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# STRESS AND STEROID REGULATION OF SYNAPTIC TRANSMISSION: FROM PHYSIOLOGY TO PATHOPHYSIOLOGY

Topic Editors
Menahem Segal, Harmen J. Krugers and Nicola Maggio

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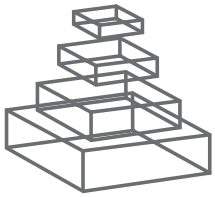

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# STRESS AND STEROID REGULATION OF SYNAPTIC TRANSMISSION: FROM PHYSIOLOGY TO PATHOPHYSIOLOGY

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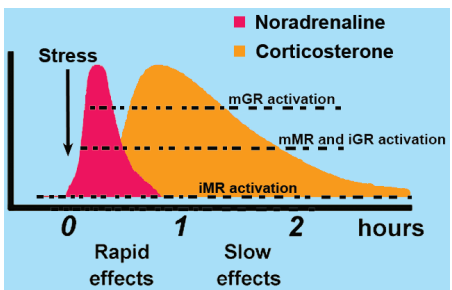


Recently, several pioneering discoveries have identified new roles of stress and steroid hormones in modulating CNS functions. Specifically, glucocorticoids, mineralocorticoids, sex hormones and neurosteroids have been shown to affect synaptic receptors and ion channels and therefore regulate in a complex manner physiological processes ranging from homeostatic to cognitive functions. Likewise, in some disorders of the nervous system, steroid hormones have been shown to play different roles: either favoring or combating the disease process.

In this Frontier Research Topic, we have put together leaders in the field to provide novel opinions on the effects of steroid hormones on synaptic transmission and plasticity from ion channels to pathophysiological processes. We expect critical reviews of the work that has been conducted recently in this area and enrich these discussions with the novel, exciting new findings.

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# Stress and steroid regulation of synaptic transmission: from physiology to pathophysiology

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Upon exposure to stressful experiences, steroid hormones, neurotransmitters, and neuromodulators are released which modulate specific processes in the brain. While the release of these compounds is believed to promote behavioral adaptation to stressful experiences, they have also been implicated in stress-related psychopathology. Extensive research in the past decade has culminated in a deeper understanding of the cellular and molecular mechanisms of how stress hormones, neurotransmitters, and neuromodulators, alone and in concert, affect the brain. This new multidisciplinary approach involving behavioral, electrophysiological, molecular, and epigenetic studies is used to elucidate the long-lasting complex effects of stress on cognitive functions in the brain. The target for the action of these mediators ranges from membrane receptors to nuclear receptors, often specific for different brain areas, affecting eventually homeostatic and various cognitive functions.

In this Frontier Research Topic, we have put together chapters written by leaders in the field that provide up-to-date summaries of the different angles of work on the effects of steroid hormones, neurotransmitters, and neuromodulators on synaptic transmission and plasticity from ion channels to pathophysiological processes. The different chapters deal with epigenetics (Hunter, 2012), which details the different nuclear targets for the long-term effects of stress. Mody and Maguire (2012) discuss the role of GABA in the feedback regulation of steroid action, Levy and Tasker summarize the current knowledge on the regulation of the HPA axis (Levy and Tasker, 2012). The main section of the Frontier Topic involves novel views on postsynaptic effects of steroid hormones, CRH, and noradrenaline on synaptic functions in the brain. These include a section on amygdala-hippocampus interaction (Li and Richter-Levin, 2012), cellular, and molecular studies on CRH effects in the hippocampus (Chen et al., 2012), effects of early life stress on metabolic functions in the brain (Bock et al., 2012), interactions between noradrenaline and corticosterone on brain function (Krugers et al., 2012), region selective effects of corticosterone in the hippocampus (Maggio and Segal, 2012), and finally, effects of corticosterone on NMDA receptor function in the hippocampus (Tse et al., 2012). Finally, a behavioral study on the interaction between gestational and adult stress (Walf and Frye, 2012) concludes the list.

