell), cytokines (IL-3, IL-4, IL-5, IL-6, TGF-β, TNF-α) and cytokine receptors (TGF-β1), chemokines, and regulatory pathways involved in the immune response to infection (NF-κB). These various immune-related functional categories were identified in both differential expression and WGCNA analysis of genes and in both KEGG and Wiki pathway databases. It is important to note that identification of specific functional pathways does not necessarily imply that such processes are occurring within the DRN in response to exercise and/or stress. Rather, pathway identification is a means of organizing the thousands of genes that are significantly differentially expressed or coexpressed in response to exercise and/or stress, by functional relationships. Genes known to be involved in B cell receptor signaling, for example, were significantly altered in the DRN in response to stress. However, B cells are only present at very low levels in a healthy brain (Anthony et al., 2003). Thus, these genes are likely performing other functions. Additionally, it is likely that the immune-associated functional categories of genes within the KEGG and Wiki pathway databases were generated from data based on peripheral immune processes, and therefore, may not be an accurate representation of immune functions within the brain. However, given the evidence that physical activity has anti-inflammatory effects peripherally that confer protection against chronic disease (Gleeson et al., 2011), elucidation of the nature of the stress-induced inflammatory response in the DRN of physically active rats is of prime importance. It is possible that exercise confers protection by dampening the stress-induced inflammatory cascade in the brain.

Overall, these data suggest that at the level of mRNA transcription in the DRN, there is a colossal response to stress. A history of physical activity, changes, but does not necessarily dampen this response. Furthermore, there is evidence of stress-induced induction of inflammatory processes, though it is not clear whether there is an overall pro-inflammatory, anti-inflammatory, or balanced response. Additionally, it is not apparent if the overall inflammatory response is different depending on physical activity status. Regardless, these data provide evidence for a stress-induced inflammatory response originating in a region of the brain implicated in stress-related mood disorders, and exemplify the importance of investigating novel theories, such as the cytokine-induced hypothesis of depression.

### NOVEL TARGETS OF EXERCISE-INDUCED STRESS RESISTANCE

The goal of this experiment was to identify novel gene targets of exercise-induced stress resistance. The "novel" component was considered to be of particular importance and therefore, the data were analyzed without the guidance of an a priori hypothesis. Differential gene expression analysis was employed to narrow the transcriptome of 17,170 genes to those most likely involved

in stress resistance. Specifically, contrasts were made between experimental groups to identify genes that were significantly differentially expressed by $p < 0.05$. The contrasts considered to be the most important for revealing exercise-induced stress resistance were the contrasts that revealed genes differentially expressed due to an exercise by stress interaction. More specifically, we were interested in identifying genes that were differentially expressed at $p < 0.05$ in response to stress depending on the physical activity status of the rat. The contrasts that provided this information were [($Sed_{Stress0}$ v. $Sed_{HC}$) v. ($Run_{Stress0}$ v. $Run_{HC}$)] and [($Sed_{Stress2}$ v. $Sed_{HC}$) v. ($Run_{Stress2}$ v. $Run_{HC}$)]. The first contrast generated a list of 1028 genes that were differentially expressed immediately following stress in physically active rats compared to sedentary rats. The second contrast generated a list of 637 genes that were differentially expressed 2 h following stress in physically active rats compared to sedentary rats. Given the large number of genes that were altered, an additional constraint was placed on the data. Only those genes that were differentially expressed at both time points were considered ($n = 169$). We reasoned that mRNA present immediately following stress and 2 h following stress was more likely to be translated into protein. The 169 genes that were differentially expressed in response to stress in physically active rats compared to sedentary rats were subjected to both KEGG and Wiki analysis, in order to organize the list of genes by functional categories. It is important to note that of the 169 genes, only 69 genes were fitted to KEGG specific functional categories, and only 26 genes were fitted to Wiki specific functional categories. Thus, it is possible that important genes were lost due to the limitations inherent in classifying genes into categories. From an a priori perspective, visual inspection of the genes not assigned to a KEGG or Wiki functional category did not reveal any compelling genes, *per se*. We chose to focus on genes classified by the Wiki database because the functional categories seemed to be tailored to more specific processes (TGF-β signaling and tryptophan metabolism pathways) compared to KEGG identified processes (metabolic pathways and pathways in cancer). From the Wiki categories, 12 genes were selected that were related to functional categories including diurnally regulated genes, tryptophan metabolism, and inflammatory-related processes. These categories were specifically chosen because the circadian system, serotonergic circuits, and inflammation have all been implicated in having a role in mood disorders. It should be noted that of the 12 genes selected from Wiki functional categories, 6 of these genes were also present in the KEGG classification of functional categories.

ANOVA analysis was performed in order to assess the differential effect of stress-induced changes within these genes in physically active compared to sedentary rats. **Table 7** provides a brief description of the function associated with the protein that each gene encodes (Safran et al., 2010).

Of particular interest are *Tdo2* and *TGF-β1* (**Figure 4**). Tryptophan is the precursor to 5-HT and therefore, serves as a regulator of 5-HT synthesis. The *Tdo2* gene encodes a major enzyme, tryptophan 2,3 dioxygenase (TDO), involved in tryptophan metabolism. TDO degrades tryptophan along the kynurenine pathway (for review, see Efimov et al., 2011). Interestingly, in rats exposed to stress, *Tdo2* mRNA expression in the DRN is differentially altered depending on physical activity status [ExS, $F_{(2,\ 38)} = 5.022$; $p = 0.0116$]. Fisher's PLSD post-hoc analyses revealed that exercise decreased baseline levels of *Tdo2* and there was no effect of stress on *Tdo2* expression in physically active rats. In contrast, sedentary rats had decreased *Tdo2* expression immediately following and 2 h post stress. Stress-induced decreases in *Tdo2* may lead to a reduction in TDO levels and decreased ability of rats to degrade tryptophan. This could contribute to the increased levels of 5-HT in the DRN following inescapable stress. Wheel running blocks stress-induced decreases in *Tdo2* mRNA expression, and thus, may prevent stress-induced

**Table 7 | Functional role of proteins encoded by genes of interest.**

| FUNCTIONAL CATEGORY | |
|---|---|
| ***Gene*** | **Protein product: function** |
| **DIURNALLY REGULATED W/CIRCADIAN ORTHOLOGS** | |
| *Klf9* | Transcription factor: can inhibit or activate transcription |
| *Ppp1r3c* | Enzyme: participates in variety of cellular processes by reversible protein phosphorylation |
| *G0s2* | Not clear: potential oncogene and regulator of latent HIV |
| **TRYPTOPHAN METABOLISM** | |
| *Aldh1a2* | Enzyme: catalyzes the synthesis of retinoic acid from retinaldehyde |
| *Tdo2* | Enzyme: catalyzes 1st and rate-limiting step in a major pathway of tryptophan metabolism, L-tryptophan > n-formyl kynurenine |
| **IMMUNE-RELATED** | |
| *Hcls1* | Substrate: role in antigen receptor signaling, potential role in regulation of gene expression |
| *Plcg2* | Enzyme: catalyze hydrolysis of phospholipids |
| *Cblb* | Ligase: negatively regulates T-cell and B-cell receptors |
| *Hck* | Enzyme: may help couple Fc receptor to activation of respiratory burst, potential role in neutrophil migration and degranulation of neutrophils |
| *IL-4* | Cytokine: forms a gene cluster w/ IL-3, IL-5, IL-13 on chromosome 5q, costimulator of DNA synthesis, induces expression of MHC II on B-cells |
| *TGF-β1* | Cytokine: controls proliferation, differentiation, regulation of other growth factors |
| *Bambi* | Receptor: related to type 1 receptors of TGF-β family |

decreases in TDO and the reduction of tryptophan metabolism in the DRN.

*TGF-β1* was an additional gene of interest. The *TGF-β1* gene encodes for the cytokine, TGF-β1. TGF-β1 is a regulator of T cells, and is typically considered to have anti-inflammatory properties. TGF-β1 has also been investigated in the context of depression. Kim et al. (2007) found increased levels of TGF-β1 in the plasma of patient's with major depression disorder and 6 weeks of treatment with antidepressants significantly decreased levels of TGF-β1. Our data suggests that *TGF-β1* mRNA in the DRN is particularly sensitive to stress ($p < 0.0001$) and differentially altered depending on physical activity status [ExS, $F_{(2,\ 38)} = 6.430$; $p = 0.0039$]. Fisher's PLSD post-hoc analysis revealed that exercise increased baseline levels of *TGF-β1* mRNA. There was a decrease in *TGF-β1* mRNA expression in physically active rats 2 h post stress. In contrast, sedentary rats had a large increase in *TGF-β1* immediately following stress. The significance of the impedance effect of exercise on stress-induced increases in *TGF-β1* mRNA levels in the DRN is unclear. Sedentary rats may have a more pro-inflammatory response to stress, and therefore, the increase in *TGF-β1* mRNA may occur in order to subdue pro-inflammatory responses. On the other hand, 5-HT treatment has been shown to increase *TGF-β1* mRNA expression in mesangial cells (Grewal et al., 1999) and therefore, increased *TGF-β1* mRNA expression in DRN cells may be due to stress-induced increases in 5-HT levels in sedentary rats. However, it is important to consider that the functional role of TGF-β1 may be different in the brain compared to peripheral tissue. Given the association of TGF-β1 with depression and antidepressant treatment, further examination of TGF-β1 is warranted.

It is important to consider that in the context of this particular analysis, the identification of novel targets of exercise-induced stress resistance was restricted to those genes that were differentially expressed due to the interaction between exercise and stress. However, it is possible that the mechanism by which exercise confers protection is not opposite of the mechanism by which stress produces negative consequences. Exercise-induced stress resistance could occur through a non-stress responsive route. Furthermore, identification of stress-resistant genes relied solely on the genes being differentially expressed in physically active rats compared to sedentary rats exposed to stress. However, genes often work in coordinated manners to carry out physiological functions. In the absence of absolute differences in gene expression, differences in the coexpression of gene networks in response to stress may underlie the protective effect of exercise. Identification of hub genes critically important to the modules detected with WGCNA will address this possibility, and may lead to the identification of additional targets. (Identification of hub genes is discussed in greater detail in Future Directions.)

## ADVANTAGES AND LIMITATIONS OF MICROARRAY ANALYSIS

The greatest advantage of microarray analysis is that it enables the simultaneous exploration of the expression of thousands of genes. Therefore, it is particularly useful in studying complex processes, such as the stress response, whereby thousands of genes are affected. When used in conjunction with laser capture microarray technology, microarray has the potential to yield whole genome expression data about an organism's response to an environmental manipulation, such as exposure to stress or voluntary exercise, in a specific region of the brain or cell type within that region. It is important to point out that microarray analysis only provides information at the level of mRNA transcription, which is not necessarily indicative of protein production. Nevertheless, transcription initiation is the most widely used means of gene regulation in eukaryotes (Cox et al., 2012), and using an exploratory approach, microarray experiments serve as an important starting screen for the identification of novel targets that can be analyzed in greater detail with other techniques (Coppola, 2011).

The exploratory approach to microarray data analysis, however, is not without its limitations. This approach relies solely on statistical significance to identify often thousands of genes that in turn, must be organized in such a way that allows for interpretation. The organization of statistically significant genes, usually by functional categories derived from bioinformatics databases, may fail to identify genes that play a crucial role in the regulation of a given process. The $5HT_{1A}$ receptor, for example, is thought to be critically involved in the mechanism by which inescapable stress produces sensitization of DRN 5-HT neurons (Rozeske et al., 2011). Using in-situ hybridization, our lab has previously shown that wheel running increases 5-$HT_{1A}$ mRNA expression in the DRN (Greenwood et al., 2003, 2005). An exploratory analysis of the current dataset did not identify 5-$HT_{1A}$ as a target of interest. However, using an a priori guided approach, ANOVA analysis of 5-$HT_{1A}$ mRNA in the DRN revealed significant main effects of exercise [$F_{(1,\ 38)} = 6.250$; $p = 0.0169$] and stress [$F_{(2,\ 38)} = 3.538$; $p = 0.0390$], but no significant interaction (**Figure 6**). Our data are consistent with the previous findings that 6 weeks of wheel running increases 5-$HT_{1A}$ mRNA expression in the DRN (Greenwood et al., 2003). We also replicated our previous report that exposure to stress decreases 5-$HT_{1A}$ mRNA expression in the DRN (Greenwood and Fleshner, 2011).

Overall, both the advantages and limitations of microarray technology are the product of the colossal dataset that a microarray experiment yields. Organization of the data is required so that statistically significant differences observed in thousands of

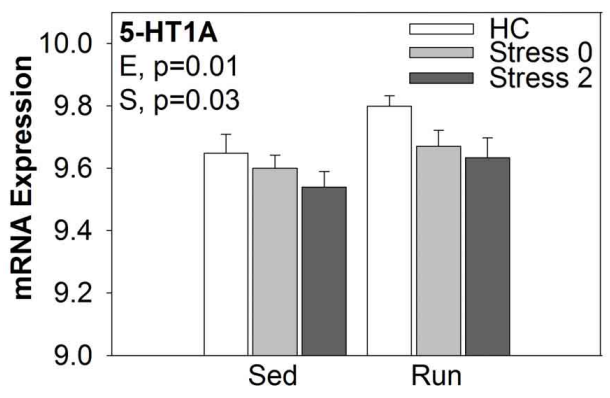


**FIGURE 6 | mRNA expression of 5-HT1A in the DRN.**

genes can be focused into more manageable and interpretable gene sets. This process of data distillation, however, is not without a price and in the process, information may be lost. Given this reality, it is of paramount importance that multiple statistical and analytical strategies are executed. The traditional analysis of differential expression should be used in addition to the more sophisticated analysis of coexpression with WGCNA. Although both approaches return long lists of genes that must be further mined, each method assesses different regulatory processes. Finally, when identifying functional categories related to genes of interest, multiple bioinformatics databases should be explored. Inconsistencies in the information returned by bioinformatics databases (Soh et al., 2010) are pervasive, and using more than one database may provide more comprehensive results.

### FUTURE DIRECTIONS

The overwhelming amount of data obtained in microarray experiments can be a challenge to manage, however, the results are powerful and provide researchers with a wealth of novel processes and genes to explore. To identify additional targets of exercise-induced stress resistance, hub genes should be identified within the modules of highly coexpressed genes detected by the WGCNA. Research suggests that a gene's position within a given network of genes, or module, is indicative of its functional significance to that module (Miller et al., 2008). More centralized genes, termed hub genes, are more likely to have critical roles in cellular function than peripheral genes (Miller et al., 2008). Therefore, centralized hub genes within the brown module may play a significant role in the effect of stress, while centralized hub genes within the black module (which was differentially altered in response to stress depending on physical activity status), may play a significant role in the protective effect of exercise against stress. Identification of hub genes within the brown and black modules could lead to the identification of additional therapeutic targets.

Further analysis of significantly regulated genes with assignment of cell type may also add richly to the dataset. More specifically, a database containing information on the genes enriched in a given cell type, such as neurons, astrocytes, and oligodendrocytes, can be used to assign cell type specificity to significantly differentially expressed or coexpressed genes (Cahoy et al., 2008). Given there was consistent evidence for inflammatory-related genes being upregulated in response to stress in the DRN, it may be insightful to identify the specific cell-type and source of this inflammatory-related mRNA expression.

Future experiments should include validating the novel targets identified with microarray analysis with PCR and/or in-situ hybridization. In-situ hybridization, in particular, could provide information on the anatomical specificity of the observed differences in mRNA expression in response to exercise and/or stress within the DRN. Additionally, novel gene targets of exercise-induced stress resistance should be tested by means of behavioral pharmacology. That is manipulation of the proteins encoded by these genes with specific agonists or antagonists in the context of inescapable stress exposure and/or exercise may reveal information on the therapeutic potential of these genes. Delivery of pharmacological agonsits of targets of upregulated "stress-resistance genes" to sedentary rats, for example, would be expected to confer protection against stress-induced behaviors in these rats in the absence of exercise.

Androgens and circadian regulationare additional topics that may warrant further investigation for having a role in the mechanisms by which exercise produces stress resistance. Our data suggest that pathways of genes related to androgen receptor signaling and diurnal regulation are differentially expressed in the DRN in physically active compared to sedentary rats following stress. Androgens promote neurogenesis (Spiritzer and Galea, 2007) and enhance cognitive function (Edinger and Frye, 2007). A recent study reported that mild exercise increased androgen synthesis in the hippocampus (Okamoto et al., 2012). It is possible that wheel running also increases androgen synthesis in the DRN and provides stress-resistance through an androgen-mediated pathway. Additionally, disruption of the circadian rhythm has been implicated in mood disorders. Interestingly, stimulation of the DRN triggers the release of 5-HT by the suprachiasmatic nucleus (SCN) (Dudley et al., 1999), the brain region responsible for the generation of circadian rhythms (Rusak and Zucker, 1979), and DRN stimulation induces circadian phase-resetting (Glass et al., 2000). Constraint of DRN 5-HT neurons in physically active rats could block stress-induced alterations in SCN output and provide stress resistance through the prevention of circadian rhythm disruption.

## CONCLUSIONS

In conclusion, when the data are organized effectively, microarray experiments have the ability to yield a rich amount of information on the molecular activities underlying physiological processes. When used in combination with laser capture microdissection, this information can be obtained from a specific region or cell type within an organism. Thus microarray technology is particularly useful in studying the neurobiological mechanisms underlying the complex pathophysiology of stress-related mood disorders. This experiment was designed to reveal novel targets by which exercise produces resistance to stress-related mood disorders, specifically within the DRN, using microarray and laser capture microdissection technology. The current data reveal evidence for different profiles of gene expression in the DRN of physically active rats exposed to stress compared to sedentary rats exposed to stress. Physically active rats have a more active and more strongly coordinated response to stress than sedentary rats. Specifically, Tdo2, a gene encoding an enzyme involved in tryptophan metabolism, may have a role in the mechanism by which exercise protects against the behavioral consequences of inescapable stress. In addition, an inflammatory-related gene encoding for the cytokine TGF-ß1 was particularly responsive to stress and this response was different depending on physical activity status. Overall, an inflammatory theme was revealed consistently across multiple analyses, suggesting a large effect of stress on inflammatory-related processes in cells of the DRN. The consequence of stress-induced inflammatory processes in the DRN should be further investigated.

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# Neural control of chronic stress adaptation

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Stress initiates adaptive processes that allow the organism to physiologically cope with prolonged or intermittent exposure to real or perceived threats. A major component of this response is repeated activation of glucocorticoid secretion by the hypothalamo-pituitary-adrenocortical (HPA) axis, which promotes redistribution of energy in a wide range of organ systems, including the brain. Prolonged or cumulative increases in glucocorticoid secretion can reduce benefits afforded by enhanced stress reactivity and eventually become maladaptive. The long-term impact of stress is kept in check by the process of habituation, which reduces HPA axis responses upon repeated exposure to homotypic stressors and likely limits deleterious actions of prolonged glucocorticoid secretion. Habituation is regulated by limbic stress-regulatory sites, and is at least in part glucocorticoid feedback-dependent. Chronic stress also sensitizes reactivity to new stimuli. While sensitization may be important in maintaining response flexibility in response to new threats, it may also add to the cumulative impact of glucocorticoids on the brain and body. Finally, unpredictable or severe stress exposure may cause long-term and lasting dysregulation of the HPA axis, likely due to altered limbic control of stress effector pathways. Stress-related disorders, such as depression and PTSD, are accompanied by glucocorticoid imbalances and structural/ functional alterations in limbic circuits that resemble those seen following chronic stress, suggesting that inappropriate processing of stressful information may be part of the pathological process.

**Keywords: glucocorticoid receptor, hypothalamo-pituitary-adrenal axis, limbic system, stress-related diseases, stress habituation, stress sensitization**

## THE PROBLEM OF CHRONIC STRESS

The organismal response to stress (defined here as a real or perceived threat to homeostasis or well-being) promotes survival via adjustments to ongoing physiological processes and behavior. The activation of multiple interacting processes, including the behavioral, autonomic, endocrine, and immune systems, produces an integrated stress response. While initially adaptive, prolonged activation of molecular pathways engaged by these systems can cause pronounced changes in physiology and behavior that have long-term deleterious implications for survival and well-being. In essence, prolonged or chronic stress changes the rules under which the body regulates homeostasis, requiring new strategies for successful adaptation. This concept lies at the heart of Selye's initial description of the "general adaptation syndrome," where after an initial "alarm" stress reaction, the organism is able to successfully manage prolonged stress for substantial periods. Only when homeostatic pressure becomes too great does the individual enter into a state of frank distress, with attendant morbidity and mortality (Selye, 1950). Importantly, the process of adaptation comes at a cost to the organism, as stress effector systems are chronically mobilized to meet the homeostatic demands of prolonged stress. Thus, stress alters the physiological milieu in a long-term manner (adaptation through change, or "allostasis") (Sterling and Eyer, 1988), and the body's response to these changes lies at the center of both successful stress resilience as well as its transition to pathology.