Altogether, these papers provide state-of-the-art insights how stress determines cellular and network function and ultimately how stress affects cognition and emotion in the brain, a subject of increasing importance in modern society.

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# Epigenetic effects of stress and corticosteroids in the brain

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Stress is a common life event with potentially long lasting effects on health and behavior. Stress, and the corticosteroid hormones that mediate many of its effects, are well known for their ability to alter brain function and plasticity. While genetic susceptibility may influence the impact of stress on the brain, it does not provide us with a complete understanding of the capacity of stress to produce long lasting perturbations on the brain and behavior. The growing science of epigenetics, however, shows great promise of deepening our understanding of the persistent impacts of stress and corticosteroids on health and disease. Epigenetics, broadly defined, refers to influences on phenotype operating above the level of the genetic code itself. At the molecular level, epigenetic events belong to three major classes: DNA methylation, covalent histone modification and non-coding RNA. This review will examine the bi-directional interactions between stress and corticosteroids and epigenetic mechanisms in the brain and how the novel insights, gleaned from recent research in neuro-epigenetics, change our understanding of mammalian brain function and human disease states.

**Keywords: epigenetics, stress, corticosteroids, glucocorticoid receptor, brain development**

## INTRODUCTION

Epigenetics, in the sense the term was originally coined by Waddington (Waddington, 1942), referred to the "interactions between genes and their products which bring the phenotype into being." Like the concept of the gene itself, much has changed with regard to epigenetics since the 1940s. At present the term refers to molecular or cellular alterations, which influence gene expression, and by extension physiology and behavior, without causing alterations to the DNA sequence itself. These alterations are generally construed to include DNA methylation, non-coding RNAs and covalent histone modifications or "marks," which include acetylation, phosphorylation, methylation, ubiquitination, and a growing host of ever more exotic moieties. These marks are written by a variety of enzymes, which interact in complex ways to alter chromatin structure and the availability of the underlying DNA for interactions with the transcriptional machinery. Epigenetic mechanisms, unlike those of the relatively static genome, are more dynamic, tissue specific and significantly from the perspective of disease, potentially *reversible*.

Epigenetic processes are active in the brain and have been linked to an increasing number of brain disorders such as Fragile X and Rett syndromes, Huntington's disease, drug abuse, schizophrenia and affective disorders (Jiang et al., 2008). Epigenetic modifications have long been thought to be involved in learning and memory, e.g., (Schmitt and Matthies, 1979), but only in the past few years have the mechanisms begun to be outlined in detail. It has also become apparent that both corticosteroids and stress have a pronounced epigenetic impact in both humans and animal models and that the relationship between the stress response and epigenetics in the brain is bidirectional. In keeping with Waddington's developmental definition of epigenetics, it is also apparent that stress and epigenetics interact selectively at a number of important neuro-developmental critical periods to influence brain and behavior not only across individual life spans but across generations as well.

## EPIGENETIC MECHANISMS

### DNA MODIFICATIONS

DNA methylation of cytosines adjacent guanines (CpG sites) is a major epigenetic mark. CpG islands, which are regions of the genome with a high concentration of CpG pairs are often located within the promoter or enhancer regions of genes. Cytosine methylation is typically a silencing mark, thus increased methylation of promoter CpG islands often reduces gene expression, while hypomethylation is usually associated with increased expression (Illingworth and Bird, 2009). DNA methylation is established developmentally, typically via DNMT3a and b and maintained throughout the lifespan of a cell, often by DNMT1. Demethylation is less well described, but it is clear that it occurs, often quite dynamically in the brain and elsewhere. It is worth noting here that the glucocorticoid receptor (GR) is known to regulate local methylation around the glucocorticoid response elements where it binds DNA (Turner et al., 2010) (see **Figure 1**). Other cytosine modifications, such as hydroxymethylation, have recently been observed in brain tissues (Kriaucionis and Heintz, 2009; Wu and Zhang, 2011) and there is evidence that the Ten-eleven translocation (TET) proteins, which act on methylcytosine to produce hydroxymethylcytosine, may be part of the de-methylation pathway, though their activity in brain and their epigenetic significance remain unclear.

### HISTONE MODIFICATIONS

Histone proteins package DNA into chromatin, which may be either tightly packed and transcriptionally silent heterochromatin, or more open and actively transcribed euchromatin. The four core histone proteins, H2A, H2B, H3, and H4 form octomers

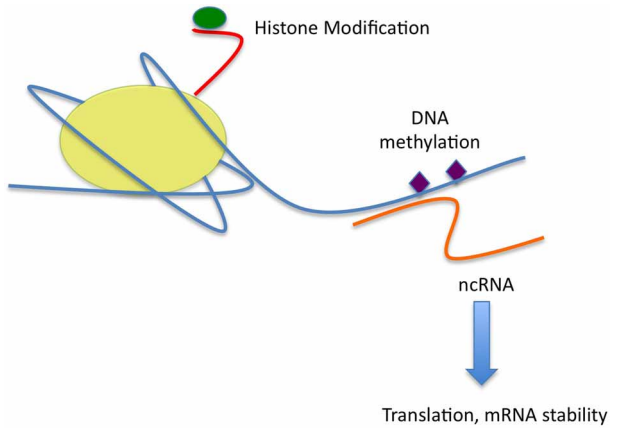


**FIGURE 1 | Figure one is a representation of the effects of stress on the three main epigenetic mechanisms as presently understood.** Stress may act to alter modifications (green) of the tails (red) of the core histone proteins of the nucleosome (yellow). Some modifications, such as acetylation, or histone 3K4 trimethylation, are associated with a loose, euchromatic state and active gene transcription. Others, such as Histone H3K9 or K27 trimethylation, are associated with dense heterochromatin and gene silencing or repressed transcription. DNA methylation (purple) is commonly associated with transcriptional repression, the function of more exotic DNA modifications, such as cytosine hydroxymethylation is a subject of intense interest, but as yet unresolved. Non-coding RNA species (orange), such as microRNAs, may alter gene transcription as well, but have effects post-transcriptionally on both mRNA stability and translation into protein.

around which DNA is wrapped. Each of the core histones has a relatively unstructured N-terminal tail which may be covalently modified at a number of residues (while the tail is the focus of much work on histone modification it should be said that covalent modifications of core of the protein are possible) (see **Figure 1**). The number of described histone modifications or "marks" is quite large (Allis et al., 2007), but thus far those which have been subject to the most examination at the level of the nervous system are histone acetylation, methylation and phosphorylation. Acetylation of histones is typically associated with a transcriptionally active state. Acetylation of histone lysine residues is achieved by histone acetyl-transferases or HATs, and the mark is erased by histone de-acetylases or HDACs. HATs and HDACs are relatively non-specific as to the specific residue they modify relative to the histone methyltransferases (HMTs) and histone de-methylases (HDMs), which tend to be both residue and valence specific. Histones lysine and arginine residues, the later may be mono- di- or tri-methylated, while the former may be mono- or di-methylated (Allis et al., 2007; Kouzarides, 2007). Each valence may be associated with a different functional state in the local chromatin, e.g., trimethylation of histone H3 lysine 4 (H3K4me3) is often associated with active gene expression, H4K20me1 is also associated with active gene expression but H4K20me3 is associated with silencing, as are H3K9me3 and H3K27me3 (Kouzarides, 2007; Ruthenburg et al., 2007a; Balakrishnan and Milavetz, 2010). Phosphorylation of histones, most often at serine residues, is controlled by a number of kinases and phosphatases, which are often involved in other cellular processes as well. Histone phosphorylations are associated with transcriptional regulation, mitotic check points and DNA damage, as well as cross talk with other histone marks (Banerjee and Chakravarti, 2011). Though histone marks were initially envisioned to comprise a relatively simple code (Jenuwein and Allis, 2001), it is apparent now that the combinatorial state of histone marks on a histone tail may be as, or more, important than the contributions of any one mark (Ruthenburg et al., 2007b).