The literature is replete with examples of the impact of stress on physiologic systems and behavior. For example, our group finds that exposure to a prolonged, unpredictable and non-habituating stress regimen (which we call "chronic variable stress," or CVS) causes marked increases in cumulative glucocorticoid secretion, sensitization of hypothalamo-pituitary-adrenocortical (HPA) responses to new stressors, decreased heart rate variability, reduced weight gain, decreased sucrose preference and increased immobility in the forced swim test (Herman et al., 1995; Ulrich-Lai et al., 2006, 2007; Jankord et al., 2011; Flak et al., 2012), suggesting functional changes across a variety of neurobehavioral systems in the brain. Whereas it can be argued that the net result of these physical and behavioral changes would be maladaptive, one has to interpret them with respect to the new context of the individual. Most responses to chronic stress are adaptive, that is, beneficial to the survival of the animal. For example, increases in corticosteroid levels promote mobilization of energy, important in times of need. Corticosteroids also inhibit systems that channel resources to functions such as growth and reproduction, not necessarily of value in the midst of physiologic challenge. Similarly, behavioral changes seen during chronic stress, while at first blush "pathological," can be argued to be beneficial in the context of chronic challenge. For example, post-stress reductions in risk assessment/behavioral withdrawal seen in so-called "anxiety" tests (e.g., elevated plus maze) (e.g., see Chiba et al., 2012) minimize risk

during periods of energetic challenge. Minimizing risk may also be linked to the switch to "habitual" behaviors observed in chronically stress rodents (e.g., see Dias-Ferreira et al., 2009; Harris et al., 2012). In a hostile environment, it may make sense to exhibit behavioral withdrawal in order to reduce threat (i.e., make oneself less available for predation). Even observed changes in immobility in the forced swim test, commonly linked to depressive phenotypes, may be interpreted as a means of conserving resources during challenging times (see Hawkins et al., 1978). Thus, while responses to chronic stress can clearly have negative consequences, one has to consider the possibility that they may solve pressing problems of the organism at the expense of future success.

Chronic stress responses represent attempts at adaptation, but as noted above can create constitute physiologic challenges in and of themselves. Excess glucocorticoid secretion can impair numerous bodily systems if extended in time; enhanced sympathetic drive can lead to cardiovascular disease; and "conservative" behavioral strategies can lessen opportunities to find new food and water sources, more secure environmental surroundings and mates. Thus, at some point, the initially "adaptive" characteristics of chronic stress reactivity can cross over to the realm of "maladaptation," defined as biological and behavioral responses that are counterproductive to the best interests of the organism. The switch from "adaptive" to "maladaptive" stress responses will be heavily dependent on the constitution of the individual, based on genetic and acquired strategies to maximize efficiency and limit overdrive of stress systems. Stress "pathologies" can arise as a result of maladaptive chronic stress responses, either as a result of systemic diseases of stress regulatory systems or pervasive activation of stress effectors in inappropriate contexts. It is important to note that the concept of "maladaptation" is not synonymous with physiologic distress. Maladaptation *per se* need not be sufficient to cause frank morbidity on its own, but may degrade the well-being of the individual or make it more vulnerable to subsequent physiologic insults.

The current review will address the problem of chronic stress, adaptation and maladaptation from the perspective of the HPA axis, perhaps the most thoroughly studied system linked to stress responses. The consequences of HPA axis activation are far-reaching, likely due to the ubiquity of glucocorticoid hormone receptors across multiple body compartments and the widespread impact of glucocorticoid hormones on gene expression. Glucocorticoid secretion is generally linked to stressful events. Consequently, glucocorticoids are often referred to as "stress hormones," a designation that undermines appreciation of their primary functions, including redistribution of energy. Indeed, so-called "stress levels" of glucocorticoid secretion can even be observed at the peak of the circadian corticosteroid rhythm, representing a flux in hormone aimed at increasing energy supplies for the active, waking hours. With this caveat in mind, consistent activation of this system constitutes both a mechanism of stress adaptation and a potential challenge for the organism, the balance of which determines resistance or susceptibility to long-term pathologies.

## STRESS ADAPTATION

To be clear, glucocorticoid responses are required for survival and adaptation. The relationship between glucocorticoid secretion and adaptation (e.g., in terms of appropriate behavioral performance) is often described as an "inverted-U" shaped curve, wherein an optimal level of glucocorticoid signaling is required to produce the most effective organismal response (De Kloet et al., 1998) (**Figure 1**). Thus, both hypo- and hyper-secretion generate poor responses, whereas an intermediate level of corticosteroids fosters superior performance. Work from De Kloet and colleagues suggests that the molecular basis of this curious phenomenon lies in the differing binding affinities and signaling characteristics of the two primary corticosteroid receptors in brain (De Kloet et al., 1998). The mineralocorticoid receptor (MR) binds low levels of glucocorticoids, and fosters cellular activation (hippocampus) and maintains basal circadian corticosteroid rhythms. The glucocorticoid receptor (GR) binds glucocorticoids across the circadian peak/stress range, and appears to inhibit hippocampal neurons and controls the magnitude of HPA axis responses to stress via negative feedback mechanisms (Reul and Dekloet, 1985; De Kloet et al., 1998). While MR and GR share virtually identical DNA binding domains, their transactivation domains are distinct, meaning that they can have very different gene targets (Datson et al., 2001, 2008). Moreover, there is evidence that the two receptors heterodimerize (Trapp et al., 1994; Nishi et al., 2004), a process that introduces the capacity to temper specific MR and GR genomic signals and perhaps introduce new types of genomic interactions.

The right arm of the inverted U-shaped curve is likely due to potential catabolic effects of glucocorticoids on physiological and

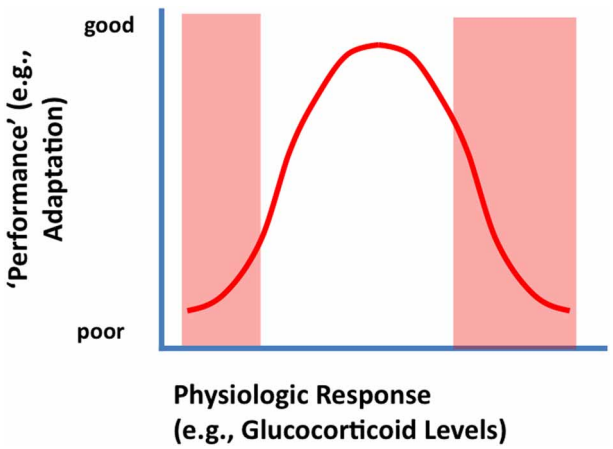


**FIGURE 1 | Inverted U-shaped relationship between physiologic or behavioral "performance' and HPA axis output (corticosterone secretion).** Secretion of glucocorticoids following stress is likely an adaptive function, supplying needed energy to meet real or potential threats. Underactive stress axis activation does not mobilize the resources needed to meet a challenge, resulting in suboptimal performance (left arm of the U-shaped curve). Excessive glucocorticoid secretion (right arm of the U-shaped curve) can cause excessive or prolonged catabolic responses, which can result in turn-off of vital stress counter-regulatory systems or energetic challenge in the CNS (see text).

cellular functions, most likely mediated by the GR. In keeping with their role in energy redistribution, glucocorticoids promote energy mobilization, including glycogenolysis, lipolysis, and proteolysis (Munck et al., 1984). Thus, these hormones promote processes that, while good for the organism in moderation, can cause long-term cellular energy depletion at high levels. In addition to effects on catabolism, glucocorticoids also inhibit processes related to growth and reproduction (Munck et al., 1984) (the organism does not need to be concerned about growing if energy reserves are being depleted). In brain, high levels of glucocorticoids can inhibit glial glucose transport (Virgin et al., 1991) and impair neuronal survival under conditions of energetic challenge (Tombaugh et al., 1992). Glucocorticoids can also inhibit expression of key neurotrophic molecules, such as brain-derived neurotrophic factor (Smith et al., 1995; Schaaf et al., 2000), and impair hippocampal neurogenesis (Gould and Tanapat, 1999). Finally, glucocorticoids down-regulate GR expression in limbic regions controlling negative feedback (e.g., hippocampus, prefrontal c) (Mizoguchi et al., 2003; Chiba et al., 2012), which limits the ability of the system to control glucocorticoid homeostasis.

Activation of glucocorticoid secretion is mediated by neuronal signals. The neuroendocrine cascade culminating in corticosteroid release is initiated by stimuli impinging on hypophysiotrophic neurons in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN). These neurons synthesize ACTH secretagogues [the most prominent of which are corticotropin-releasing hormone (CRH) and arginine vasopression (AVP)] that are released into the hypophysial portal circulation (median eminence) and transported to the anterior pituitary gland. Corticotropes respond to CRH and AVP by releasing ACTH, which is released into the systemic circulation and causes synthesis and release of glucocorticoids at the level of the adrenal gland. Glucocorticoid secretion is self-limited, undergoing end-product feedback inhibition via binding GRs in multiple brain regions as well as the pituitary gland (Keller-Wood and Dallman, 1984; Myers et al., 2012b). The net result is an HPA axis "stress response" that has a rapid onset and more gradual wane, with glucocorticoid secretion generally peaking in 15–30 min and lasting up to several hours, depending on the severity of the stressor (**Figure 2**). The shape of the response underscores its adaptive value in the short-term, and limits the possible negative effects of a prolonged glucocorticoid response.

Stimulation of the HPA axis occurs in reaction to or in anticipation of stress (Herman et al., 2003; Ulrich-Lai and Herman, 2009). Physiological threats (systemic stressors) initiate largely reflexive responses that can be triggered without conscious perception. However, anticipation of threat requires the organism to interpret the significance of multi-modal sensory information with respect to previous experience. Thus, stimuli that predict adversity (psychogenic stressors) can generate an HPA axis response in the absence of an existing physiologic insult. The relevance of the anticipatory glucocorticoid response hinges on the predicted need for adaptive hormonal secretion in order to redistribute resources (e.g., energy) to meet the challenge (Herman et al., 2003; Ulrich-Lai and Herman, 2009). Appropriate activation of the HPA axis by acute stress is critical, as inappropriately

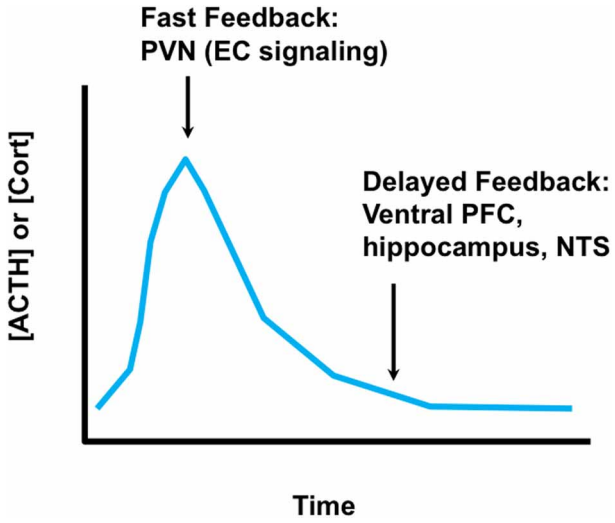


**FIGURE 2 | Time domains of glucocorticoid feedback.** Fast glucocorticoid feedback occurs in the realm of minutes to seconds, and likely involves membrane effects of glucocorticoids on endocannabinoid synthesis at the PVN. Delayed feedback controls return of glucocorticoid levels to baseline, and likely involves glucocorticoid feedback signals at multiple brain regions, including the prefrontal cortex, ventral subiculum (hippocampus) and possibly the nucleus of the solitary tract. It is not clear whether delayed feedback is genomic, non-genomic or a combination of both.

low reactivity can hinder physiological resilience and cognitive processes (e.g., learning and memory) (Diamond et al., 1992; Reber et al., 2007). However, many of the effects of glucocorticoids that are beneficial for short-term survival can be counterproductive or even deleterious if prolonged. Therefore, the activation and inhibition of glucocorticoid release is a temporally regulated process involving rapid neuronal activation and efficient inhibition.

Control of glucocorticoid secretion is accomplished by multiple mechanisms. The first line of defense is rapid shut-off of ACTH release by glucocorticoids secreted in response to stress. Rapid inhibition is almost certainly non-genomic, and is mediated in part by direct feedback onto PVN CRH neurons and at the pituitary (John et al., 2004; Tasker and Herman, 2011). At the PVN, so-called "fast feedback" is mediated by membrane actions of glucocorticoids, which cause local mobilization of endocannabinoids and subsequent inhibition of excitatory afferent input (Di et al., 2003; Evanson et al., 2010). Fast feedback inhibits ACTH release within minutes, and affects the amplitude of the stress response (Keller-Wood and Dallman, 1984). Glucocorticoids also act via the MR to rapidly enhance the excitability of hippocampal and basolateral amygdala neurons via a non-genomic mechanism. Both regions are upstream of the PVN, and thus binding via the MR may impact HPA drive via synaptic mechanisms (Pasricha et al., 2010). The eventual shut-off of the HPA axis stress response (return to baseline) is thought to be mediated by feedback working through limbic circuitry (Jacobson and Sapolsky, 1991; Sapolsky et al., 1991; Boyle et al., 2005; Furay et al., 2008). Shut-off occurs in the

time realm of genomic actions, and may reflect cellular changes in pathways impinging on the PVN. However, it remains possible that delayed shut-off may be mediated at least in part by non-genomic signaling.

Intracellular GR (and MR) signaling is subject to modulation by other proteins, which may affect function in the context of stress. For example, the GR-binding factor FK506 binding protein 51 (also known as FKBP5) reduces glucocorticoid binding affinity and GR nuclear translocation (Binder, 2009). In PFC, chronic stress enhances expression of FKBP5, which is correlated with impaired GR nuclear trafficking and reduced expression of GR-regulated genes (Guidotti et al., 2012). Stress-related changes in expression of FKBP5 (as well as other GR binding proteins) may represent a mechanism for reduced feedback efficacy and prolonged HPA axis responses (Mizoguchi et al., 2003).

Chronic stress causes a marked reorganization of the central components of the HPA axis. Numerous studies document increases in CRH and AVP expression in parvocellular PVN neurons (Herman et al., 1995; Makino et al., 1995), consistent with increased response capacity of the central limb of the HPA axis. Chronic stress also causes marked structural plasticity in CRH neurons. Glutamatergic and NE terminal appositions on CRH somata and dendrites increase with chronic stress, consistent with enhanced excitatory drive (Flak et al., 2009). There is also evidence that PVN GABAergic signaling is impaired following chronic stress. Chronic variable stress exposure causes decreases in GABA-A receptor subunit mRNAs, which would be predicted to diminish the potential for inhibition of the HPA axis (Cullinan, 2000). At the synaptic level, chronic stress decreases miniature inhibitory post-synaptic potentials in PVN neurons (Verkuyl et al., 2004), consistent with decreased inhibitory innervation. In addition, recent studies suggest that stress causes a reversal of the cellular chloride gradient in parvocellular PVN neurons (Hewitt et al., 2009), essentially negating the inhibitory impact of GABA on post-synaptic neurons. Neuroplastic responses likely reflect increased demand upon the CRH neurons, serving to maintain response capacity if confronted with additional stressors.

Hyperactivity of the PVN may be linked to alterations in glucocorticoid feedback in brain. Numerous studies indicate that chronic stress decreases expression of GR in the prefrontal cortex (PFC) and hippocampus (Mizoguchi et al., 2003; Chiba et al., 2012). Lesion and stimulation studies indicate that both of these regions play a key role in inhibition of HPA axis stress responses, working by way of excitation of inhibitory relays into the PVN [e.g., in the bed nucleus of the stria terminalis (BST), dorsomedial hypothalamus and peri-PVN regions, among others] (Herman et al., 2003; Ulrich-Lai and Herman, 2009). Local administration of glucocorticoids into the PFC reduce HPA axis responses to stress (Diorio et al., 1993), also consistent with an important role in feedback regulation. This conclusion is further supported by studies demonstrating that forebrain deletion of the GR (including the PFC and hippocampus) (Boyle et al., 2005; Furay et al., 2008) or local knock-down of GR in the PFC (McKlveen et al., 2013) enhance HPA axis stress responses. Thus, chronic stress-induced reductions in PFC and hippocampal GR may remove an important brake on the HPA axis, resulting in down-stream changes in PVN excitability.

It is important to note that glucocorticoids also affect behavioral processes that modulate the impact of stress on the organism. Behavioral analyses indicate that glucocorticoids cause changes in learning strategy, wherein mice switch from spatial learning to stimulus-response learning in the context of acute stress or corticosterone. The switch in learning strategy is blocked by an MR antagonist, suggesting effects mediated by the MR (Schwabe et al., 2010). In addition, mice overexpressing MR fail to extinguish conditioned fear, consistent with impaired behavioral flexibility (Harris et al., 2012). Animals with forebrain deletion of GR or knockdown of GR in the PFC have increased immobility in the forced swim test (Boyle et al., 2005; McKlveen et al., 2013), supporting a role for GR in behavioral strategy selection in this context.

In contrast to the PFC and hippocampus, amygdalar structures appear to be positively regulated by chronic stress. Expression of CRH in the central nucleus of the amygdala (CeA) is increased under conditions of chronic stress or glucocorticoid excess (Makino et al., 1994, 1999; Shepard et al., 2000, 2003). Moreover, glucocorticoid implants in the CeA increase corticosterone responses to acute stress, suggesting a positive glucocorticoid feedback effect mediated by this region (Shepard et al., 2003). The CeA is linked to excitation of the HPA axis, mediated by inhibition of inhibitory relay neurons innervating the PVN (including the BST and dorsomedial hypothalamus, which are also implicated in inhibition by the PFC and hippocampus) (Herman et al., 2003). Thus, in addition to impairing feedback inhibition, chronic stress may permit feed-forward activation of the PVN by way of the amygdala.

The impact of chronic stress is also evident at the pituitary and adrenal. Chronic stress exposure causes up-regulation of proopiomelanocortin mRNA expression and protein content (Shiomi et al., 1986), consistent with enhanced capacity for release of ACTH. At the adrenal, chronic variable stress causes cellular hypertrophy and hyperplasia in the zona fasciculata of the adrenal cortex, which causes elevated responsiveness to ACTH (Ulrich-Lai et al., 2006). The PVN, pituitary and adrenal changes occur with the context of relatively small changes in resting glucocorticoid secretion, consistent with modulation of the overall capacity of the HPA axis to respond (rather than a pronounced and prolonged basal hypersecretion). The peripheral changes likely reflect the overall cumulative impact (severity) of the stress regimen, as mild or habituating regimens may not be sufficient to cause frank changes at the brain, pituitary, and adrenal level [e.g., attenuated stress-induced adrenal hypertrophy (Flak et al., 2012) and deceased induction of PVN vasopressin mRNA expression (Gray et al., 2010)].

Neurocircuit mechanisms underlying generation of chronic stress-induced HPA hyperdrive remain to be determined. In general, lesions of brain regions known to be involved in inhibition or excitation of acute stress reactivity do not affect the development of HPA-relevant chronic stress symptoms. For example, lesions of the ventral subiculum exacerbate responses to acute stress, but do not affect basal glucocorticoid secretion, adrenal hypertrophy or thymic atrophy following chronic stress (Herman and Mueller, 2006). Moreover, lesions of the medial and central amygdala, putative stress excitatory regions, do not attenuate chronic stress

responses (Prewitt and Herman, 1997; Solomon et al., 2010). Thus, it is not clear that regions mediating acute stress responses are required for development of chronic stress-related HPA axis dysfunction.

## HABITUATION TO REPEATED STRESS EXPOSURE

Successful adaptation to chronic stress is a dynamic process that is dependent on the attributes of the stress exposure, such as severity, modality and duration. Stress "habituation" is thought to be an important adaptive response to repeated challenge, wherein responses to a given stressor decrease upon repeated exposure and thus reduce the overall physiological burden (e.g., cumulative effects of glucocorticoid secretion) with time. Animals can generally habituate to repeated stressors (Grissom and Bhatnagar, 2009). This is evident by a marked reduction in HPA axis activation with repeated exposure to the same stimulus (**Figure 3**). Habituation is observed after exposure to a wide array of stimuli, ranging from mild (e.g., novel environment) to severe (limb and head immobilization) (Campmany et al., 1996; Grissom and Bhatnagar, 2009). The rate of habituation is dependent on the severity of the stressor (Garcia et al., 2000). Habituation is likely mediated by diminution of central responses to the stressor; for example, activation of c-fos expression in the medial parvocellular PVN is markedly reduced in the PVN following repeated restraint stress (Girotti et al., 2006). Habituation limits the overall physiological impact of stress, in terms of effects of repeated stress on energy balance (i.e., minimal weight loss), adrenal function (i.e., no adrenal hypertrophy) and cumulative corticosterone exposure (small decrements in thymus weight, associated with small episodic increases in corticosterone exposure) (Flak et al., 2012).