### NON-CODING RNA

Non-coding or ncRNA is another contributor to the stock of epigenetic mechanisms that may be at play in the brain. A variety of different functional types of RNA fall under the rubric of ncRNA, the best known being microRNAs (miRNA), which regulate mRNA levels by a variety of means (see **Figure 1**). Of course, there are a variety of other short ncRNA species and long non-coding RNA (lncRNA, ncRNA longer than 200 bases), which plays a number of roles in regulating gene activity and chromatin structure as well. To date miRNAs are best established as epigenetic factors in the nervous system. However, both lncRNAs and snoRNAs, which are involved in the processing of ribosomal RNA, have been implicated in disorders such as Alzheimer's disease and Prader–Willi syndrome (Esteller, 2011) and it is likely that novel species of ncRNA will be linked to nervous system function and dysfunction with greater and greater frequency in coming years, as they have in cancer research, which historically, has been closer to the leading edge of epigenetics than the neurosciences.

## EPIGENETIC EFFECTS STRESS AND CORTICOSTEROIDS IN DEVELOPMENT

It has been known for some time that stressful manipulations in early life contribute to changes in stress reactivity that persist into adulthood. More severe interventions like maternal separation (MS) having a sensitizing effect on the stress axis and milder ones such as neonatal handling promoting a more resilient phenotype (Francis and Meaney, 1999). Many of these manipulations alter the expression of the GR or other stress responsive genes such as AVP, CRH or BDNF. One of the most interesting examples of the contribution of early environment to epigenetic alterations in stress responsiveness is the effect of natural variations in maternal nursing, licking and grooming behavior on the behavioral and endocrine responses to stress in adult offspring described in a series of papers produced in collaboration between the Meaney and Szyf labs at McGill University. Meaney established that variations in arch backed nursing (ABN) and licking and grooming (LG) were stable within individual mothers and that these correlated with behavioral differences in their adult offspring (Champagne et al., 2003). Further, they demonstrated that high LG-ABN mothers produced offspring with higher hippocampal GR and lower levels of hypothalamic CRH and consequently lower HPA activation in response to stress (Liu et al., 1997; Francis et al., 1999). Remarkably, these effects persisted across generations in a non-genomic fashion, prompting some to wonder if a partial rehabilitation of Lamarck was in order.

The mechanism by which these epigenetic effects were transmitted was first described in detail in a paper by Weaver (Weaver et al., 2004). He found that the adult offspring of low LG-ABN

mothers showed higher levels of DNA methylation of the GR 1–7 promoter (corresponding to the human 1-F promoter) (Turner and Muller, 2005) in the hippocampus than those of High LG-ABN mothers. Cross fostering of pups to high LG-ABN dams was found to reverse the change in the methylation of this region, demonstrating that the epigenetic change was responsive to maternal input. The methylation was most strongly targeted to the NGFI-A (also known as *egr*1) response element within the GR 1–7 promoter, and chromatin immunoprecipitation (ChIP) studies in 6 day old pups revealed lower NGFI-A binding in this region in low LG-ABN pups than in high, and this difference does persist into adulthood, though hippocampal levels of NGFI-A are the same. In addition to lower DNA methylation, high LG-ABN offspring showed higher levels of histone acetylation at the GR 1–7 promoter suggesting that there is a persistent epigenetic marking of this locus during the neonatal critical period (Weaver et al., 2004, 2007). As histone de-acetylase inhibitors (HDACi) can both increase histone acetylation and reduce DNA methylation (Cervoni and Szyf, 2001), Weaver infused the HDACi trichostatin A into the brains of adult rats and successfully reversed both the deficits in hippocampal GR expression and HPA stress reactivity found in low maternal LG-ABN animals (Weaver et al., 2004), thus demonstrating for the first time the potential *reversibility* of the epigenetic consequences of stress. Provocatively, McGowan and collaborators found that similar alterations occurred in suicides with a history of childhood abuse (McGowan et al., 2009), though other groups have failed to replicate this finding in major depressives (Alt et al., 2010) it remains highly interesting.