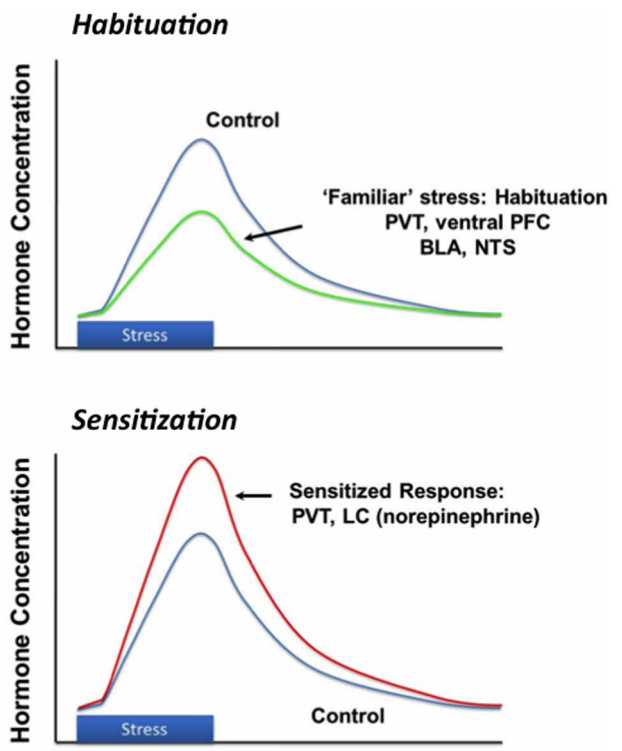


**FIGURE 3 | Stress habituation and facilitation.** Repeated exposure to the same stressor results in progressive diminution of response magnitude, thought to be mediated by structures such as the prefrontal cortex (PFC) and paraventricular thalamus (PVT). Exposure to a new stressor after either homotypic or hetertypic stressors causes a larger than normal ("sensitized' or "facilitated' response), which may be mediated by enhanced drive from the basolateral amygdala (BLA), PVT or locus coeruleus (LC).

Changes in cumulative glucocorticoid exposure may be in itself a factor in the habituation process. The addition of a stress response atop the normal circadian rhythm adds to the "total" level of glucocorticoids seen by the organism, which may modulate ongoing excitability of key regulatory sites and thereby limit responsiveness. Habituation to repeated stress is blocked by pre-stress injections of an MR antagonist (Cole et al., 2000), suggesting that the process may be regulated in part by glucocorticoid signaling via the MR. Unlike variable or "severe" stress regimens [e.g., chronic social stress, chronic unpredictable stress (Chao et al., 1993; Herman et al., 1995)] repeated restraint stress does not down-regulate GR and MR mRNA expression (hippocampus) (Girotti et al., 2006), suggesting that neural feedback mechanisms remain intact. While an elevated feedback signal may be relevant to habituation, it does not constrain the HPA response to new stressors, which can be as great or greater than responses seen in stress-naïve animals (see below) (Akana et al., 1992; Marti et al., 1994).

The CNS mechanisms regulating stress habituation appear to involve limbic forebrain circuitry. Functional studies indicate that local inactivation of the ventral PFC region prior to restraint stress blocks the development of habituation to subsequent exposure, suggesting activation is necessary for the process (Weinberg et al., 2010). The basolateral amygdala also appears essential for HPA axis habituation, as local blockade of beta-adrenergic receptors after daily stress exposure attenuates reductions in ACTH and corticosterone release observed following repeated restraint (Grissom and Bhatnagar, 2011). It is important to note that the ventral prefrontal cortex has rich connections with basolateral amygdala (McDonald et al., 1999; Vertes, 2004), and thus it is possible that the two work in concert to promote habituation. Other limbic forebrain regions may also play a role in the habituation process; for example, diminished HPA axis responses to repeated noise exposure is associated with a significant increase in c-fos mRNA activation in the orbitofrontal cortex, in contrast to decreases seen in other regions of the frontal cortex (including the PFC) (Campeau et al., 2002). Like other limbic cortices, the orbitofrontal cortex has potential polysynaptic connections with subcortical limbic stress effector pathways (Price, 2007), and increased engagement may play a role in dampening stress responses.

There is also evidence for control of habituation at the level of the limbic thalamus. Work from Bhatnagar and colleagues implicate the paraventricular thalamus (PVT) in habituation of the HPA axis stress response. The PVT is a midline thalamic nucleus that interconnects with several limbic stress-regulatory regions, including the medial PFC, basolateral amygdala and bed nucleus of the stria terminalis. Lesions of the PVT block habituation

of HPA axis responses to repeated restraint (Bhatnagar et al., 2002). Moreover, PVT lesions reduced the efficacy of dexamethasone feedback actions on stress-induced HPA activation following repeated but not acute restraint exposure (Jaferi et al., 2003), suggesting that effects on habituation may be linked to glucocorticoid signaling. In support of this hypothesis, local blockade of GR and MR in the PVT prevent habituation without affecting responses to acute stress (Jaferi and Bhatnagar, 2006), consistent with glucocorticoid feedback effects mediated by this region.

The habituation process may involve generalized reductions in activation of sensory signaling pathways. For example, repeated exposure causes marked reduction in restraint induced c-fos mRNA activation of primary sensory cortices and thalamic sensory relays (in addition to the PVN and limbic stress circuits), suggesting that habituation may be in part due to reduced strength or salience of perceived sensory cues (Girotti et al., 2006).

It is also possible that stress habituation is mediated by structural alterations along stress integrative circuits. For example, repeated brief restraint, a treatment that generally causes habituation, causes retraction of basal dendrites of prefrontal cortical neurons (Brown et al., 2005), which may impact down-stream regulation of HPA axis responses. More prolonged restraint-exposure paradigms (6 h/day, 21 days) produces dendritic retraction and spine loss in the PFC (Vyas et al., 2002; Cook and Wellman, 2004; Radley et al., 2006, 2008) as well as retraction in subfield CA3 of the hippocampus (Magarinos and McEwen, 1995), while causing increased dendritic complexity in the basolateral amygdala (Vyas et al., 2002). Chronic restraint also increases branching of GABAergic interneurons in the PFC (Gilabert-Juan et al., 2012), suggestive of enhanced inhibition. Structural alterations may affect the excitability of neurons, which can then alter the overall balance of limbic inputs to neurons controlling stress responsiveness.

It is important to note that habituation is not limited to repeated stressors. Chronic unpredictable or variable stress regimens also decrease physiological responses over time, although not to the same extent as homotypic regimens. For example, numerous studies indicate that the impact of chronic variable stress on body weight is most profound during the initial 1–3 days of exposure, with values plateauing thereafter (Tamashiro et al., 2007). In addition, our group has shown that chronic variable stress (CVS)-induced corticosterone hypersecretion is significantly reduced from the first to the second week of exposure (unpublished observations). Adrenal hypertrophy and thymic atrophy plateau between 1 and 2 weeks of CVS, suggesting that the impact of chronic unpredictable stress (Paskitti et al., 2000), at least in terms of the HPA axis, is not progressive.

## CHRONIC STRESS SENSITIZATION

It should be emphasized that the "stress habituated" state does not reflect return to normal physiologic status. Habituating regimens (e.g., repeated brief restraint) result in long-term changes in CNS stress circuits, including up-regulation of CRH expression in the PVN, even in the context of reduced corticosterone responses to individual restraint sessions. As noted above, despite habituation to homotypic stressors, novel stressors will induce a disproportionately large HPA axis stress response relative to acutely stressed controls (**Figure 3**) (Akana et al., 1992; Marti et al., 1994). Increases in CRH gene expression and enhanced HPA axis responding indicate that the underlying sensitivity of the HPA axis is increased, even though the response to the repetitive stimulus is diminished.

The PVT may mediate sensitization (facilitation) of responses to novel stressors in animals habituated to a homotypic stressor. The PVT is one of a handful of brain regions showing enhanced Fos induction following repeated stressor exposure (e.g., cold) (Bhatnagar and Dallman, 1998). As was the case with habituation, sensitization is blocked by lesions of the PVT, suggesting an important role for this region in registering stressor chronicity (Bhatnagar and Dallman, 1998). Control of HPA axis sensitization by the PVT appears to be mediated by neuropeptidergic circuits. Cholecystokinin (CCK) appears to be released in the PVT during the process of sensitization and is important in limiting the magnitude of sensitization (Bhatnagar et al., 2000). Conversely, PVT activation via the orexin pathway is required for full elaboration of stress sensitization (Heydendael et al., 2011).

Sensitization also involves noradrenergic neurons of the locus coeruleus (LC) and/or nucleus of the solitary tract (NTS). In cortex, repeated stress exposure sensitizes norepinephrine (NE) release following novel stressors (Nisenbaum and Abercrombie, 1993). Moreover, increased PVN responsiveness to NE is observed following chronic cold exposure, and appears to be required for HPA axis sensitization (Pardon et al., 2003; Ma and Morilak, 2005). Repeated homotypic stress also increases expression of tyrosine hydroxylase expression (rate limiting enzyme in NE synthesis) in the LC (Angulo et al., 1991; Mamalaki et al., 1992; Melia et al., 1992), suggesting that increased biosynthetic capacity in limbic forebrain-projecting norepinephrine neurons may play a role in the sensitization process.

Sensitization is also characteristic of models that minimize habituation, such as CVS. ACTH and corticosterone responses to novel stressors are augmented in CVS animals (e.g., see Ulrich-Lai et al., 2007). As noted, habituating and non-habituation stress regimens differ with respect to baseline endpoints (body weight, resting corticosterone, adrenal weight and/or thymus weight), but data comparing effects on the magnitude of sensitization are lacking. Thus, it is not known whether or not unpredictable regimens elicit more profound sensitization of the HPA axis.

Unpredictable stress regimens may also be relevant for understanding the lasting impact on the individual. To address this issue, we tested the long-term impact of a CVS regimen on neuroendocrine as well as behavioral outcomes. Our data indicate that CVS induces a late-emerging and long-lasting HPA axis hyporesponsivity to novel stressors (Ostrander et al., 2006), as well as impaired extinction of fear conditioning and enhanced freezing responses to reminder cues (McGuire et al., 2010). These findings indicate that chronic unpredictable stress exposure may impair resilience to future stressful experiences.

## SUCCESSFUL ADAPTATION vs. "PATHOLOGY"

Both habituating and non-habituating stress regimens present a challenge to the organism, and both cause sensitization of stress responses. A critical difference between the two is the cumulative

impact on the body and brain. In habituating stress regimens, the ability to reduce stress axis activation over time suggests that the glucocorticoid burden may not be sufficient to cause maladaptative physiological or behavioral consequences, or that it will either take longer for cumulative damage to occur.

Maintained or enhanced stress responses to non-habituating regimens are likely linked to both severity and predictability. As noted above, stress regimens that vary in intensity habituate at different rates (Garcia et al., 2000). One could posit that a stress regimen of sufficient intensity may be able to completely block the habituation process and lead to maladaptive consequences. In addition, regimens that vary stressors or have uncertain outcomes (e.g., social stressors) may not sufficiently engage habituation mechanisms, thus allowing physiological or behavioral responses to persist for extended periods of time. It is likely that both factors are involved in determining the net impact of chronic stress exposure on the individual.

Mechanisms underlying the transition from adaptation to pathology are poorly understood. Part of the problem in defining this progression lies in determining when responses meant to be adaptive "cross over" into the realm of maladaptation. For example, at what point does elevated glucocorticoid secretion start to take a toll on the brain and body? The answer likely lies at the level of the individual, and is dependent on numerous processes including hormone clearance, MR and GR expression levels, interactions of bound receptors with nuclear co-activators and co-repressors, genetic predispositions and epigenetic modifications of receptor targets. From a neural perspective, the progression from "adaptive" to "maladaptive" is likely dependent on the degree to which the brain engages physiological responses in an appropriate context. It is appropriate to mount a glucocorticoid response in response to an imminent threat; however, engaging the HPA axis chronically or in response to innocuous cues is not.

Importantly, context plays a role in dictating how tissues respond to glucocorticoids. Equivalent levels of glucocorticoids can exert different effects on cellular function depending on stress history. For example, in hippocampus, the same dose of corticosterone down-regulates mTOR expression in chronically stressed animals, but not controls (Polman et al., 2012). mTOR is an important cell signaling pathway that is involved in neuroplasticity and subsequent control of mood (Li et al., 2010). Thus, the function of the mTOR pathway will be markedly different in the context of stress, which subsequently affects behavioral and physiological responses. These data suggest that glucocorticoids may have exaggerated impact when an organism is exposed to stress, even if absolute levels of hormone are not elevated to so-called "pathophysiologic" levels.

Animal studies tracking the adaptation/pathology transition are difficult to design, due to the lack of accompanying self-reports signifying emotional or physical discomfort characteristic of human stress-related disorders. We have attempted to address this issue by comparing neural activity in habituating (repeated restraint) vs. non-habituating (CVS) models, as the latter exhibits a more severe HPA axis "phenotype," in terms of baseline corticosterone secretion, adrenal hypertrophy and thymic involution (Flak et al., 2012). The CVS regimen also induces behavioral changes suggestive of altered cognition (increased immobility in the forced swim test) and hedonic processing (decreased sucrose preference) (Ulrich-Lai et al., 2007; Jankord et al., 2011). Using FosB staining as a marker of long-term activation, we demonstrated that both repeated restraint and CVS procedures increase the number of activated neurons in key stress regulatory areas, including the ventral medial prefrontal cortex and the dorsomedial hypothalamus, the latter a structure linked to integration of endocrine, autonomic and behavioral stress responses. However, exposure to CVS caused FosB activation in regions not affected by repeated restraint, including the posterior hypothalamus and the NTS, both of which project to the PVN. Moreover, CVS-induced FosB expression in the medial PFC was increased significantly beyond that seen following homotypic stress exposure. In contrast, unpredictable stress did not result in decreased activation of any structures showing FosB induction by repeated restraint, suggesting that regions are not "de-recruited" (Flak et al., 2012). Thus, it is evident that unpredictable stress recruits regions not engaged by the habituating regimen, consistent with usage of distinct circuits under the two conditions.

The regions "recruited" by chronic unpredictable stress are of substantial importance in integration of stress responses across multiple modalities. The posterior hypothalamus is involved in coordinating defensive behaviors and autonomic responses to stressors (Shekhar and Dimicco, 1987; Lisa et al., 1989). The PH also sends excitatory projections to the parvocellular PVN (Ulrich-Lai et al., 2011), and recent data from our group suggests that activation of the PH potentiates HPA axis responses to stress (Myers et al., 2012a). The NTS is traditionally thought of as an autonomic regulatory region, but also participates in HPA axis activation by both catecholaminergic and non-catecholaminergic neurons (Ulrich-Lai and Herman, 2009), and appears to play a role in regulation of anxiety-related behaviors (via GLP-1) (Kinzig et al., 2003). Both regions receive afferents from the ventral PFC (Vertes, 2004), and may together may comprise a neural circuit responsible for perpetuation of stress responses in the face of prolonged severe or unpredictable stress (**Figure 4**).

The enhanced HPA axis drive seen in chronic unpredictable models is likely driven by uncontrollability and uncertainty regarding outcomes. Maier's work elegantly demonstrates that controllability over a stressor (shock) can protect against the development of helplessness behavior, social inhibition and behavioral withdrawal seen following inescapable stress (Maier and Watkins, 2010). Importantly, brain regions controlling development of helplessness overlap with those recruited during exposure to unpredictable stress (i.e., ventral divisions of the medial PFC) (Maier and Watkins, 2010). Thus, continued drive of the HPA axis may be mediated by engagement of the same (or parallel) circuits that control behavioral responses to unpredictability.

Numerous psychiatric disorders are associated with dysregulation of stress responses. A substantial subpopulation of depressed individuals exhibit glucocorticoid dyshomeostasis, manifest primarily as disrupted cortisol rhythms and resistance to negative feedback (Sachar et al., 1973; Carroll, 1982; Wong et al., 2000).

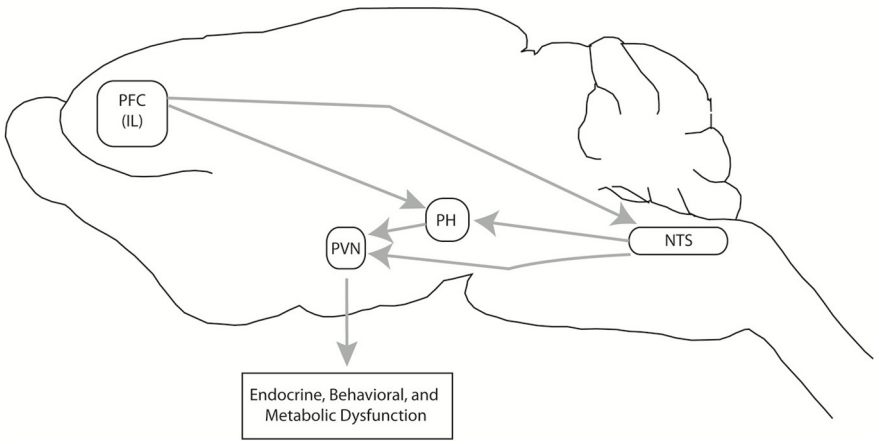


**FIGURE 4 | Chronic unpredictable stress-recruited circuitry.** Exposure to chronic variable stress (CVS) selectively recruits chronic cellular activation (in terms of FosB expression) in a discrete set of interconnected brain regions, including the prefrontal cortex (PFC), nucleus of the solitary tract (NTS), and the posterior hypothalamic nucleus (PH). The PH projects to both the PH and NTS, and both PH and NTS project to the PVN, creating the opportunity for PFC-PVN regulation in parallel, in series, or both. Via this pathway, chronic stress can increase drive to the PFC and subsequently cause glucocorticoid-mediated endocrine, behavioral, and metabolic dysfunction. This figure is reprinted from Flak et al. (2012), with permission.

In fact, depression is associated with long-term HPA axis activation, manifest as adrenal hypertrophy (Amsterdam et al., 1987, 1989) as well as somatic changes indicative of increased glucocorticoid burden [e.g., accelerated bone loss (Gold and Chrousos, 2002)]. It is postulated that depression-related hypercortisolemia causes glucocorticoid "resistance" by down-regulating GRs, thereby "freeing" the HPA axis from feedback control of downstream stress effectors (Pariante, 2004). Accordingly, depression may be an example of "out of context" corticosteroid secretion, wherein hormone release is not aligned with a true threat and is increased out of proportion with the actual objective impact of the stressor (relative to non-depressed individuals). Thus, to some extent, depression shares attributes with what may be considered a disorder of chronic stress regulation.

Conversely, PTSD is linked to low cortisol levels, which appears to be a heritable trait (Yehuda, 2009; Radley et al., 2011). In this case, enhanced glucocorticoid feedback may impair cortisol responses that are essential for normal processing of stressful information: in effect, patients are on the "left arm" of the inverted U-shaped curve relating hormone action and performance. A role for low "trait" cortisol in PTSD is supported by recent studies showing efficacy of exogenous corticosteroids in reducing symptoms in the clinic (Suris et al., 2010).

Chronic stress also causes pathological problems that extend beyond the realm of affective disorders. For example, there is a wealth of data linking chronic stress to age-related neurodegeneration and cognitive decline, likely mediated by glucocorticoid hypersecretion (see Lupien et al., 1999; Landfield et al., 2007). Chronic stress is also linked to somatic pathologies, including cardiovascular disease (see Steptoe and Kivimaki, 2012) and the metabolic syndrome (see Tamashiro et al., 2011), among others. Indeed, accumulating evidence suggests that stress has broad impact on virtually all physiological systems, with glucocorticoids serving as a contextual signal that is superimposed atop specific cellular processes.

Perhaps the key to understanding stress pathology lies in consideration of context. In depression, for example, physiological and behavioral symptoms, e.g., helplessness, anhedonia, HPA axis dysfunction and cardiovascular pathology, all mimic those induced by chronic stress regimens in animal models. The key difference between the two is the context in which these responses occur: as argued above, these behaviors and physical reactions may be entirely appropriate when an animal (or person) is confronted with environmental or physical adversity. In human depressives, the link with "actual" stress is less evident, raising the possibility that processes underlying the disorder essentially permit stress related behaviors and physical reactions to occur in the absence of threat. In depressed patients, the prefrontal cortex and amygdala, two critical components of stress circuitry, show abnormal activation patterns without any clear threat present (Drevets, 2000; Mayberg et al., 2005), suggesting the potential for engagement of responses out of context. Mounting situationally inappropriate stress responses may in turn contribute to cumulative damage associated with maladaptive aspects of stress responses (e.g., excess glucocorticoid action).