The MS model of early life stress, where pups are separated from their dam for several hours a day during the first two weeks of life, has also been demonstrated to have an epigenetic impact. MS, like low LG-ABN rearing, produces HPA hyperactivity in adults, partly by increasing expression of the adrenocorticotrophin (ACTH) gene, *pomc*, in the anterior pituitary. As ACTH release is driven by the release of AVP and CRH from the hypothalamus into the pituitary portal circulation, Murgatroyd and collaborators examined the effect of MS on AVP and CRH gene expression. They found that MS increased AVP, but not CRH expression in the hypothalamus, and that the increase was associated with DNA hypomethylation in an AVP enhancer region which appears to be the major binding site for the methyl CpG-binding domain protein MeCP2 (Murgatroyd et al., 2009). Notably, MeCP2 is also associated with the primary pathology of Rett syndrome (Kriaucionis and Bird, 2003), and to play a role in the regulation of the expression of stress responsive genes such as BDNF (Chen et al., 2003; Martinowich et al., 2003). It has also been demonstrated that MS, like the LG-ABN model can produce transgenerational epigenetic effects, though, in contrast to the later model, some of the changes in DNA methylation appear to be passed on in the male germ line (Franklin et al., 2010). Another early life stress model, using stressed and abusive dams, showed that the pups reared under these conditions showed reduced levels of BDNF expression in the prefrontal cortex, which correlated with DNA hypermethylation at the activity dependent exon IV promoter. The investigators were able to reverse this effect by infusing the DNA methylation inhibitor zebularine (Roth et al., 2009).

Prenatal stress exposure also has an impact on stress reactivity in adulthood. Chronic exposures produce exaggerated corticosterone responses to stress and a number of deficits in hippocampal structure and function (Fujioka et al., 1999; Lemaire et al., 2000; Coe et al., 2003), while milder exposures produce a more resilient phenotype, resembling neonatal handling (Fujioka et al., 2001, 2006). Mueller and Bale have recently shown that prenatal stress results in increased DNA methylation at the GR 1–7 promoter in the hippocampus and reduced methylation at the CRH promoter in the hypothalamus and central amygdala of adult male animals with corresponding alterations in gene expression. Similar changes were not observed in females, and the sex difference correlates with differences in the expression of a number of genes in the placenta, including the DNA methyltransferases DNMT1 (Mueller and Bale, 2008). Some of the epigenetic effects of prenatal stress in this model were passed on to the F2 generation via a mechanism that appears to involved the expression of miRNAs which target the β-glycan gene (Morgan and Bale, 2011). This result is particularly worthy of note not only because a similar process appears to occur in humans (Oberlander et al., 2008), but because it suggests that at least some behavioral and cognitive sex differences may have and epigenetic rather than a genetic origin. That the effects of prenatal stress may be reversible via post-natal handling (Lemaire et al., 2006) speaks to the possibility that structured behavioral interventions, e.g., (DiCorcia and Tronick, 2011) may have significant translational utility in reducing the incidence of adult mental health issues, many of which show significant associations with maternal stress and depression (Talge et al., 2007; Brand and Brennan, 2009).

## EPIGENETIC EFFECTS OF STRESS DURING ADULTHOOD

The hippocampus has received much attention both from researchers who study stress as well as those who study learning and memory. In animal models it is in fact quite difficult to discern the difference between learning and memory tasks like the Morris water maze or fear conditioning, and acute stressors such as the forced swim test, see for instance (Trollope et al., 2012). As a perusal of the Allen Brain atlas will show, the hippocampus shows high expression levels for a large number of epigenetic enzymes, so it is unsurprising that both stress and memory formation have been shown to utilize epigenetic mechanisms at the level of the hippocampus.

Fear conditioning is associated with a variety of short and long-term epigenetic changes. Miller and Sweatt showed that fear conditioning causes increased expression of the DNA methyltransferases DNMT3A and DNMT3B and that the inhibition of these enzymes impaired the consolidation of fear memories (Miller and Sweatt, 2007). Further, they found that fear conditioning altered DNA methylation on the *reelin* and *PP1* genes, both of which have an influence on memory in other models (Miller and Sweatt, 2007), as well as methylation of the BDNF gene (Lubin et al., 2008). PP1 is notable in that one of its activities seems to be removing phosphorylations from histone H3 at serine 10, and that this seems to be the basis for its role in long-term memory (Koshibu et al., 2009, 2011). Another series of studies established a role for histone acetylation in both long-term recall of fear conditioning and spatial memory. These studies

began with the observation that environmental enrichment (EE), which helped to rescue memory deficits in and inducible neurodegenerative mouse model (CK-p25), also increased levels of histone acetylation in the brain. Utilizing HDAC inhibitors alone the investigators were able to replicate the effects of EE on memory and demonstrate an increase in synapse formation as well (Fischer et al., 2007). A subsequent study established that HDAC2 was the major neuronal class I HDAC and the HDAC responsible for modulating memory and synaptic plasticity, via a surprisingly select number of genes, including glutamate receptor subunits and BDNF (Guan et al., 2009), the later observation, replicating the work of Bredy (Bredy et al., 2007). These findings provide the outlines of a complex set of interactions between memory, stress, or fear, a number of different epigenetic actors and long-term plasticity of the brain and behavior.

With regard to explicit examinations of the effects of stress upon epigenetic modifications in the brain one of the earliest findings was that of Bilang-Bleuel, who found that forced swim stress produced a significant increase in phospho-acetylation of Histone H3, at serine 10 and lysine 14 (H3S10p-K14ac) respectively, in the dentate gyrus of the hippocampal formation (Bilang-Bleuel et al., 2005). This combination of histone marks is associated with a transcriptionally active chromatin state (Cheung et al., 2000; Clayton et al., 2000), and had been previously observed in the brain after treatment with a variety of neurotransmitter receptor agonists (Crosio et al., 2003). Work building on this initial finding established that a similar induction was produced by novelty stress and that in both cases the phenomenon was *N*-Methyl-D-aspartate (NMDA) receptor dependent, and associated with c-Fos induction in the same cells which showed the H3S10p-K14ac signal (Chandramohan et al., 2007, 2008). Another study demonstrated that inhibitory input from GABAergic neurons acted as a break on the up-regulation of H3S10p-K14ac (Papadopoulos et al., 2011). Given the long association between the actions of glucocorticoid and glutamatergic signaling in the effects of stress on the hippocampal formation, e.g., (McEwen, 1999), it is encouraging that the H3S10p-K14ac story appears to require both actors, though through the novel intermediaries of Elk-1 and MSK1. GR appears to interact directly with these proteins, promoting their phosporylation via NMDA receptor activation of the ERK-MAPK pathway and that this activation plays a role in the formation of the memory of the event (Reul et al., 2009; Gutierrez-Mecinas et al., 2011). Voluntary exercise, which is typically protective against the negative sequelae of stress, actually increases the levels of H3S10p-K14ac after both novelty and swim stress, suggesting that this may be part of an adaptive stress response rather than a pathological one (Collins et al., 2009).