## PERSPECTIVE

Considerable progress has been made in understanding neural circuits and processes responsible for chronic stress habituation, sensitization and pathology. However, the neural trigger that differentiates successful from unsuccessful coping remains elusive. Uncovering processes underlying the transition from adaptation and pathology bears consideration of what constitutes "adaptation" and "pathology," as many responses and behaviors that may appear "maladaptive" make perfect sense in the appropriate context. Indeed, context-inappropriate physiological and emotional responses are hallmarks of stress-related disorders (depression, PTSD). Moving forward, animal models will need to incorporate an appreciation of the relevance of physiologic and

behavioral endpoints to the normal repertoire of the organism under conditions of challenge, and develop testing conditions that can more clearly query how chronic stress can generate situationally inappropriate responses reminiscent of human pathology.

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# Female vulnerability to the development of depression-like behavior in a rat model of intimate partner violence is related to anxious temperament, coping responses, and amygdala vasopressin receptor 1a expression

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Exposure to violence is traumatic and an important source of mental health disturbance, yet the factors associated with victimization remain incompletely understood. The aim of the present study was to investigate factors related to vulnerability to depression-like behaviors in females. An animal model of intimate partner violence, which was previously shown to produce long-lasting behavioral effects in females as a result of male partner aggression, was used. The associations among the degree of partner aggression, the long-term consequences on depressive-like behavior, and the impact of the anxious temperament of the female were examined. In a separate group, pre-selected neural markers were evaluated in the amygdala and the lateral septum of females. Expression was examined by analyses of targeted candidate genes, serotonin transporter (*slc6a4*), vasopressin receptor 1a, (*avpr1a*), and oxytocin receptor (*oxtr*). Structural equation modeling revealed that the female's temperament moderated depressive-like behavior that was induced by cohabitation aggression from the male partner. More specifically, increased floating in the forced swim test following male aggression was most apparent in females exhibiting more anxiety-like behavior (i.e., less open arm exploration in an elevated plus-maze) prior to the cohabitation. Aggression reduced *slc6a4* levels in the lateral septum. However, the interaction between partner aggression and the anxious temperament of the female affected the expression of *avpr1a* in the amygdala. Although, aggression reduced levels of this marker in females with high anxiety, no such pattern was observed in females with low anxiety. These results identify important characteristics in females that moderate the impact of male aggression. Furthermore, these results provide potential therapeutic targets of interest in the amygdala and the lateral septum to help improve post-stress behavioral pathology and increase resilience to social adversity.

**Keywords: vulnerability indicators, resilience indicators, domestic violence, vasopressin receptor subtype 1a, serotonin, anxiety, social stress, individual differences**

## INTRODUCTION

It has long been recognized that individuals who are exposed to adverse situations present with varying psychopathological outcomes, depending on factors such as individual characteristics and socio-environmental milieu that may help buffer the effects of adversity. These factors have been captured in a variety of bio-psycho-social models that have flourished in recent years.

Intimate partner violence is one of the most common forms of violence against women (Watts and Zimmerman, 2002). It is often associated with chronic post-traumatic stress disorder and depressive outcomes (Beydoun et al., 2012), both of which are related to the intensity of aggression by the partner (Cascardi et al., 1999). However, little is known about the underlying mechanisms that confer vulnerability to lasting emotional consequences. The main aims of the present report are to identify neural alterations that accompany symptoms caused by cohabitation with an aggressive partner and to determine whether anxious temperament may play a role in determining the consequences of such social adversity.

A critical development in our understanding of the consequences of stressful experiences is the recognition that one's interpretation of and coping with an experience may be more important than the event itself in determining the individual's psychopathological outcome (Lazarus and Folkman, 1984). Several predisposing vulnerabilities to a negative psychological outcome have been identified, comprising numerous and diverse aspects of cognitive and emotional styles (e.g., reviewed in Elwood et al., 2009). Anxiety-related aspects appear to play an important role in the psychopathological outcome. For example, high anxiety interacts with trauma exposure in the elicitation of anxiety-related distress (Larsson et al., 2008). Among women, anxiety sensitivity has been found to interact with negative

life events, predicting increased posttraumatic stress symptoms (Feldner et al., 2008), particularly dysphoria (Elwood et al., 2009).

Perpetrator characteristics appear to incompletely explain the negative interactions and the clinical psychopathological outcome in human couples. For example, negative emotionality, including anxious reactions, can be associated with not only perpetration of abuse but also victimization (Moffitt et al., 2001; Robins et al., 2002). Emphasizing the dyadic nature of human relationships, Moffitt et al. (2001) suggested that both partners need to be taken into account to understand processes that define relationship quality, and ultimately, to improve prevention and treatment of psychopathological outcomes. Mechanisms underlying this dyadic relationship are further examined here.

A rat model of intimate partner violence, based on male exposure to peripubertal stress, was recently introduced by our laboratory (Cordero et al., 2012; Márquez et al., 2013). In investigating the neural mechanisms involved in exposure to acts of aggression in a controlled fashion, this model may help elucidate the vulnerabilities to psychopathological outcomes in victims of intimate partner violence, who are arguably relatively less studied than perpetrators. A further benefit of this rat model is the absence of assortative mating, a common issue in human studies and a potential confound for understanding causal events in this dyadic relationship (e.g., Frisell et al., 2012).

We have previously reported that in this model, females exposed to more aggressive partners developed behaviors that were reminiscent of abused and depressed women (Cordero et al., 2012). This pattern was accompanied by alterations in the dorsal raphe nucleus (Cordero et al., 2012), the main source of serotonin and a critical region in emotionality (Lowry et al., 2005). Here, in the context of an aggressive experience, we sought to examine the hypothesis that an anxious temperament would increase the level of depressive-like behavior induced by the stressful experience (Sandi and Richter-Levin, 2009) by affecting coping behaviors. In order to further examine the molecular substrates of resilience and vulnerability to depressive-like symptoms, we also investigated potential changes in gene expression in selected targets of the dorsal raphe nucleus, namely, the amygdala, and the lateral septum. The amygdala is a key node in the neural circuit that is engaged by fear and threat assessment (Mahan and Ressler, 2012), and the lateral septum is commonly associated with emotionality and social behaviors, including affiliative responses in humans (Sheehan et al., 2004; Moll et al., 2012). Candidate genes of interest implicated in depression and social affect were examined, including the serotonin transporter *slc6a4* and receptors for the neuropeptides oxytocin (*oxtr*) and vasopressin [the main receptor, *avpr1a*; for reviews, see Beck (2008); Neumann and Landgraf (2012)]. Overall, this work examines how individual differences in anxious temperament among females relate to gene activity in the brain with respect to resilience to the long-term effects of aggression victimization.

## MATERIALS AND METHODS

In order to address issues of resilience, novel analyses of unpublished data from an earlier study using our intimate partner violence model (Cordero et al., 2012) are presented here, accompanied by a new cohort of subjects for neural marker assays. The methods for the intimate partner violence model and accompanying analyses have been previously published in detail (Cordero et al., 2012).

### SUBJECTS

Wistar Han rats were obtained from Charles River Laboratories (Lyon, France). Animals were maintained under controlled conditions (12-h light/dark cycle; lights on at 7:00 a.m.; 22 ± 2°C). Food and water were available *ad libitum*. With the exception of the home cage interactions (see details below), animal testing occurred during the first half of the animals' light phase. All procedures conformed to the Swiss National Institutional Guidelines on Animal Experimentation and were approved through a license by the Swiss Cantonal Veterinary Office Committee for Animal Experimentation.

Adult female virgin rats (12 weeks old) were screened for anxiety-like behavior using the elevated plus-maze (Pellow et al., 1985), a test that is widely used to evaluate animals' anxiety-related behaviors. Females were subsequently assigned to a male according to their weight, in order to preclude pairings with larger size differences that could affect the male–female interaction. After 21 days of cohabitation with a male, subjects either (1), after parturition and weaning, were housed in groups of 3 for 1 week and behaviorally characterized for lasting consequences on emotionality ($n = 41$), as indicated by the depressive-like behavior measured in the forced swim paradigm (see details below), or (2) were processed for brain extraction ($n = 24$; see details below).

### ELEVATED PLUS MAZE

The elevated plus-maze consists of two opposing open arms (each one measuring 45 × 10 cm) and two closed arms (each one measuring 45 × 10 × 50 cm) that extend from a central platform (10 × 10 cm) that is elevated 65 cm above the floor. The rats were placed individually on the central platform, always facing the same enclosed arm, and were allowed to freely explore the maze for 5 min. The parameters that were evaluated with the video tracking system (Ethovision 3.1.16, Noldus, Wageningen, Netherlands) were the total distance traveled (cm) and time spent (s) in the open and closed arms, the frequency of entries into each type of arm, and the velocity (cm/s). The floor of the apparatus was washed after each test with a 1% acetic acid solution to remove odors left by the previous subject. The anxiety-like behavior used to assign females in an unbiased fashion to the stress groups in the previous report (Cordero et al., 2012) was measured as the percentage of time spent in the open arms of the elevated plus-maze in a test conducted prior to cohabitation.

### MALE–FEMALE COHABITATION

Adult male virgin rats (12 weeks old) each cohabitated with a female. The focus here is on absolute levels of aggression directed at the female, and since in the previous study (Cordero et al., 2012) subjects in each group, control, and peripubertally stressed, exhibited some aggressive behaviors, presently no distinction is made regarding the origins of the male behavior, which will not be further discussed.

During male–female cohabitation, the home cage was changed three times (once per week) at approximately 17–19 h.

The cage change is an arousing experience that stimulates social interactions, and for this reason, it was used as the starting time for behavioral observations. At 1-week intervals, immediately upon entering each new fresh cage, social interactions were video recorded for 30min. The duration of attacks, lateral threats, upright, and keep down behaviors exhibited by the male toward the female, and the time during which the female displayed defensive-submissive behavior (either freezing or being in a supine position under the male) were scored by an experimenter who was blinded to the treatment conditions and assisted by a computer program (The Observer 5.0.25, Noldus, Wageningen, Netherlands). The measure of aggression is the summed percentage of attacks, lateral threats, upright, and keep down behaviors exhibited by the male partner. Aggression and female defensive-submissive behaviors were averaged across the three cohabitation week samples. Animals were not visibly wounded by these behaviors, except for superficial scars on a few females.

### FORCED SWIM TEST

For this test, we used an adapted version of the original rat forced swim test (Porsolt et al., 1978), in which a passive, floating behavioral response is thought to indicate depressive-like behavior. Rats were individually placed for 15 min in a plastic beaker (25 cm diameter, 46 cm deep) filled with water (25 ± 1°C) to a height of 30 cm. The rats were then removed from the tank, gently dried with a towel, returned to their home cages, and then returned to the water 24 h later for 5 min. The total duration of floating was measured for each forced swim test session. Rats were considered to show floating (immobility) behavior when they did not struggle, only making the movements necessary to keep their heads above water. The water was changed after each session, and the cylinder was cleaned to avoid the influence of alarm pheromones that were left behind by the previous animal. For the present purpose, for a pure measure of floating, we analyzed the behavior of the rats on the first day of the forced swim test, since the rats had experienced prior stress that can facilitate immobility, without the need for a water pre-exposure (Borsini et al., 1989).

### GENE EXPRESSION

Fresh brains were removed, and over ice coronal slices produced with a razor blade, the lateral septum and the extended amygdala quickly dissected using fine curved forceps (slices respectively approximately −0.4 to 1.6 and −3.6 to 2.12 mm from bregma, *cf.* **Figure 1**; Paxinos and Watson, 1997). Tissue samples were quickly placed in RNAse free cryotubes, flash-frozen in liquid nitrogen, and stored at −80°C until further processing. RNA was isolated using the RNAqueous-Micro kit (Ambion, Applied Biosystems, Rotkreuz, Switzerland). Following ethanol precipitation and quantification with Nanodrop (Thermo Fisher Scientific, Wohlen, Switzerland), cDNA was synthesized using the Superscript VILO kit (Invitrogen, Basel, Switzerland), and quantitative real-time PCR reactions (Applied Biosystems 7900HT) were conducted in triplicate using Power SYBR Green PCR Master Mix and primers for *slc6a4*, *avpr1a*, and *oxtr* designed to be complementary to each gene of interest [Microsynth (Balgach, Switzerland; see sequences in **Table 1**)]. Gene expression was normalized to the internal ribosomal reference genes *rps-18* and *rps-29*, and the analyses were conducted with qBase 1.3.5 (Hellemans et al., 2007) using the comparative cycle threshold method, yielding [delta][delta]Ct = [delta]Ct,sample—[delta]Ct,reference. The efficiency of all of the primer pairs was confirmed by performing reactions with serially diluted samples. The specificity of all of the primer pairs was confirmed by analyzing the dissociation curve.

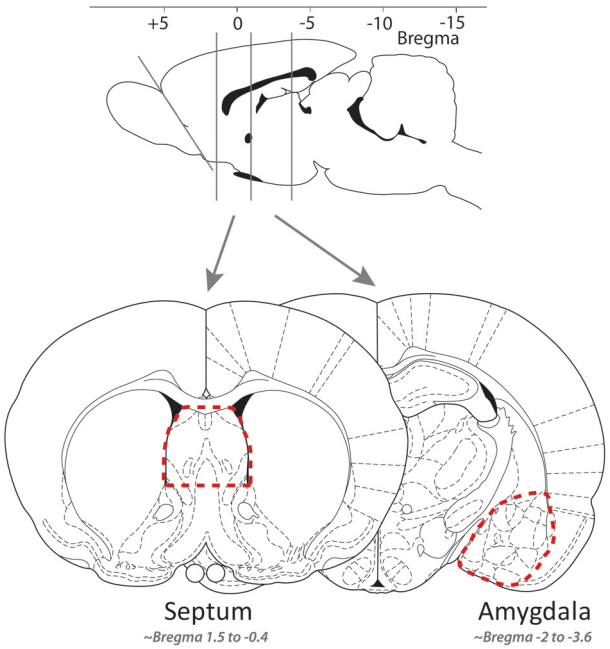


**FIGURE 1 | The approximate location of the coronal slices along the rostro-caudal axis (top) and of the tissue isolated for the lateral septum and amygdala samples [bottom; adapted from Paxinos and Watson (1997)].**

**Table 1 | Primer sequences for the gene expression analyses of the selected candidates.**

| | |
|---|---|
| *slc6a4* F | AAACGGGTGCATTTCCATATG |
| *slc6a4* R | GGCGTAACCAATGCCTTTGA |
| *avpr1a* F | AACGAACAGCACTGGGATGTG |
| *avpr1a* R | GGAATGAATCTGATGGATTTGGAA |
| *oxtr* F | CATCACCTTCCGCTTCTATGG |
| *oxtr* R | ATGCCCACCACCTGCAAGTA |
| *rps-18* F | TCATGCAGAACCCACGACAA |
| *rps-18* R | TCACGTCCTTCTGTCTGTTCAAG |
| *rps-29* F | GCCGCGTCTGCTCTAACC |
| *rps-29* R | GCACATGTTCAGCCCGTATTT |

*F, forward; R, reverse.*

### STATISTICAL ANALYSES

#### *Behavior*

Bivariate correlations, Mann–Whitney non-parametric comparisons, Student *t*-tests, and univariate analyses of variance (ANOVA) were conducted using SPSS (Statistical Package for the

Social Sciences) software (Zürich, Switzerland). Preliminary analyses were conducted by producing "low"and "high" subgroups based on a median split of behavioral measures, examining male aggression and female elevated plus-maze exploration (all subjects were included).

Using a structural equation modeling approach, an interactional model was applied (Amos 17.0, SPSS, Zürich, Switzerland). Multivariate approaches decompose the variable relationships into separate components by *concurrently* accounting for each variable under investigation (unlike bivariate analyses). The form of the model tested examined how characteristics of each subject in the dyad interacted to determine the consequences for the female. Specifically, we examined whether female anxiety-like behavior moderated the effect of partner aggression on defensive-submissive behavior and whether those variables together determined the depressive-like outcome. For this moderation analysis, behavioral measures were treated as continuous rather than discrete, categorical variables because of the greater statistical power that this approach provides (cf. Lazic, 2008). Because of their degree of distribution non-normality, a square root transformation was applied to partner aggression and female defensive-submissive values (Tabachnik and Fidell, 1996). Data for the independent (predictor) and the proposed moderating variables were standardized [*Z*-score; as recommended in Frazier et al. (2004)], and an interaction term was obtained by calculating their product. In the model, a significant relationship between the interaction term and the dependent variable indicates moderation. Covariances between each open arm exploration and male aggression with their product-term were omitted after observing their non-significance, and the final model that was tested is presented. Model fit indices were the comparative fit index (CFI), the root mean square error of approximation (RMSEA), and $\chi^2$. Typically, a good-fitting model that is a plausible representation of the underlying data structure is expected to have a non-significant $\chi^2$, CFI $\geq$ 0.90–0.95 and RMSEA $<$ 0.05 ($p$ close $>$ 0.05; Tabachnik and Fidell, 1996). Finally, the moderation was plotted using the Stats Tool Package, with values $\pm$1 standard deviation from the mean representing low vs. high levels (Gaskin, 2012).

### *Gene expression*

For univariate gene expression analyses, expression values were normalized to the control group to visualize the fold change. For Two-Way analyses of variance, subgroups were identified according to a median split on behavioral measures, examining male aggression (relatively "low" vs. "high") and elevated plus-maze exploration (relatively "low" vs. "high"; all subjects were thus included).

## RESULTS

### BEHAVIORAL PATTERNS ASSOCIATED WITH THE DEPRESSIVE-LIKE OUTCOME OF EXPERIENCING LONG-TERM AGGRESSION

In order to understand factors conferring resiliency to the development of depressive-like symptomatology, we examined whether male aggression elicited female defensive-submissive behavior and whether, the temperament of the female prior to cohabitation played a role in the elicitation of such behavior. Preliminary analyses were based on subgroups of female anxiety-like behavior, operationally defined as less exploration of the open arms of the plus-maze ("low" vs. "high," mean percent ($\pm$S.E.M.) = 22.3($\pm$1.8) and 6.2($\pm$0.8), with $n = 20$ and 21, respectively; $U = 0$, exact $p < 0.001$). First, female anxiety-like behavior, was found to be associated with enhanced aggression by the male partner (**Figure 2**; $U = 123.0$, exact $p < 0.05$). Second, partner aggression ("low" vs. "high", mean percent ($\pm$S.E.M.) = 3.0($\pm$0.3) and 10.3($\pm$1.0), with $n = 20$ and 21, respectively; $U = 0$, exact $p < 0.001$), as expected, elicited defensive-submissive behavior in the females (**Figure 3A**; $U = 79.0$, exact $p < 0.001$) and led to increased depressive-like behavior, as measured by the observation of floating by the females in the forced swim paradigm [**Figure 3B**; $t_{(39)} = -2.86, p < 0.01$].

We next proceeded to examine the relationships among all of these variables concurrently using a structural modeling approach. As a usual first step with this approach, we present the correlations among female anxiety-like behavior, male partner aggression, female defensive-submissive behavior, and female depressive-like behaviors (**Table 2**). We found that aggression by the male partner was significantly negatively correlated with

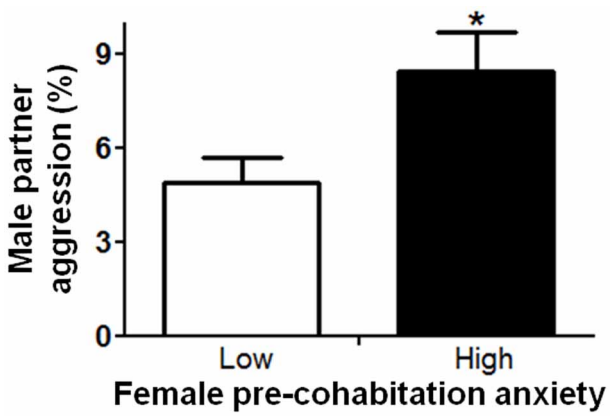


**FIGURE 2 | Female anxiety-like behavior, as observed on the elevated plus-maze prior to cohabitation, was associated with increased aggression by the male partner.** The mean ± standard error of the mean are presented. $^*p < 0.05$.

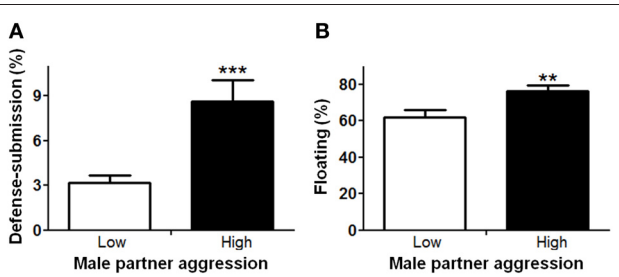


**FIGURE 3 | Higher aggression by the male partner during cohabitation significantly increased (A) defensive-submissive behaviors and (B) depressive-like behavior, as determined by floating behavior.** The mean ± standard error of the mean are presented. $^{**}p < 0.01$; $^{***}p < 0.001$.

**Table 2 | Bivariate correlations for the behavioral measures used in the model.**

| | | EPM open arm (% pre-cohabitation) | Partner aggression $\left(\sqrt{\%}\right)$ | Defensive-submissive behavior $\left(\sqrt{\%}\right)$ | Floating (%) |
|---|---|---|---|---|---|
| EPM open arm (% pre-cohabitation) | *r* | – | **−0.44**** | −0.10 | −0.23 |
| | *p* | | *0.004* | *0.546* | *0.150* |
| Partner aggression $\left(\sqrt{\%}\right)$ | *r* | | – | **0.48**** | **0.34*** |
| | *p* | | | *0.001* | *0.030* |
| Defensive-submissive behavior $\left(\sqrt{\%}\right)$ | *r* | | | – | −0.16 |
| | *p* | | | | *0.327* |
| Floating (%) | *r* | | | | – |
| | *p* | | | | |

*EPM, elevated plus-maze; r, Pearson correlation (values in bold font when significant); p, two-tailed statistical significance (italics); significant p-values, *$p < 0.05$; **$p < 0.01$; $n = 41$.*

the percentage of time spent in the elevated plus-maze open arms before cohabitation. Aggression by the male partner was also, as one would expect, significantly positively correlated with female defensive behavior and with the extent of floating behavior.

In contrast, none of the correlations were significant between any of the open arm, female defensive, or floating behaviors, as shown in **Table 2**. Therefore, these correlational data suggest that female temperament and defensive behaviors correlate independently with male aggressive behavior. Therefore, female temperament and defensive behaviors could be considered separate factors, according to this correlational analysis.

### MODELING THE BEHAVIORAL FACTORS CONFERRING RESILIENCE TO DEPRESSIVE-LIKE BEHAVIOR

The structural equation modeling analysis teased apart some additional, putatively causal behavioral relationships (**Figure 4**). We sought to verify whether females' anxious temperament affected coping abilities in the face of social adversity (**Figure 4A**). As shown in **Figure 4B**, in addition to showing that correlational effects were maintained, such as the predictive ability of male partner aggression on increased defensive behavior ($\beta = 0.67$, $p < 0.001$) and subsequent floating behavior ($\beta = 0.44$, $p < 0.05$), as well as the covariation of male partner aggression with elevated plus-maze anxiety ($\varphi = -0.36$, $p < 0.05$), effects of anxiety on female defensive-submissive behavior and, in turn, effects of female defensive behavior on floating behavior emerged. While the extent of pre-cohabitation open arm exploration, i.e., low anxiety (1) marginally predicted a lower propensity for extensive floating ($\beta = -0.27$, $p = 0.08$), (2) it significantly contributed to the extent of the defensive-submissive behavior ($\beta = 0.40$, $p < 0.001$), (3) which in turn buffered against subsequent floating behavior ($\beta = -0.37$, $p < 0.05$). A differential defensive-submissive response to partner aggression that depended on female anxious temperament is revealed by the significant interaction term ($\beta = 0.32$, $p < 0.01$). This moderation portion of the model is graphically presented in **Figure 4C**, showing that the percentage of time in the open arms significantly dampened (or vice versa, low anxiety-like behavior amplified) the extent of defensive-submissive behavior elicited by partner aggression.

### REGIONAL GENE EXPRESSION PATTERNS ASSOCIATED WITH BEHAVIORAL VULNERABILITY TO DEPRESSIVE-LIKE SYMPTOMS

In order to examine patterns of gene expression associated with the effects of male partner aggression and female anxiety-like behavior, subgroups were produced based on a median split for each variable ["low" vs. "high" anxiety-like behavior, mean percent (±S.E.M.) = 23.9(±1.8) and 7.0(±1.7), $t_{(22)} = 6.8$, $p < 0.001$]; and male partner aggression, respectively 3.6(±0.5) and 11.3(±1.7), $t_{(22)} = -4.3$, $p < 0.001$; $n = 5$–6 for each of the four subgroup combinations. As shown in **Figure 5A**, partner aggression led to a significant reduction of *slc6a4* expression in the lateral septum (Two-Way ANOVA on regional gene expression $F_{(1, 19)} = 8.4$, $p < 0.01$). In contrast, there were no main effects of partner aggression on either *avpr1a* or *oxtr* expression in the lateral septum (all $p > 0.25$; for amygdala, all $p > 0.13$, respectively **Figures 5B,C**). There was no main effect of anxiety-like behavior on any of the genes examined in the lateral septum (**Figures 5A–C**; all $p > 0.26$) or the amygdala (**Figures 5D–F**; all $p > 0.27$). Anxiety and male partner aggression interacted in one region, the amygdala, for only *avpr1a* expression (Two-Way ANOVA on regional gene expression, $F_{(1, 17)} = 11.7$, $p < 0.005$; both other genes, $p > 0.14$; for lateral septum, all $p > 0.26$). More specifically, as shown in **Figure 5E**, high anxiety females exhibited augmented *avpr1a* expression in the amygdala when partner aggression was relatively low ($p < 0.01$), and while partner aggression reduced the expression of this gene in high anxiety females ($p < 0.005$), in low anxiety females, *avpr1a* expression was not affected by partner aggression (other comparisons, $p > 0.11$).

## DISCUSSION

Intimate partner violence is often associated with depressive outcomes (Beydoun et al., 2012), and the present study sought to uncover behaviors and neural markers that are associated with resilience to partner aggression. Using a rodent model of intimate partner violence and combining behavioral data and modeling (Castro et al., 2010), the current study revealed that individual differences in anxiety-like behavior were related to the outcome of partner aggression on ensuing depressive-like symptoms by dampening defensive-submissive coping behaviors. Furthermore, when investigating potential alterations of relevant genes in brain

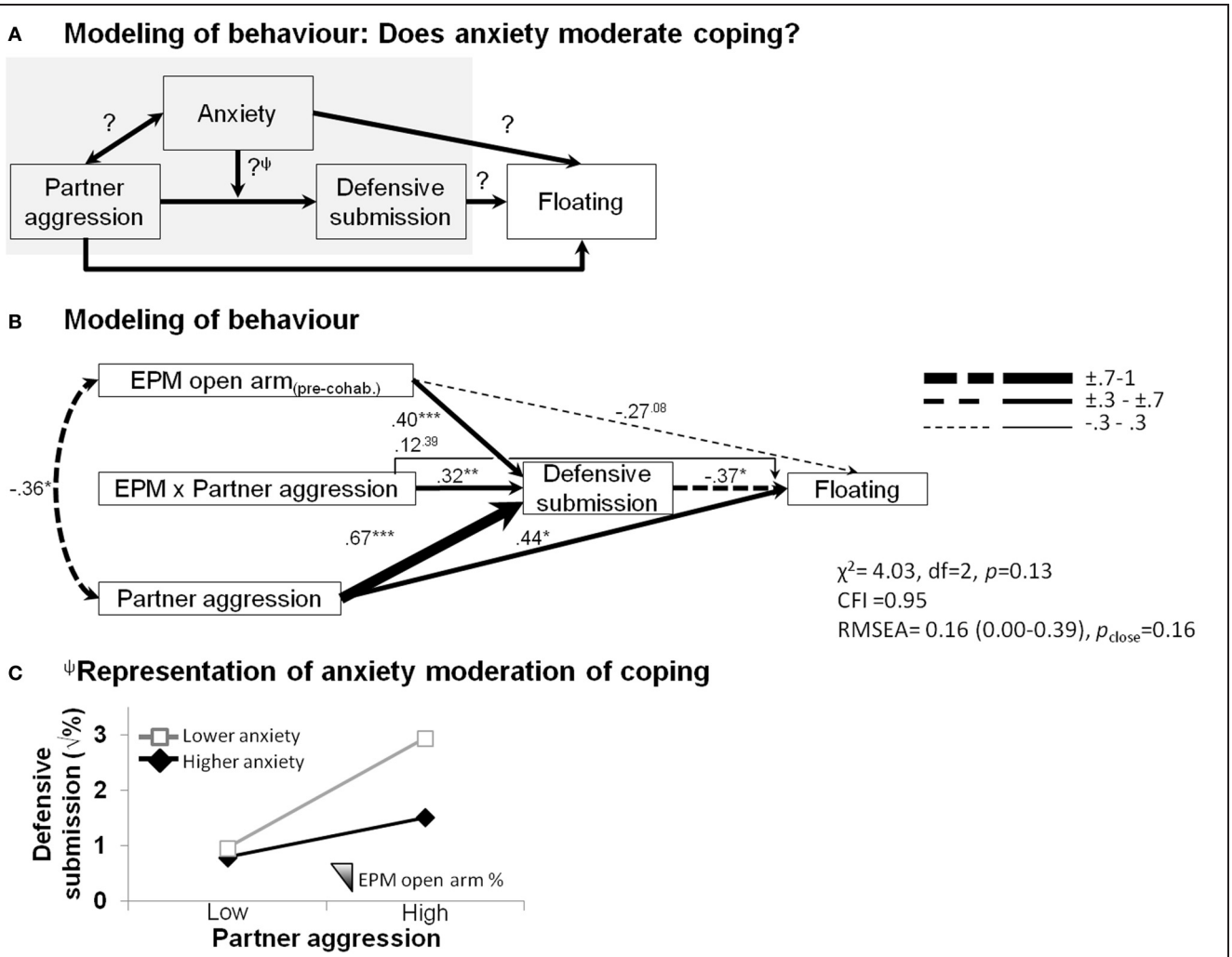


**FIGURE 4 | Structural equation modeling of behaviors of each partner predicting female depressive-like outcome.** Anxiety moderates the impact of partner aggression on the development of depressive-like floating behavior. **(A)** Integration of the behavioral patterns using structural equation modeling. Note that the result of the moderation analysis$^{\Psi}$ is graphically presented in **(C)**. **(B)** Partner aggression (1) predicted more floating, a depressive-like outcome; (2) significantly covaried with the female propensity to explore the open arms of the elevated plus-maze, as observed before the onset of the cohabitation; (3) accounted for a substantial proportion of the defensive-submissive response of the female; (4) prolonged floating was associated with less sustained defensive-submissive behaviors; and (5) the extent of which was dampened by the anxious disposition of the female (moderation shown by a significant product-term). The model fit indices are presented ($\chi^2$, chi-square; CFI, comparative fit index; RMSEA, root mean square error of approximation). Path strengths are shown with their statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and are represented by an incremental line thickness, as shown in the figure. **(C)** Focus on the representation of the moderation of the outcome by anxiety. Although, defensive-submissive behaviors are equivalent at lower aggression levels and they overall increase when faced with a more aggressive partner, this increase is dampened by pre-existing female anxious-like temperament (*less* open arm exploration in the elevated plus-maze, EPM).

regions that are essential for the regulation of anxiety and social behaviors (i.e., the lateral septum and the amygdala), we found that while male partner aggression led to reduced expression of the serotonin transporter *slc6a4* in the lateral septum regardless of female anxiety, male partner aggression led to reduced vasopressin receptor 1a (*avpr1a*) expression in the amygdala only in high anxiety females. These findings identify the lateral septum as a brain region that, regardless of female anxiety is associated with vulnerability to depressive-like symptoms elicited by aggression exposure, as indicated by the reduced expression of a key gene in the serotonergic pathway. Importantly, our results indicate that anxious temperament can influence the outcome of aggressive cohabitation. The vulnerability of this at-risk population to depressive-like symptoms was found to be associated with alterations in amygdala vasopressinergic signaling.

The results of the modeling analysis imply that females that are less avoidant of the open arm in the elevated plus-maze subsequently behave in a such way that facilitates their subordination to an aggressive male partner, as evidenced by less male partner aggression and more defensive-submission (freezing or supine posture under the male). Such "agreeable," "complementary" behavior (respectively, Moskowitz, 1994; Tiedens and

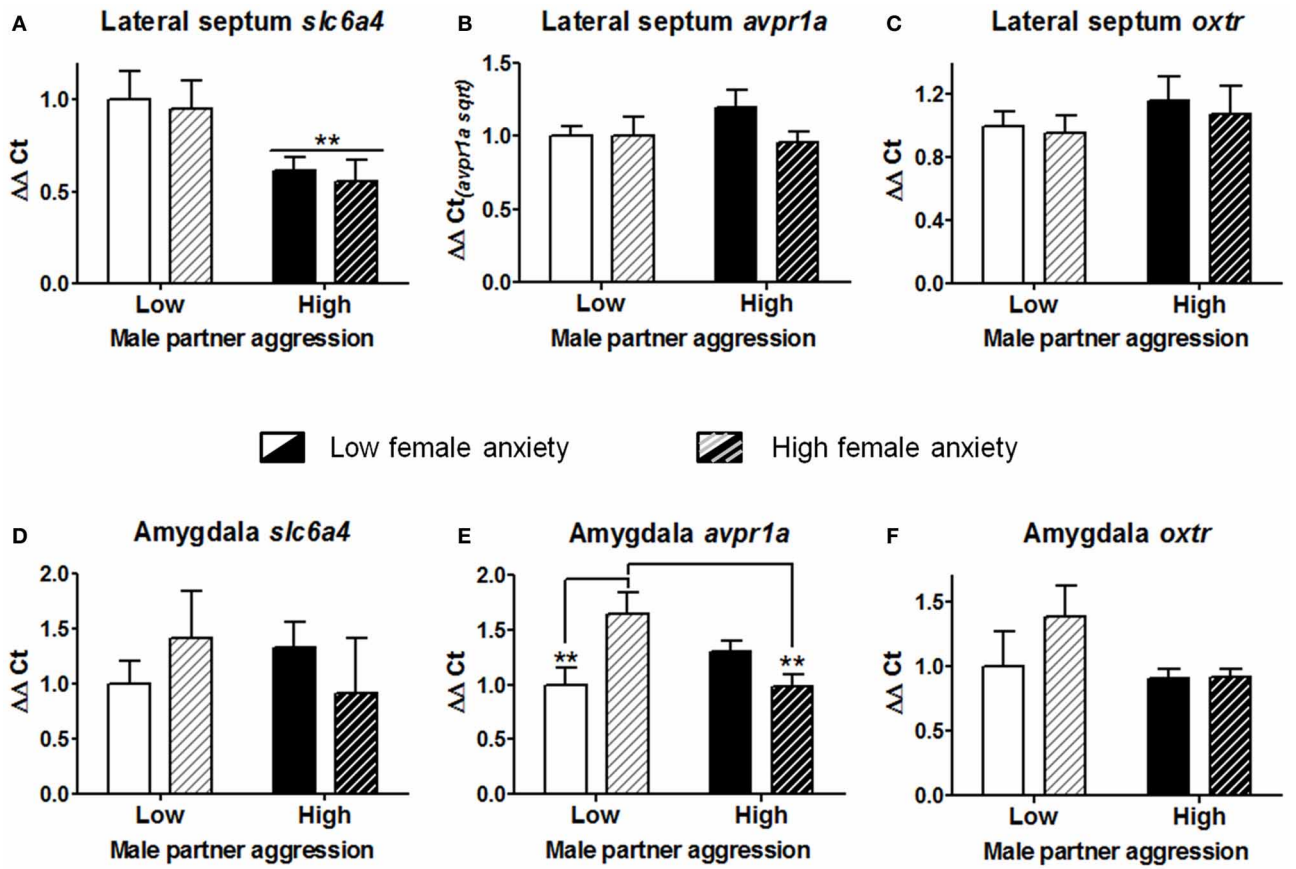


**FIGURE 5 | Gene expression analyses of the serotonin transporter (*slc6a4*), vasopressin receptor 1a (*avpr1a*), and oxytocin receptor (*oxtr*) for the lateral septum (A–C) and the amygdala (D–F).** Cohabitation with an aggressive male partner reduced the expression of *slc6a4* in the lateral septum of the females. In the amygdala, partner aggression and female anxiety-like predisposition interacted to determine the *avpr1a* expression. Females that had exhibited lower exploration of the open arms in the elevated plus-maze (i.e., "high" vs. "low" anxiety) exhibited a higher level of expression of this gene, yet this augmentation was blunted with sustained partner aggression. In contrast, no significant alterations were observed for *oxtr* in these regions. The mean ± standard error of the mean are presented. Sqrt, square root.

Fragale, 2003) may mitigate the risks of living with an aggressive partner, and buffer from future depressive-like symptoms. The development of subordination appears in this case to be an adaptive strategy, and with respect to "proactive vs. reactive"/"hawk vs. dove" strategic coping patterns, these females may present characteristics of the latter: low risk-taking, non-aggressive, and cautious yet thorough in their environment appraisal (Koolhaas et al., 1999; Korte et al., 2005).

The present reciprocal partner influence of the behaviors of both the perpetrator and victim in our model is in line with clinical findings, further supporting the view that both partners need to be taken into account to understand a relationship and ultimately to improve prevention and treatment of psychopathology (Moffitt et al., 2001). A benefit of the rat model described here is the absence of assortative mating, which is a common issue in human studies and a potential confounder for the understanding of causal events in this dyadic relationship (Rhule-Louie and McMahon, 2007).

The association reported here with floating behavior in the forced swim paradigm may be particularly relevant to understanding vulnerability to the development of depressive-like symptomatology following intimate partner violence, particularly in terms of learned helplessness. Although, there is conflicting evidence on the association of disturbances of affect, such as depression and anxiety, with ensuing victimization [positive relationship; Kim and Capaldi, 2004; Amar and Gennaro, 2005; Lehrer et al., 2006; but no association in Raiford et al. (2007), Fergusson et al. (2008)], learned helplessness may mediate the relationship between violence and symptoms of both depression and post-traumatic disorder (Bargai et al., 2007).

Depression, conditioned defeat and learned helplessness have been associated with dorsal raphe function (Maier and Watkins, 2005; Goswami et al., 2010; Hammack et al., 2012). As there are direct dorsal raphe serotonergic projections to the lateral septum (Steinbusch, 1981; Risold and Swanson, 1997), a reduction in the serotonergic signaling regulator *slc6a4* (not in *maoa*, data not shown) in this region may be related to the disturbances that we previously reported for this neurotransmitter system in the dorsal raphe of females exposed to aggressive males (Cordero et al., 2012). In that previous study, females cohabitated either with a

control or an aggressive male. Ten weeks after the cohabitation, dorsal raphe subregions were differentially affected, with females exhibiting reduced levels of serotonergic cell activation at baseline (ventrolateral) but increased levels upon exposure to an unfamiliar male (dorsal and caudal; Cordero et al., 2012), respectively previously associated with either anti-depressant or -panic properties, vs. anxiogenesis, as recently described in a review of the literatures of human imaging and post-mortem analyses as well as that of animal models (imaging, pharmacological, and lesion work on anxiety-, panic-, and depressive-like behavior, as respectively interpreted from the consequences of e.g., social or non-social inescapable stress, hypercapnia, and forced swim test consequences; Hale et al., 2012). Here, of particular interest is the reduced baseline activation in the ventrolateral subregion, one associated with anti-depressant properties. Lateral septum serotonin has also been proposed to be protective against depression (Sheehan et al., 2004), and it receives the majority of its dorsal raphe inputs from the ventrolateral subregion (Kanno et al., 2008).

The serotonin transporter plays an important role in emotion and social relations [reviewed by Canli and Lesch (2007)], and interestingly, a gene variant conferring low *slc6a4* function was reported to increase depression resulting from abuse in pregnant women (Scheid et al., 2007). The current finding of reduced serotonin transporter expression in the lateral septum of females subjected to increased aggression is consistent with the proposal [in review by Sheehan et al. (2004)] that chronic stress would reduce lateral septum activity, which could occur via increased serotonin or blunting of its reuptake. Such modulation of serotonin in the lateral septum would lead to its inhibition, and lower activity of the lateral septum has been associated with increased fear and learned helplessness-like behavior (Sheehan et al., 2004).

Altogether, these observations indicate that this system would be a prime candidate for the alleviation of the psychopathological symptoms associated with intimate partner violence. Among the potential mechanisms for the sustained changes found in our study in *slc6a4* expression could be epigenetic changes that are induced by exposure to aggression. Methylation of *slc6a4* from peripheral blood can yield reduced mRNA expression (Philibert et al., 2007) and reduced *in vivo* brain serotonin synthesis (Wang et al., 2012), in association with the affective outcome of traumatic events. Promoter methylation levels of the *slc6a4* gene may either protect or confer vulnerability to unresolved loss or related post-traumatic stress disorder, according to genetic variants at different sites (respectively, Van IJzendoorn et al., 2010; Koenen et al., 2011). Finally, although the administration of the serotonin precursor tryptophan has been found to increase agreeableness in men, it is less consistently so in women (aan het Rot et al., 2006; Young et al., 2007). Informing this gender discrepancy the present results may suggest for women the consideration of the vasopressinergic system, in association with anxious temperament.

A role for anxiety in modulating the engagement of the amygdala with social adversity would be consistent with evidence in mouse lines exhibiting anxiety differences, where higher anxiety was associated with enhanced social avoidance after repeated social defeat (Savignac et al., 2011). While serotonin transporter gene variants conferring reduced function have been associated with amygdala basal hyperactivity and hyper-reactivity to perceived threat in males and females (Hariri et al., 2002; Canli et al., 2006), no differences were observed in the expression of this gene in the amygdala in the present study. In contrast, alterations were observed for the *avpr1a* gene. Notably, the vasopressinergic system has also been associated with depression, as well as anxiety. Vasopressin activates the hypothalamus-pituitary-adrenal axis and can exert anxiogenic properties [reviewed in Engelmann et al. (2004)]. Strikingly, vasopressin functional effects appear to depend on the socio-emotional context. Therefore, although peripheral vasopressin was associated with positive social couple relations in a non-depressed sample (Gouin et al., 2012), in depressed samples, it was found to positively correlate with the extent of the disorder, and in particular with the anxious vs. non-anxious depression subtype (Van Londen et al., 1997; De Winter et al., 2003).

In the amygdala, *avpr1a* is present mainly in the central nucleus [female and male voles, (Insel et al., 1994); male rats, (Veinante and Freund-Mercier, 1997)]. Anxiety and *avpr1a* expression in the amygdala have been associated in females. Lactating dams from rat lines bred for high anxiety exhibit more aggressive behavior than those bred for low anxiety. The aggression is associated with greater release of vasopressin in the central amygdala nucleus, which is dependent on *avpr1a* (Bosch and Neumann, 2010). It should also be noted that in addition to aggression, alterations in central amygdala vasopressin receptor 1a gene expression may be related to post-partum maternal behaviors in rats (Caughey et al., 2011) and humans (Bisceglia et al., 2012); however, any potential vasopressin effects on maternal behavior were found to be unrelated to the dam's anxiety in rats (Bosch and Neumann, 2008).

It should be noted that the lateral septum and the amygdala are interconnected (Risold and Swanson, 1997), may reciprocally modulate each other, and are both part of what has been termed a "social behavior neural network" (Newman, 1999). Our results suggest that such a putative regulatory loop may be disturbed in females that develop depressive-like symptoms following their experience with an aggressive partner *via* amygdala vasopressin signaling, particularly those with a more anxious temperament at baseline.

Therefore, the present findings provide potentially useful insight for the development of clinical intervention for trauma caused by intimate partner violence. These findings emphasize the importance of accounting for individual differences in temperament to uncover substrates of vulnerability to social adversity, which may prove useful in addressing variability in responses to psycho- and pharmacotherapy. Manipulations of the proposed targets in the amygdala and the lateral septum could help increase resilience and promote recovery from social trauma. Integrating this information into basic research and ultimately clinical practice may prove fruitful in evaluating treatment opportunities and improving translational success.

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# Resilience in shock and swim stress models of depression

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Experimental models of depression often entail exposing a rodent to a stressor and subsequently characterizing changes in learning and anhedonia, which may reflect symptoms of human depression. Importantly, not all people, and not all laboratory rats, exposed to stressors develop depressed behavior; these "resilient" individuals are the focus of our review. Herein we describe research from the "learned helplessness" and "intermittent swim stress" (ISS) models of depression in which rats that were allowed to control the offset of the aversive stimulus with a behavioral response, and in a subset of rats that were not allowed to control the stressor that appeared to be behaviorally and neurochemically similar to rats that were either naive to stress or had controllability over the stressor. For example, rats exposed to inescapable tailshock, but do not develop learned helplessness, exhibit altered sensitivity to the behavioral effects of $GABA_A$ receptor antagonists and reduced *in vitro* benzodiazepine receptor ligand binding. This pattern suggested that resilience might involve activation of an endogenous benzodiazepine-like compound, possibly an allostatic modulator of the $GABA_A$ receptor like allopregnanolone. From the ISS model, we have observed in resilient rats protection from stressor-induced glucocorticoid increases and immune activation. In order to identify the neural mediators of these correlates of resilience, non-invasive measures are needed to predict the resilient or vulnerable phenotype prior to analysis of neural endpoints. To this end, we found that ultrasonic vocalizations (USVs) appear to predict the resilient phenotype in the ISS paradigm. We propose that combining non-invasive predictive measures, such as USVs with biological endpoint measures, will facilitate future research into the neural correlates of resilience.

**Keywords: shock and swim stress, $GABA_A$ receptor, neurosteroids, resilience, ultrasonic vocalizations**

Depression is a widespread disorder resulting in significant suffering for the patient and their families (Nestler et al., 2002; Knol et al., 2006). Although great strides have been made in the last 50 years toward improving antidepressant pharmacotherapies (Berton and Nestler, 2006; Drevets et al., 2008), fewer than one half of the people prescribed antidepressant drugs respond favorably to treatment and remain refractory (Southwick et al., 2005; Berton and Nestler, 2006). Exposure to stressors over the lifespan is a good predictor of risk for depression, and the prevailing view is that depressed mood is the result of an interaction between stressors and genetic factors (Caspi et al., 2003, 2010; Southwick et al., 2005). However, understanding an individual's stress history and genetic risk is not sufficient for understanding vulnerability to depression, as many people experience chronic or severe stress without ever developing major depression. Thus, many individuals are resilient to depression. The goal of the work reviewed here was to identify behavioral and neural characteristics of resilience with the hope of illuminating previously unappreciated candidates for therapeutic drug development and preventative therapies.

It is important to note at the outset that we conceptualize resilience broadly. For instance, an individual may appear to be resilient because of previous experiences that rendered the individual *resistant* to the stressor's consequences, because of an inherent capacity to *recuperate* after trauma, or because of their ability to *mitigate* the physiological or psychological consequences of a stressor by implementing effective coping strategies (for further discussion see Fleshner et al., 2011). Our research into the neural correlates of resilience began in the early 1980's in the laboratory of Dr. Steven Maier using the "learned helplessness" model of depression (Maier et al., 1973; Maier and Seligman, 1976). Early research by Maier and Seligman (1976) demonstrated that the controllability of a stressor was one of the most important predictors of stressor consequences on behavior. Initial studies focused on instrumental shuttle escape learning in dogs and rats (Overmier and Seligman, 1967; Maier et al., 1973), but have since expanded to include activity measures (Jackson et al., 1978; Drugan and Maier, 1983), food competition dominance (Rapaport and Maier, 1978), rewarding effects of drugs (Will et al., 1998), and social behavior (Short and Maier, 1993; Christianson et al., 2009). In each of these cases, exposure to unpredictable and inescapable shocks (inescapable stress, IS) resulted in behavioral changes that reflect aspects of anxiety and depression. However, if the shocks were escapable

(escapable stress, ES) by means of performing a behavioral wheel-turn response, then behavior appeared normal in subsequent tests. Thus, the controllability of the stressor determined whether the subject would appear resilient or vulnerable. These, "stressor controllability effects" have been reviewed elsewhere (Maier and Watkins, 2005, 2010).

## STRESSOR CONTROLLABILITY AND STRESS-INDUCED ANALGESIA

Studies of stressor controllability typically employ a "triadic design," which permits the experimenter to manipulate only the variable of control while the amount and number of shocks are equal (**Figure 1**). At the time we began studying stressor controllability, the major focus was on identifying the mechanisms that caused learned helplessness; specifically, the shuttle escape learning deficit that occurs only after IS exposure. It was hypothesized that exposure to IS caused a change in central analgesia systems, such that subsequent exposure to footshocks in the shuttle escape task would not be sufficiently motivating because of an enhanced analgesia (see Maier, 1986 for a review of behavioral stress-induced analgesia studies). Interestingly, we found a very different activation of pain inhibition systems during the stress experience depending on whether the rats experienced ES vs. IS. IS induced a long-lasting analgesia mediated by endogenous opioids. Importantly, ES also induced analgesia, but it was much shorter and independent of endogenous opioids (**Figure 1**; Maier et al., 1982; Drugan et al., 1985a). This indicated that stressor controllability determined what type of pain inhibition systems were activated in response to stress. It was hypothesized that the non-opioid form of analgesia observed as a result of ES would enable the coping behavior, whereas the opioid analgesia observed after IS would inhibit behavioral responses (Maier, 1986).

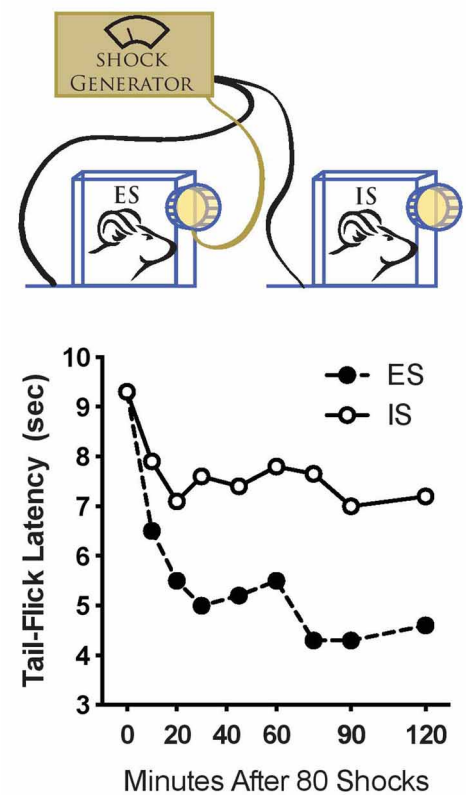


**FIGURE 1 | Stressor controllability and resilience to tailshock. Top:** A schematic diagram of the stressor controllability experiment. Rats are assigned to either escapable stress (ES), inescapable stress (IS), or no stress. Rats in the ES group receive a series (usually 80–100 trials) of tailshocks while restrained in a chamber with a wheel that is connected to a controlling computer. Rats in the ES group may escape from shocks by turning the wheel. Rats in the IS group are "yoked" to the ES rat such that shock is terminated for the IS when the wheel turn response is made. The wheel in the IS chamber is not connected to the computer, so these rats do not have control over shock onset or offset. Importantly, each rat in the pair receives exactly equal tailshock. **Bottom:** Stressor controllability and analgesia. Rats were exposed to 80 trials of ES or IS, and pain tolerance was quantified using the tail-flick reflex to heat applied to the tail at various times after stress. Both ES and IS rats displayed analgesia immediately after stress. However, analgesia persisted for 2 h after IS while ES rats recovered (adapted from Drugan et al., 1985a).

## STRESSOR CONTROLLABILITY AND $GABA_A$

We were intrigued about the identity of this coping-induced analgesia that could orchestrate such a differential reaction to the stress. Mineka and colleagues (1984) noted that ES vs. IS rats exhibited very different behaviors in between shocks. In fact, they reported greater fear responses associated with the IS vs. ES context. Thus, we tested whether the stressor-induced analgesia was dependent upon fear or anxiety experienced during shock exposure. Indeed, administration of the anxiolytic benzodiazepine, chlordiazepoxide (Librium), before IS prevented the development of learned helplessness and long-lasting analgesia (Drugan et al., 1984). Conversely, administration of the anxiogenic β-carboline, FG 7142, in lieu of IS exposure, produced a learned helplessness-like shuttlebox escape deficit (Drugan et al., 1985b). Together these studies provided evidence that mitigating anxiety might be necessary for the prevention of learned helplessness effects that occurred in ES rats.

Since benzodiazepines and β-carbolines act at the $GABA_A$ receptor (Braestrup et al., 1980; Paul and Skolnick, 1982), this was an obvious place to start our systematic investigation. The hypothesis to be tested was that ES would facilitate GABAergic tone (mimicking an anxiolytic agent), while IS would interfere with GABAergic tone (similar to an anxiogenic agent). We first utilized bicuculline-induced seizures as a behavioral assay of $GABA_A$ receptor function after ES or IS. If ES increased $GABA_A$ function, then significantly greater concentrations of the $GABA_A$ antagonist would be required to induce seizure and vice versa for IS. This is precisely what occurred with rats exposed to ES protected from either bicuculline or picrotoxin-induced seizures, while IS rats showed an increased susceptibility to seizure (**Figure 2**; Drugan et al., 1985c, 1994). The functional significance of this change in GABAergic sensitivity was revealed in that stressor controllability altered the hypnotic and ataxic effects of several central nervous system depressants. More specifically,

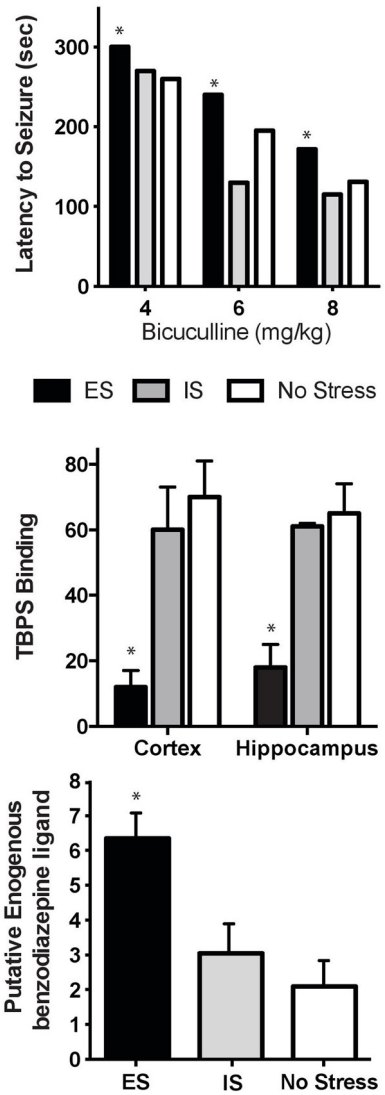


**FIGURE 2 | Stressor Controllability and Central Benzodiazapine/ $GABA_A$ receptor. Top:** Rats were exposed to ES, IS, or no stress, and then administered increasing doses of bicuculline 2 h after stress. Rats with prior ES appeared to be protected from seizures with a significant delay in the onset of clonus seizure symptoms compared to unstressed rats [ES had a significantly longer latency compared to no stress controls ($*p < 0.05$). Adapted from Drugan et al., 1985c]. **Middle:** Mean (+SEM) [$^{35}$S]TBPS receptor binding (2 nM, fmol/mg protein brain tissue) 2 h after stress. Exposure to ES significantly reduced competitive binding of the benzodiazepine receptor ligand ($*p < 0.05$, adapted from Drugan et al., 1994). **Bottom:** Mean (+SEM) brain levels of benzodiazepine receptor agonist molecules (ng/mg brain tissue) 2 h after stress. Prior ES significantly increased levels of endogenous benzodiazepine receptor ligands ($*p < 0.05$, adapted from Drugan et al., 1994).

immediately or 2 h following inescapable shock stress, we observed an enhanced reactivity to both ethanol and midazolam compared to non-shocked controls. However, exposure to escapable shock did not change the reactivity to these minor tranquilizers (Drugan et al., 1992, 1996). Thus, it appeared that providing rats with a coping mechanism altered central GABAergic function. Protection against picrotoxin-induced convulsions, and its association with brain benzodiazepine receptor occupancy, has been well-established (Duka et al., 1979; Braestrup et al., 1982; Mennini and Garattini, 1982; Paul et al., 1982). It was hypothesized that ES would stimulate the release of endogenous ligands for the benzodiazepine binding site on the $GABA_A$ receptor and, thereby, result in allosteric changes to the $GABA_A$ site. Thus, after ES, fewer binding sites would be available for the experimenter-administered bicuculline. Indeed, *in vitro* radioligand binding assays of the $GABA_A$ receptor in rats following ES or IS revealed that rats exposed to ES exhibited decreased [$^{35}$S]T-butylbicyclophosphorotionate (TBPS) binding to the picrotoxin site on the $GABA_A$ receptor in cortex and hippocampus tissue when compared to IS rats and controls (Drugan et al., 1994).

As with clinical depression, not all rats exposed to IS develop learned helplessness. More specifically, a portion of rats initially exposed to IS learn to escape in the shuttlebox test. Rats were exposed to IS and then tested for shuttle escape performance 24 h later. The shuttle escape task requires that the rat shuttle from one side of the apparatus to the other to terminate the footshock. Rats that exhibit learned helplessness typically "fail" this task, and subsequently the experimenter terminates the shocks, typically after 30 s. An arbitrary criterion was established to split rats into "fail" and "learn" groups based on a median split in average escape latencies. Thus, two non-overlapping distributions of rats were identified, and this pattern has been reported by numerous groups (Chi et al., 1989; Drugan et al., 1989a,b; McIntosh and Gonzalez-Lima, 1994; Minor et al., 1994; Koen et al., 2005). Furthermore, the behavioral profiles of these rats remained the same when challenged with an IS 2 or 4 weeks later, and were subsequently tested for shuttle escape performance. This indicated that this initial stress reactivity was a rather stable trait (Drugan et al., 1989b). We then tested the $GABA_A$ binding in rats that were divided into "failers" and "learners" as above. The brains of the group that learned (e.g., were resilient) showed an increased binding of [$^3$H] muscimol to the $GABA_A$ receptor in cerebral cortex, but a reduction in [$^{35}$S]TBPS binding to the benzodiazepine binding site on the $GABA_A$ receptor (Drugan et al., 1993). These findings, and those from rats exposed to ES, suggested that the resilience to tailshock involved activation of an endogenous benzodiazepine-like ligand. To our knowledge, this was the first evidence that ES, or resilience to IS, activated endogenous neural machinery that could mitigate the anxiety and analgesia evoking consequences of shock exposure *per se*.

## STRESSOR CONTROLLABILITY AND ENDOGENOUS ANXIOLYTIC FACTORS

The search for endogenous ligands for the benzodiazepine/$GABA_A$ receptor began in the late 1970's and has continued into the present. Putative endogenous ligands include purines (Skolnick et al., 1978, 1980), hemoglobin metabolites (Ruscito and Harrison, 2003), diazepam binding inhibitor (DBI; Costa et al., 1994), octadecaneuropeptide (ODN; Do-Rego et al., 2001), neuroactive steroids such as 3 alpha, 5 alpha-tetrahydrodeoxycorticosterone (THDOC; Majewska et al., 1986), and brain-derived neurosteroids such as pregnenolone (Jung-Testas et al., 1989). Although the ES-induced endogenous ligand

remains unknown, there is evidence in support of neurosteroids. First, the effects on benzodiazepine/$GABA_A$ binding reviewed above occur in adrenalectomized rats, indicating that the endogenous anxiolytic factor is not a product of the adrenal gland (i.e., a neuroactive steroid such as THDOC; Drugan et al., 1993). Second, evidence exists from a study in which we observed a proactive interference of ES on a subsequent spatial memory task, an effect that depended on neurosteroid synthesis (Healy and Drugan, 1996). There is clear evidence that *de novo* steroid synthesis can occur in the brain (Jung-Testas et al., 1989). Pregnenolone is the primary precursor of steroid hormone biosynthesis in adrenal tissue (Hechter et al., 1951; Brown et al., 1979; Jung-Testas et al., 1989), yet this substance is found in the brain of rats or monkeys with either surgical or pharmacological removal of peripheral steroid secretion (Robel et al., 1987). Given this information, we hypothesized that both ES exposure as well as resistance to IS effects may be the result of the release of a positive modulatory, brain derived neurosteroid. One such candidate is the A-ring-reduced metabolite of progesterone, 3 alpha-hydroxy-5 alpha-pregnan-20-one (allopregnanolone). Importantly, this substance inhibits [$^{35}$S]TBPS binding (Gee et al., 1987) and has anticonvulsant (Belelli et al., 1989), and anxiolytic properties (Crawley et al., 1986). Finally, stress-induced increases of allopregnanolone have been observed for 1 h following 5–10 min ambient swim stress in sham as well as adrenalectomized rats (Purdy et al., 1991). Allopregnanolone represents a novel target for therapeutic drug development. Testing the hypothesis that stress-induced synthesis and release of allopregnanolone contributes to resilience is the focus of future research.

## PROBING THE GENERALITY OF STRESSOR CONTROLLABILITY EFFECTS IN A NOVEL INTERMITTENT SWIM STRESS PARADIGM

The tenacious pursuit of neural mechanisms mediating stressor controllability effects by Maier and colleagues has provided very exciting insight into the pathophysiology of stress related disorders and novel treatments. However, the vast majority of reported research into stressor controllability has utilized tailshock as a stressor. Although tailshock has some advantages as a stimulus (i.e., it is aversive without producing tissue damage, it can be precisely administered, rats readily learn to escape it, and it does not lead to habituation) one might wonder if the results from studies of controllability and resilience to tailshock generalize to stressor exposure *per se.* To this end, we developed an intermittent swim-stress (ISS) version of the stressor controllability paradigm in which rats are exposed to brief, unpredictable forced swims that can be terminated in the escapable swim group by pressing a lever hanging in the middle of the swim chamber. ISS is conducted in a plastic chamber with a false floor that can be raised and lowered into a tank of water; the idea was to create an intermittent version of the forced swim test (FST) that is widely used [Porsolt et al., 1977, 1979; see Brown et al. (2001), or Drugan et al. (2005) for a photo of the ISS apparatus], with the only difference being the temperature of the water between paradigms.

As with shock, the controllability of swim determines the behavioral outcome. Twenty-four hours after stress, rats exposed to inescapable, but not escapable ISS, display increased immobility during a 5 min FST (Drugan et al., 2005). We have also reported a learned helplessness-like, escape learning deficit in rats exposed to inescapable ISS in something we termed the "swim escape test" (SET). The SET places rats in the swim apparatus with a lever positioned at the surface of the water during a trial. The rats are required to press the lever once (fixed-ratio; FR-1) during the first five trials and twice (FR-2) during the subsequent trials in order to escape from the forced swim. The instrumental requirements were developed to be identical to the learned helplessness shuttlebox escape test (Maier et al., 1973). Again, similar to inescapable tailshock, some of the rats previously exposed to ISS fail to learn the escape response, while others do learn and appear to be resilient (Christianson and Drugan, 2005). We hope to extend the generality of findings from the learned helplessness, tailshock stress paradigm to stress *per se* by determining whether the neural substrates such as the medial prefrontal cortex, found to be important in the work of Maier and colleagues (see Maier and Watkins, 2010 for review) apply to controllability of swim stress. Rearing and housing conditions also play a role in subsequent resilience and vulnerability with early weaning and isolation, but not maternal separation, predicting more depressive-like behavior (i.e., increased mean swim time in the SET). Similarly, maternal contributions also play a role; pups from dams that displayed increased anxiety-like behavior had longer swim times in the SET and were more likely to be classified as vulnerable (Stiller et al., 2011). Thus, the ISS model provides the same empirical features as the tailshock paradigm: the behavioral consequences depend on stressor controllability and rats can be identified as stress-resilient or -vulnerable based on the performance of an escape test.

## IMMUNE CORRELATES OF RESILIENCE AND VULNERABILITY IN THE INTERMITTENT SWIM STRESS PARADIGM

Since conducting the pharmacology and behavioral studies discussed above in the shock paradigm, our interests and technologies have evolved. With the ISS model, we became interested in examining the consequences of stressor exposure on immune endpoints as inflammatory processes are implicated in the pathophysiology of depression (Raison et al., 2006; Dantzer et al., 2008). Using a median split in SET performance to identify resilient and vulnerable samples after ISS, stress vulnerable rats exhibited increased post-SET plasma corticosterone (CORT) concentrations, and enhanced T-cell proliferation in response to conconavalin-A (Con-A) compared to stress resilient rats (Levay et al., 2006; Stiller et al., 2011). Because rats that learn to escape would experience significantly less exposure to swim than those that failed, and accordingly less exposure to cold water, core body temperature was assessed in rats following the SET. Not surprisingly, rats that exhibited poor escape learning and long escape latencies (i.e., vulnerable) exhibited greater hypothermia than rats with good escape learning and short escape latencies (i.e., resilient). This difference amounted to an additional 7.4 min of total swim time (11.7 vs. 4.3 min), which led to a 2.0°C decrease in body temperature (Levay et al., 2006). Unfortunately, the results from endocrine and immune measures (after SET) are confounded by both hypothermia and differential exposure to swim.

Although these ISS results are encouraging, there is a potential confound in that the groups are typically measured following a subsequent test, which is a stressor itself (e.g., social interaction, social defeat, shuttlebox performance, and SET). The tests themselves may change brain chemistry and mask the "true" changes due to stress resistance interfering with the identification of neural processes underlying resilience. In addition, the subjects in tests, such as shuttlebox escape, receive unequal amounts of stress (e.g., less shock in subjects that learn and more shock in those that fail). Similarly, in the SET, rats show differential escape behavior, which results in a different amount of swim stress exposure among all rats prior to extraction of the brain to look at neural changes. For example, this makes any brain changes observed following these tests uninterpretable, because of the potential confound of differential stress exposure during the test causing the differences and not stress resistance *per se*. This drawback is not unique to the ISS paradigm. Many of the strategies used in the field to investigate resilience involve experimentally manipulating the environment in some fashion and a behavioral assay to identify resilient vs. vulnerable populations (see Russo et al., 2012, for recent review). In many cases the assay for vulnerability, in our case the SET or the shuttlebox test, introduce a confounding variable that make subsequent analyses of brain, immune, or other endpoints difficult to interpret. However, in the Drugan et al. (1993) study, the $GABA_A$ receptor changes were observed in resilient rats that were given 3–4× the number of escape trials in an effort to equilibrate shock exposure to the vulnerable rats. Nonetheless, the pattern of the shock exposure still differed between vulnerable (longer duration shocks on each trial) and resilient (shorter duration shocks on each trial), and this still may influence brain changes.

## ULTRASONIC VOCALIZATIONS AS A CORRELATE OF RESILIENCE

In order to conduct post stress analyses on resilient and vulnerable populations, one would need a tool that reliably predicts performance in assays like the SET and shuttlebox that is both non-stressful and non-invasive. To this end, we began to record ultrasonic vocalizations (USVs) emitted by rats during ISS exposure. USVs are emitted by rats following significant environmental events and can be used as an "on-line" measure of emotional status (Knutson et al., 2002). USVs have been used to indicate exposure to cold (Blumberg and Stolba, 1996), or as a correlate of fear behavior in developmental studies (Brunelli and Hofer, 1996; Dichter et al., 1996). USVs are categorized based on a combination of frequency and duration dimensions (Litvin et al., 2007). Adult rats emit "22-kHz" and "50-kHz" USVs (Brudzynski et al., 1993; Panksepp et al., 1998). Fifty-kilohertz calls are associated with positive or approach situations (Knutson et al., 1998; Panksepp et al., 1998), whereas 22-kHz USVs occur in response to aversive stimuli (Tonoue et al., 1986; Blanchard et al., 1991; Miczek et al., 1991, 1995; Knapp and Pohorecky, 1995; Brudzynski, 2001; Swiergiel et al., 2007) and during defensive/submissive behavior (Thomas et al., 1983; van der Poel and Miczek, 1991). Thus, 22-kHz USVs tend to be emitted during distress and correlate with negative affect (Brudzynski et al., 1993; Panksepp et al., 1998), although exceptions have been noted (Barfield and Geyer, 1972, 1975; van der Poel and Miczek, 1991). However, a recent finding suggested that USVs might be a predictor of stress resilience. Jelen et al. (2003) reported that rats emitted 22 kHz USVs in the presence of a safety signal rather than a danger signal. This laboratory investigation confirmed observations in the field where rodents emitted USVs when they were in a position of safety, yet observed the approach of a predator (Litvin et al., 2007).

Given this information, USVs were recorded throughout ISS exposure and two distinct populations of rats were identified. One group produced many long duration USVs, while another made very few USVs with shorter durations (**Figure 3**; Drugan et al., 2009). Upon subsequent exposure to the SET, rats in the first group appeared to be resilient with good escape learning, while the rats in the later group did not learn to escape (**Figure 3**; Drugan et al., 2009). Thus, the high number and

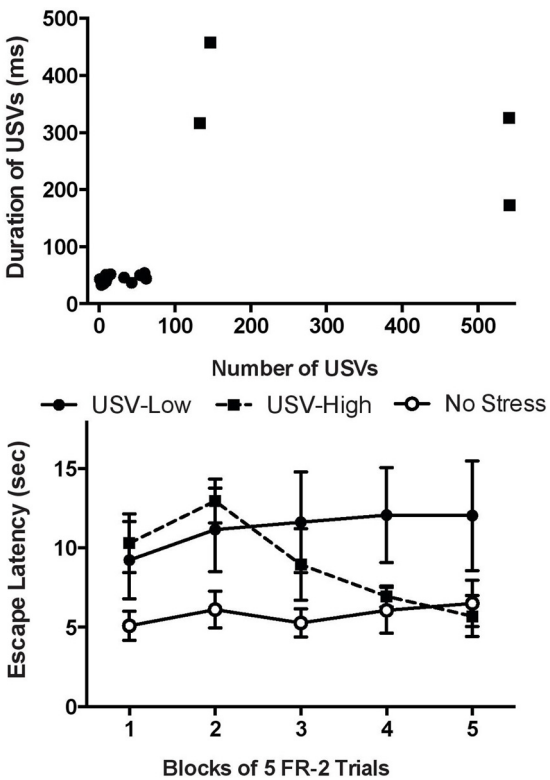


**FIGURE 3 | Predicting resilience in the Swim Stress Paradigm. Top:** A scatter plot characterizing the ultrasonic vocalizations (USVs) of the rats in the swim stress group recorded during stress exposure. Rats were categorized as USV-High rats (black squares) or USV-Low (black circles). USV-High rats produced a large number of long-duration vocalizations while USV-Low rats emitted few, short-duration vocalizations. Plotted are the number of ultrasonic vocalizations vs. the average duration of the vocalizations for each rat. **Bottom:** Mean (±SEM) time to escape in the swim escape test (SET). Rats in the USV-Low group displayed a detriment in learning while the USV-High group appeared resilient and performed similarly to the no stress control group. The no stress group was exposed to the swim stress apparatus, but was never exposed to swim (adapted from Drugan et al., 2009).

long-duration USVs made during ISS appear to be a good predictor of resilience in the SET. An important next step in this research line is to determine if USVs also predict resilience in other behavior endpoints such as forced swim or anxiety behaviors. Consistent with our initial report, several recent studies have used USVs to predict behavior in a drug self-administration paradigm (Maier et al., 2012; Meyer et al., 2012). The data encourage continued assessment of USVs to establish this as a useful tool to forecast subsequent stress reactivity. As noted, such a tool will eliminate the confounding effect of post-stress tests used to behaviorally identify resilient and vulnerable populations. By using USVs as a predictor, the experimenter can be sure that both resilient and vulnerable populations have received identical stressor exposure.

## CONCLUSIONS AND FUTURE DIRECTIONS

The results of the research reviewed support a few points that should inform continued research into the neural correlates of resilience. Foremost is the importance of understanding the confounding influence of the behavioral test employed to identify resilient vs. vulnerable populations of subjects. Some of the work we have reviewed must be considered in light of these confounds. The behavioral and biological endpoints quantified were assessed after rats performed differentially in tasks that would result in different exposure to stressful stimuli—such as footshock or forced swimming. Our work, in concert with that of others, has built support for emitted USVs as a way to forecast resilient populations so that neural or physiological endpoint measures may be conducted without introducing additional stressors—a non-invasive measure of stress reactivity. Also important is the possible role of endogenous modulators of the $GABA_A$ receptor, such as allopregnanolone in stress resilience. Questions remain regarding the central sites of synthesis and action of this neurosteroid during ES, whether behavioral correlates of resilience (e.g., USVs) are causally linked to neurosteroid actions, and whether stress inhibitory neural circuits interact with the neural loci that support production of USVs. These are the focus of ongoing research and should broaden our understanding of resilience and provide new avenues for antidepressant development.

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# Coping changes the brain

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One of the earliest and most consistent findings in behavioral neuroscience research is that learning changes the brain. Here we consider how learning as an aspect of coping in the context of stress exposure induces neuroadaptations that enhance emotion regulation and resilience. A systematic review of the literature identified 15 brain imaging studies in which humans with specific phobias or post-traumatic stress disorder (PTSD) were randomized to stress exposure therapies that diminished subsequent indications of anxiety. Most of these studies focused on functional changes in the amygdala and anterior corticolimbic brain circuits that control cognitive, motivational, and emotional aspects of physiology and behavior. Corresponding structural brain changes and the timing, frequency, and duration of stress exposure required to modify brain functions remain to be elucidated in future research. These studies will advance our understanding of coping as a learning process and provide mechanistic insights for the development of new interventions that promote stress coping skills.

**Keywords: stress, coping, exposure therapy, neuroplasticity, neuroimaging, learning, emotion regulation**

## INTRODUCTION

Diverse therapeutic and preventive interventions utilize intermittent stress exposure to enhance the development of stress coping skills. Intermittent exposure to stress is, for example, an aspect of resiliency training for people that work in conditions where performance in the face of adversity is required, e.g., medical and military personnel, aviators, police, firefighters, and rescue workers (Meichenbaum, 2007; Stetz et al., 2007). Exposure therapy for anxiety disorders likewise teaches patients to imagine a graded series of stress-inducing objects or situations, and then encourages interaction with these stressors *in vivo*. These procedures promote learning and provide opportunities to practice stress coping skills (Tryon, 2005; McNally, 2007; Craske et al., 2008). Here we consider how learning as an aspect of coping in the context of stress exposure therapy changes the brain. This perspective builds on evidence that learning reflects experience-dependent neuroadaptations in brain regions that mediate cognitive, motivational, and emotional aspects of physiology and behavior (Poldrack, 2000; Posner and DiGirolamo, 2000; Dolan, 2002; Pascual-Leone et al., 2005).

Initially, we conducted a *PUBMED* (www.ncbi.nlm.nih.gov/pubmed) search using the terms "exposure therapy" AND "brain" to identify 49 published reports. Excluding review papers and empirical studies of animal models, obsessive compulsive disorder, and interventions not related to exposure reduced the search results to eight reports. Seven additional studies cited in the initial eight reports were subsequently found to be appropriate for inclusion in the review, bringing the total to 15 studies listed in **Table 1**. Treatment efficacy was established in all 15 studies based on behavioral outcomes not addressed in the summaries presented below except for relevant brain-behavior correlations identified by various neuroimaging techniques. Nine of the 15 studies examined patients with specific phobias and six studies examined patients with post-traumatic stress disorder (PTSD). Seven of the nine studies of phobias focused on fear of spiders and we begin by reviewing these reports in chronological order.

## SPIDER PHOBIA

Paquette et al. (2003) compared healthy non-phobic controls ($n = 13$) with spider phobics ($n = 12$) before and after exposure therapy in a functional magnetic resonance imaging (fMRI) study of blood oxygenation as a measure of neural activity. Exposure therapy entailed guided mastery training, education for correcting spider misbeliefs, and exposure to pictures of spiders, films of spiders, and real spiders. During the initial scan session and prior to therapy, responses to spiders vs. butterflies were greater in dorsolateral prefrontal cortex and the parahippocampal gyrus in spider phobics compared to non-phobic controls. After exposure therapy, responses to spiders were diminished in right dorsolateral prefrontal cortex and right parahippocampal gyrus compared to pre-treatment fMRI data. These findings suggest that exposure therapy results in decreased demands on brain regions that mediate cognitive strategies involved in self-regulation (prefrontal cortex) and de-conditioning of traumatic memories (hippocampus).

Straube et al. (2006) randomized patients with spider phobia to exposure therapy ($n = 14$) or a wait list control condition ($n = 14$), and compared these patients to healthy non-phobic controls ($n = 14$) using fMRI. Exposure therapy entailed training to enhance cognitive restructuring and exposure to pictures of spiders, touching tarantula spider skin, and handling real spiders. Before exposure therapy, responses to spiders vs. neutral

**Table 1 | List of studies in the review.**

| Citation | Disorder | Exposure therapy duration | Brain imaging modality | Interval between scans | Preplanned brain regions | Exploratory brain regions |
|---|---|---|---|---|---|---|
| Paquette et al., 2003 | Spider phobia | Multiple sessions 4 weeks | fMRI | 6 weeks | PFC* | Frontal gyrus, fusiform gyrus, occipital gyrus, parahippocampal gyrus, parietal cortex |
| Straube et al., 2006 | Spider phobia | Multiple sessions 2 days, 4–5 h/day | fMRI | 2 weeks | Amygdala, ACC*, fusiform gyrus*, insula*, parahippocampal gyrus, PFC*, thalamus* | Basal ganglia, central gyrus, cuneus, frontal gyrus, lingual gyrus, occipital gyrus, parietal gyrus, precentral gyrus, precuneus, temporal gyrus |
| Goossens et al., 2007 | Spider phobia | Single session 4–5 h | fMRI | 2 weeks | Amygdala*, ACC*, insula* | Occipital cortex |
| Schienle et al., 2007 | Spider phobia | Single session 4 h | fMRI | 2 weeks | Amygdala, ACC, fusiform gyrus, insula*, OFC*, parahippocampal gyrus, PFC | Angular gyrus, frontal gyrus, lingual gyrus, occipital gyrus, parietal gyrus, supramarginal gyrus |
| Leutgeb et al., 2009 | Spider phobia | Single session 4 h | EEG | 1 week | n/a | n/a |
| Leutgeb et al., 2012 | Spider phobia | Single session 4 h | EEG | 1 week | n/a | n/a |
| Hauner et al., 2012 | Spider phobia | Single session 2–3 h | fMRI | 2 hours/week/6 months follow up | Amygdala* | ACC, frontal gyrus, fusiform gyrus, insula, lingual gyrus, occipital cortex, parietal lobe, PFC, temporal gyrus |
| Nave et al., 2012 | Snake phobia | Single session 2–3 h | fMRI | 2 weeks | Amygdala, ACC, frontal gyrus*, insula, OFC | n/a |
| Furmark et al., 2002 | Public speaking phobia | Multiple sessions 9 weeks | PET | 9 weeks | Amygdala*, hippocampus* | Temporal cortex |
| Lindauer et al., 2005 | PTSD | Multiple sessions 4 months | sMRI | 4 months | Amygdala, hippocampus, parahippocampal gyrus | n/a |
| Felmingham et al., 2007 | PTSD | Multiple sessions 8 weeks | fMRI | 6 months | Amygdala, ACC* | Frontal gyrus, hippocampus, parietotemporal gyrus, postcentral gyrus, temporal gyrus |
| Rabe et al., 2008 | PTSD | Multiple sessions 8–12 weeks | EEG | 3 months | n/a | n/a |
| Lindauer et al., 2008 | PTSD | Multiple sessions 4 months | SPECT | 4 months | n/a | Frontal gyrus |
| Roy et al., 2010 | PTSD | Multiple sessions 6 weeks | fMRI | 2 months | Amygdala*, ACC, hippocampus | Frontal gyrus, PFC, subcallosal gyrus |
| Adenauer et al., 2011 | PTSD | Multiple sessions 12 weeks | MEG | 4 months | n/a | Parietal cortex, occipital cortex |

*Brain regions are specified for preplanned comparisons and post-hoc exploratory analyses. Asterisks signify a statistically significant exposure therapy treatment effect for the preplanned comparisons. Abbreviations: ACC, anterior cingulate cortex; OFC, orbitofrontal cortex; PFC, prefrontal cortex; PTSD, post-traumatic stress disorder; EEG, electroencephalography; fMRI, functional magnetic resonance imaging; PET, positron emission tomography; sMRI, structural magnetic resonance imaging; SPECT, single-photon emission computed tomography; n/a, not applicable.*

pictures were greater in the anterior cingulate cortex, insula, and left extrastriate visual cortex of phobic patients compared to non-phobic controls. Conversely, non-phobic controls showed greater responses in the left amygdala, bilateral parahippocampal gyrus, and pre- and post-central gyri. After exposure therapy, responses to spiders were diminished in the anterior cingulate cortex, insula, left dorsal medial prefrontal cortex, thalamus, and left precuneus in the exposure therapy group compared to wait list controls. The exposure therapy group also exhibited greater responses in the right cuneus relative to wait list controls. Within-subjects comparisons confirmed post-treatment reductions in the response to spiders in anterior cingulate cortex, insula, and left thalamus. Non-phobic controls were not scanned twice so their responses from the initial scan session were compared to second scan responses for the exposure therapy and wait list groups. No significant differences were observed between non-phobic controls and the exposure therapy group after treatment, but the wait list group continued to exhibit greater activity in the anterior cingulate cortex and right insula compared to healthy controls. These results together suggest normalized anterior cingulate and insula activity in phobic patients successfully treated with exposure therapy.

Goossens et al. (2007) examined patients with spider phobia ($n = 16$) before and after exposure therapy, and compared these patients to healthy non-phobic controls ($n = 14$) using fMRI. Exposure therapy entailed education for correcting spider misbeliefs and exposure to drawings of spiders, photographs of spiders, and real spiders. During the initial scan session and prior to therapy, responses to spiders vs. neutral pictures were greater in the left amygdala, anterior cingulate cortex, and insula in phobics compared to non-phobic controls. After exposure therapy, group differences were no longer discerned in the amygdala, anterior cingulate cortex, or insula. Within-subjects comparisons confirmed post-treatment reductions in the left amygdala, anterior cingulate cortex, and insula. Furthermore, percent change in the amygdala response to spiders vs. neutral pictures positively correlated with therapy-related outcome measures of fear and anxiety. These findings support earlier suggestions that exposure therapy normalizes the activity of corticolimbic brain circuits.

Schienle et al. (2007) randomized patients with spider phobia to exposure therapy ($n = 14$) or a wait list control condition ($n = 12$), and compared these patients to healthy non-phobic controls ($n = 25$) using fMRI. Exposure therapy consisted of a single 4-h session of progressive gradual approach tasks involving a live spider. During the initial scan session and prior to therapy, greater responses to spiders vs. neutral pictures were discerned in the left amygdala and fusiform gyrus for phobic patients compared to non-phobic controls. Conversely, non-phobic controls exhibited greater activity in the right inferior parietal gyrus, inferior frontal gyrus, anterior cingulate, medial orbitofrontal cortex, and right dorsolateral prefrontal cortex compared to phobic subjects. After exposure therapy, greater responses to spiders were discerned in bilateral medial orbitofrontal cortex in successfully treated patients compared to wait list controls. Within-subjects comparisons confirmed post-treatment right orbital frontal increases and also revealed reduced activity in the right insula. Additional analyses revealed a positive correlation between the reduction of experienced anxiety and somatic panic symptoms and decreased activation in the right amygdala and left insula. The reduction of arousal ratings was also positively correlated with a decreased right amygdala response.

In a follow-up study using the same experimental design, Schienle and colleagues randomized patients with spider phobia to exposure therapy ($n = 22$) or a wait list control condition ($n = 23$), and compared these patients with healthy non-phobic controls ($n = 20$) in an electroencephalography (EEG) study (Leutgeb et al., 2009). Event related potentials were extracted for three well characterized and temporally precise time windows following spider vs. neutral stimulus onset. Before exposure therapy, larger parietal P300 and early-late positive potential (early-LPP) amplitudes were discerned in response to spiders vs. neutral pictures in phobics compared to non-phobic controls. After exposure therapy, greater central late-late positive potential (late-LPP) amplitudes were discerned in response to spiders for successfully treated patients compared to wait list controls. Parietal P300 and early-LPP amplitudes remained unchanged from pre-treatment levels. Schienle and colleagues found similar LPP enhancement in a recent EEG study of children with spider phobia (Leutgeb et al., 2012) and suggest that exposure therapy alters neural markers of attention allocation by reducing attentional avoidance and changing the way spiders are perceived.

Hauner et al. (2012) examined 12 patients with spider phobia before and after exposure therapy using fMRI. Exposure therapy entailed a 14-step series of progressive approach tasks with a live tarantula spider conducted during a single 2–3 h session. During the initial scan session and prior to therapy, responses to spiders vs. neutral pictures were greater in right amygdala, anterior cingulate cortex, insula, and ventral medial prefrontal cortex, while diminished responses were discerned in right dorsolateral prefrontal cortex. After successful treatment, self-reported reductions in fear induced by spider pictures were accompanied by increased activity in right dorsolateral prefrontal cortex with decreased activity in amygdala, anterior cingulate cortex, insula, and ventral medial prefrontal cortex. Additionally, increased responses to spiders vs. neutral pictures were observed immediately after treatment in right superior parietal cortex.

Six months after treatment, Hauner et al. (2012) found that in patients free from phobic symptoms responses to spiders were similar to those observed immediately after exposure therapy for amygdala and limbic brain regions. However, dorsolateral prefrontal cortex responses were diminished relative to responses observed immediately after exposure therapy, and diminished responses were also discerned in bilateral ventral visual cortex. These findings indicate that brain changes can occur long after completion of exposure therapy and suggest a time-limited role for top-down prefrontal control of amygdala and limbic brain systems.

## SNAKE PHOBIA

Nave et al. (2012) conducted a double-blind placebo controlled trial of exposure therapy combined with D-cycloserine in patients with snake phobia using fMRI. Patients were randomized to placebo plus exposure therapy ($n = 9$) or D-cycloserine plus exposure therapy ($n = 7$) to determine whether D-cycloserine

enhances treatment efficacy. Exposure therapy entailed a 13-steps series of progressive gradual approach tasks involving a live snake. After exposure therapy, responses to snakes vs. neutral pictures were diminished in right dorsal prefrontal cortex in both the D-cycloserine and placebo groups compared to pre-treatment fMRI data. Other prefrontal brain regions showed qualitatively different responses in the exposure therapy plus D-cycloserine vs. placebo conditions but the significance of these findings is not obvious because patients in both treatment conditions showed equivalent reductions in snake phobia severity. In the placebo group, additional analyses revealed a positive correlation between the reduction of snake phobia severity and decreased snake-elicited activation in right/middle frontal gyrus, right insula/inferior frontal gyrus, bilateral medial orbitofrontal cortex, left orbitofrontal cortex, and bilateral amygdala. In the D-cycloserine group, affective ratings scores were negatively correlated with perigenual cingulate cortex activation and positively correlated with left ventrolateral prefrontal cortex activation.

## PUBLIC SPEAKING ANXIETY

In a study of public speaking anxiety by Furmark et al. (2002), patients were randomized to exposure therapy ($n = 6$), citalopram medication ($n = 6$), or a wait list control condition ($n = 6$). Exposure therapy entailed training to enhance cognitive restructuring, homework assignments, and interaction with stressful simulated public speaking situations. All patients were scanned using positron emission tomography (PET) with oxygen 15-labeled $H_20$ as an indicator of regional cerebral blood flow (rCBF) while presenting a short speech to a 6–8 person audience. After exposure therapy or the citalopram treatments, rCBF responses to public speaking were diminished in the amygdala, hippocampus, and anterior and medial temporal cortex compared to pre-treatment PET data. Pre- vs. post-treatment changes in rCBF were not observed in the wait list control condition. Between-group comparisons confirmed that rCBF reductions occurred in both treatment conditions compared to the wait list control condition, with particularly strong responses discerned in right temporal lobe regions. Additionally, favorable outcomes at 1-year follow-up were associated with greater initial suppression of the subcortical rCBF response to public speaking in the periaqueductal gray area, left thalamus, and bilateral amygdala. These results suggest that both pharmacologic and exposure-based therapies may act through a common mechanism to attenuate amygdalar-limbic hyperactivity and diminish public speaking anxiety.

## POST-TRAUMATIC STRESS DISORDER

In addition to serving as an effective treatment for phobic disorders, exposure therapy is often used to treat patients with PTSD. In the only structural brain imaging study that met our search criteria, Lindauer et al. (2005) investigated structural brain changes in patients with PTSD related to personal violence or exposure to accidents or disasters randomized to exposure therapy ($n = 9$) or a wait list control condition ($n = 9$). These patients were compared to trauma-experienced, but non-PTSD controls ($n = 14$) using structural MRI to measure the volume of specific brain regions. Before exposure therapy, PTSD patients had significantly smaller total, right, and left hippocampal volumes, and larger total and left parahippocampal gyrus volumes compared to traumatized, non-PTSD controls corrected for total brain volume variation. Volumetric changes were not discerned in any brain region after exposure therapy for PTSD.

Felmingham et al. (2007) examined eight patients with personal assault or motor vehicle accident-related PTSD before and after exposure therapy using fMRI. Imaginal exposure was combined with training to enhance cognitive restructuring. Before exposure therapy, responses were greater in right postcentral gyrus, right middle temporal gyrus, and left superior temporal gyrus in response to fearful vs. neutral stimuli. After exposure therapy, responses to the same fearful stimuli were increased in bilateral anterior cingulate cortex, left middle temporal gyrus, right inferior frontal gyrus, left parietotemporal gyrus, and right hippocampus. Post-treatment increases in anterior cingulate cortex activity were positively correlated with symptom improvements, whereas amygdala activity was negatively correlated with symptom improvements.

Rabe et al. (2008) examined patients with motor vehicle accident-related PTSD or subsyndromal PTSD randomized to exposure therapy ($n = 17$) or a wait list control condition ($n = 18$) using EEG. Imaginal exposure, writing exposure, and *in vivo* exposure were combined with cognitive restructuring education and relaxation training. After exposure therapy, diminished right anterior brain responses to trauma-related vs. neutral pictures were discerned in treated patients compared to wait list controls. Right anterior brain responses were correlated with outcome measures of PTSD severity.

In a follow-up to their initial brain structural imaging study described above, Lindauer et al. (2008) randomized patients with PTSD related to personal violence or exposure to accidents or disasters to exposure therapy ($n = 10$) or a wait list control condition ($n = 10$), and compared these patients to trauma-experienced but non-PTSD controls ($n = 15$) using single-photon emission computed tomography (SPECT) to measure rCBF. Imaginal exposure was combined with psychoeducation, writing tasks, and training to enhance cognitive restructuring. Before exposure therapy, greater rCBF responses were discerned in the right insula and right dorsolateral prefrontal cortex in PTSD patients compared to traumatized non-PTSD controls. After exposure therapy, decreased rCBF responses to trauma-related imaginal exposure were discerned in right dorsolateral prefrontal cortex in treated patients compared to wait list controls. The effect of treatment on outcome measures of PTSD severity correlated positively with rCBF changes in the left superior temporal gyrus and middle frontal gyrus.

Roy et al. (2010) examined military service members with PTSD randomized to either imaginal ($n = 8$) or virtual reality ($n = 7$) exposure therapy using fMRI. Imaginal exposure involved imaginary recall of the traumatic experience in progressively greater detail, while virtual reality exposure included interaction with trauma-relevant virtual visual environments with multi-sensory stimulation, i.e., tactile vibrations and the smell of cordite during virtual explosions. Due to high rates of dropout and resulting small sample sizes, imaginal vs. virtual

reality therapy could not be compared. By collapsing across these two treatment conditions, a diminished post-treatment amygdala response to negative, but not neutral, stimuli on the Affective Stroop Test was discerned relative to pre-treatment data.

Adenauer et al. (2011) randomized PTSD patients to exposure therapy ($n = 16$) or a wait list control condition ($n = 18$) in a magnetoencephalography (MEG) study. Due to subject dropout and other exclusion criteria, post-treatment MEG data analysis included 11 exposure therapy subjects and eight wait list controls. Exposure therapy entailed a narrative analysis focused on the detailed reconstruction of each patient's traumatic memory. During the initial scan session and prior to therapy, no group differences were discerned in cortical responses to aversive vs. neutral pictures. After exposure therapy, greater responses were discerned in superior parietal cortex for treated patients compared to wait list controls. Additionally, within-subjects comparisons revealed greater left occipital cortical responses to aversive vs. neutral stimuli after exposure therapy compared to pre-treatment MEG data. Adenauer and colleagues discuss these findings as evidence that exposure therapy decreases attentional avoidance and enhances voluntary control over previously avoided traumatic memories.

## FUNCTIONAL BRAIN CHANGES

All but one study that met our search criteria used functional brain imaging modalities to examine how stress exposure changes the brain. Four of the 14 functional studies used EEG or MEG, which both have high temporal resolution but are spatially restricted to cortical surface potentials spanning 1–2 cm. The remaining 10 functional studies used PET, SPECT, or fMRI, which all have limited temporal resolution but are able to target both cortical and deeper brain structures with millimeter spatial resolution.

The temporal precision of MEG and EEG allows for the characterization and dissociation of attentional brain network processes that occur within milliseconds of stimulus presentation. Two relevant event-related potentials, the P300 and the LPP, have been shown to reflect increased attention toward motivationally relevant stimuli. The P300 peaks between 300 and 500 ms following stimulus onset and has been linked to automatic attention processes, while the LPP typically peaks between 500 and 3000 ms of stimulus onset and has been associated with controlled attention and emotion regulation (Olofsson et al., 2008; Dunning and Hajcak, 2009). The spider phobia EEG studies report enhanced LPP amplitudes and no change in P300 amplitudes in response to spiders in treated patients (Leutgeb et al., 2009, 2012). The stability of P300 amplitudes suggests that exposure therapy does not change automatic attention processes. Enhancement of LPP amplitudes likely reflects reduced attentional avoidance to arousing stimuli as patients learn through repeated exposures that feared consequences do not occur and that avoidance is unnecessary for anxiety reduction. Adenauer et al. (2011) similarly attribute their MEG findings to brain mechanisms involved in reducing attentional avoidance.

In the functional studies based on PET, SPECT, and fMRI, preplanned comparisons and exploratory analyses converged on five regions of interest, i.e., amygdala, prefrontal cortex, anterior cingulate cortex, insula, and hippocampus (**Table 2**). All of these regions are known to be involved in emotion regulation and resilience. In studies of healthy humans, for example, cognitive efforts aimed at reducing negative emotions decrease amygdala responses determined by fMRI (Ochsner et al., 2002, 2004). Exposure therapy likewise diminished amygdala responses in four different studies (Furmark et al., 2002; Goossens et al., 2007; Roy et al., 2010; Hauner et al., 2012) and none of the studies reported a post-treatment increase (**Table 2**). Four studies also consistently noted exposure therapy-induced down regulation of the insula

**Table 2 | Summary of functional brain changes.**

| Citation | Disorder | Amygdala | ACC | Hipc | Insula | PFC |
|---|---|---|---|---|---|---|
| Paquette et al., 2003 | Spider phobia | – | – | ↓ | – | ↓ |
| Straube et al., 2006 | Spider phobia | – | ↓ | – | ↓ | ↓ |
| Goossens et al., 2007 | Spider phobia | ↓ | ↓ | – | ↓ | – |
| Schienle et al., 2007 | Spider phobia | – | – | – | ↓ | ↑ |
| Hauner et al., 2012 | Spider phobia | ↓ | ↓ | – | ↓ | ↑↓ |
| Nave et al., 2012 | Snake phobia | – | – | – | – | ↓ |
| Furmark et al., 2002 | Public speaking phobia | ↓ | – | ↓ | – | – |
| Felmingham et al., 2007 | PTSD | – | ↑ | ↑ | – | ↑ |
| Lindauer et al., 2008 | PTSD | – | – | – | – | ↓ |
| Roy et al., 2010 | PTSD | ↓ | – | – | – | – |

*Preplanned comparisons and exploratory analyses converged on five most common regions of interest in the functional studies based on PET, SPECT, and fMRI. Post-treatment increases (↑), decreases (↓), or no-change (–) in functional activity are shown for each region. Abbreviations: ACC, anterior cingulate cortex; Hipc, hippocampus; PFC, prefrontal cortical regions; and PTSD, post-traumatic stress disorder.*

(Straube et al., 2006; Goossens et al., 2007; Schienle et al., 2007; Hauner et al., 2012). This region integrates somatic signals for interoceptive awareness and participates in networks needed to determine stimulus salience and attentional focus (Paulus and Stein, 2006).

Prefrontal cortex plays a key role in top-down control of amygdala activity and thereby regulates behavioral responses to emotionally salient events (Salzman and Fusi, 2010; Todd et al., 2011). In animal models, prefrontal control of amygdala activity mediates learned extinction of conditioned fear (Gottfried and Dolan, 2004; Delgado et al., 2006), and fMRI studies of humans have identified inverse correlations between increased prefrontal and decreased amygdala responses to emotional stimuli (Ochsner et al., 2002, 2004). In this review, we found only a single study that specifically reported increased prefrontal and decreased amygdala responses to emotional stimuli after stress exposure therapy (Hauner et al., 2012). Furthermore, five of seven studies (**Table 2**) that identified exposure therapy-induced changes in prefrontal activity actually reported post-treatment decreases in prefrontal responses (Paquette et al., 2003; Straube et al., 2006; Lindauer et al., 2008; Hauner et al., 2012; Nave et al., 2012).

Additional inconsistencies were evident in anterior cingulate cortex and hippocampus (**Table 2**). Three studies reported exposure therapy-induced down regulation of anterior cingulate cortex activity (Straube et al., 2006; Goossens et al., 2007; Hauner et al., 2012) in keeping with its role in assessing the salience of emotional information (Etkin et al., 2011), but one study noted post-treatment increases anterior cingulate cortex responses (Felmingham et al., 2007). Two studies reported that exposure therapy decreases hippocampal responses to subsequent presentations of emotional stimuli (Furmark et al., 2002; Paquette et al., 2003), but one study reported increased post-treatment hippocampal responses (Felmingham et al., 2007).

One possible explanation for the discrepancies noted above is that exposure therapy may have different neurobiological effects in patients with different specific phobias or patients with PTSD related to warfare trauma vs. motor vehicle accidents. Inconsistencies may also arise from differences between studies in the duration of exposure therapy which ranged from single sessions spanning a few hours to multiple sessions spanning many weeks (**Table 1**). Likewise, variation in the content and type of stress exposure during each exposure session may contribute to inconsistent results as demands on specific brain regions will vary in a task-specific manner. Additionally, many studies incorporate various neutral stimuli from the International Affective Picture System but no clearly neutral standard yet exists. Brain responses to spiders vs. butterflies, for example, are likely to differ from responses to spiders vs. other "neutral" stimuli such as snails, household items, or geometric objects.

Discrepancies in functional brain changes may also arise from differences in the timing of brain scans relative to the exposure therapy sessions (**Table 1**). Exposure therapy effects are dynamic and may emerge with varying temporal delays, and resulting brain changes may be long-lasting or transient depending on the specific brain functions affected. For example, dorsolateral prefrontal cortex responses to spider stimuli were increased immediately after exposure therapy in the fMRI study by Hauner et al. (2012), but 6 months later the same brain region showed a diminished response to the same spider stimuli. The investigators indicate that the initial post-treatment brain scan but not the 6-months follow up scan overlapped with exposure-induced engagement of corticolimbic regions that were transiently involved in learning and memory consolidation.

## STRUCTURAL BRAIN CHANGES

Despite evidence that brain functions rely on structural scaffolding and communication across distributed networks of neurons (Mesulam, 1990; Kolb and Whishaw, 1998; Citri and Malenka, 2008; Singer, 2009) we found only a single study designed to determine whether stress exposure therapy induces structural changes in corticolimbic brain circuits (Lindauer et al., 2008). Stress exposure therapy effects were not detected in this study but other forms of learning are known to induce structural changes in the brain (Gaser and Schlaug, 2003; Draganski et al., 2004; Zatorre et al., 2012). Remarkably, these changes can occur rapidly as only a few min of practicing a whole-body balancing task increases gray matter volumes in frontal and parietal brain regions with gray matter expansion maintained for up to several weeks (Taubert et al., 2010).

Experience-dependent brain changes have also been reported for white matter tissue determined by diffusion tensor imaging (Bengtsson et al., 2005; Scholz et al., 2009). Diffusion tensor imaging measures diffusion-driven displacements of water molecules. Water diffusion is less restricted in the direction of white matter axon bundles than in the perpendicular direction, and measures of this anisotropy can be used to characterize microstructural properties that are sensitive to alterations in white matter myelination and axonal integrity (Le Bihan, 2003, 2006). As an insulator of axons, myelin modifies nerve conduction velocities and, in turn, increases or decreases the functional synchrony of organized neural networks (Szeligo and Leblond, 1977; Salami et al., 2003; Yamazaki et al., 2010). Future investigations of brain changes induced by stress exposure therapy may therefore benefit by combining functional and structural brain imaging modalities to determine whether functional outcomes reflect structural changes and vice versa.

## LIMITATIONS

All studies have their limitations and our analysis of stress coping-induced brain changes is no exception. Each of the 15 studies reviewed here exposed patients to stressful conditions that, on average, diminished subsequent measures of anxiety. Coping in the context of stress exposure therapy was further promoted in several of the studies by professional training to enhance cognitive restructuring, relaxation, guided mastery, and diverse forms of psychoeducation. The extent to which specific training procedures modify the neurobiological effects of concomitant exposure to stress remains to be determined.

Additional studies are needed to assess whether stress coping-induced brain changes spontaneously occur in healthy humans as well as patients with disorders beyond those considered here. Nine of the 15 studies examined patients with specific phobias

and the remaining six studies examined patients with PTSD. Seven of the nine studies of phobias focused on fear of spiders. Despite the potentially promising extension of principles derived from exposure therapy to explain empirically supported treatments for depression (Tryon and Misurell, 2008), we failed to find a published study of exposure therapy-related brain changes in this patient population.

Sample sizes are limited in all 15 studies and high dropout rates are prevalent in the studies of PTSD. Additionally, some patients with PTSD were comorbid for depression and/or were taking medications. Several of the studies compared brain imaging data from patients scanned both before and after exposure therapy with data acquired during single scan sessions for healthy controls. Patient post-treatment comparisons with controls are therefore confounded with potential test/re-test effects. The various brain imaging modalities considered in our review differ in their strengths and limitations as summarized above. The simultaneous application and integration of diverse brain imaging modalities within a single study may provide a more comprehensive understanding of stress coping-induced changes in the brain.

## SUMMARY AND CONCLUSIONS

In summary, the literature on brain changes induced by learning as an aspect of coping in the context of stress exposure therapy highlights functional neuroadaptations in brain regions that mediate emotion regulation and resilience. Corresponding structural brain changes and the duration, frequency, and timing of stress exposure required to modify brain functions now remain to be elucidated in detail. Such studies will provide mechanistic insights for the development of new interventions that enhance the adaptive aspects of coping with stress. The neuroscience of coping is an untapped resource for the development of new interventions because little is known about the neurobiology of learning to cope with stress.

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