Social defeat stress, which represents one of the stronger models of human depression in terms of ethological and face validity (Nestler and Hyman, 2010), has a clear epigenetic component as well, as was first demonstrated in the Nestler laboratory (Tsankova et al., 2006). They found that chronic social defeat profoundly increased the levels of the repressive histone mark H3 lysine 27 dimethyl at promoter regions of the BDNF gene, while treatment with antidepressants produced and increase in activating marks such as histone H3 acetylation and histone H3 lysine four dimethylation (Tsankova et al., 2004, 2006). Subsequent studies found associations between chronic cocaine and social stress and HDAC5 (Renthal et al., 2007), as well as an antidepressant effect of HDAC2 in the social defeat model (Covington et al., 2009), the latter of particular interest given its importance in memory and the effects of EE mentioned above. Chronic social defeat also induced DNMT3a expression in the accumbens, while chronic cocaine reduced it (LaPlant et al., 2010). In the paraventricular nucleus of the hypothalamus resilience to social stress has been found to correlate with the DNA methylation status of the CRH gene (Elliott et al., 2010), further evidence that DNA methylation also plays a role in stress and stress resilience. The Nestler group also found connections between anti-depressant activity, resilience to social defeat and changes in the repressive histone H3 lysine 9 and 27 methylations in the nucleus accumbens (Wilkinson et al., 2009; Covington et al., 2011). The H3K9 di-or tri-methyl mark has also been shown to change in response to cocaine administration in the accumbens (Maze et al., 2010) and increase in response to acute restraint stress or chronic fluoxetine in the hippocampus (Hunter et al., 2009). The later study also demonstrated stress dependent changes in both the H3K4me3 and H3K27me3 marks. The fact that both cocaine and stress effect the same marks follows from the link between cocaine's reinforcing effects and corticosteroids (Piazza et al., 1991). Thus, interventions that increase levels of H3K9 di-or tri-methyl in the limbic system, appear to promote resilience to stress and depression like behavior in animal models. Indeed, the methyl donor SAMe has been shown to have anti-depressant effects in humans (Miller, 2008), though whether this is due to an effect on epigenetic modifications is not yet clear.

The effects of stress on non-coding RNA activity and the regulation of the stress axis by ncRNA in the brain, have received less attention than DNA methylation and histone modification, but the few studies thus far completed demonstrate that the epigenetic actions of RNA are also likely to be a significant part of the effects of stress upon the brain. The GR is the target of a number of miRNAs (Turner et al., 2010). Uchida was the first to observe that the miRNA, miR-18a was involved in region and strain specific regulation of GR expression and stress responsiveness in Fischer 344 rats (Uchida et al., 2008). Another miRNA, miR-124a was soon added to the list of negative regulators of GR expression (Vreugdenhil et al., 2009). Both acute and chronic stress have been shown to regulate the expression of miR-134 and miR-183 in the hippocampus and amygdala and these miRNAs in turn regulate the splicing of acetylcholinesterase, and may thus fine tune the activity of the cholinergic system in response to stress (Meerson et al., 2010). Acute and chronic stress also appear to regulate miR-34 in the amygdala, where it reduces anxiety by reducing the expression of the CRHR1 receptor (Haramati et al., 2011). Acute stress selectively regulates let-7a, miR-9 and miR 26-a/b in the frontal cortex, but not the hippocampus of mice (Rinaldi et al., 2010). Regionally and temporally specific regulation of miR-186 and miR 709 was found in the hippocampus, prefrontal cortex and cerebellum of rats stressed for either 2 or 4 weeks and miR-186 were found to regulate the expression of the *Eps-15* gene (Babenko et al., 2012). Mongrain found that a sleep deprivation stress caused significant changes in the expression

of 10 miRNAs in the mouse brain, as seven of these did not change in adrenalectomized mice, it is probable they are regulated by corticosteroids (Mongrain et al., 2010). While the relations of whole classes of ncRNA's to stress and the stress axis remain to be explored, it can be said that ncRNA has a clear relation to the epigenetic tuning of the stress response and will likely provide a novel avenue to understanding stress and its associated pathologies.

## CONCLUSIONS

Though the study of the interactions between the stress axis and the epigenome remains in its early stages, its promise is already evident. It is already clear that stressful interactions with the environment induce regionally and developmentally specific changes in behavior and in brain structure and function. It is also apparent that many of these changes are potentially reversible via environmental or pharmacologic interventions.

As most of the studies rehearsed here have focused on regional changes in epigenetic marks, in no small part due to the technical difficulties involved with ChIP in small tissue samples, future studies examining sub-regional differences known to be behaviorally significant, such as comparisons between the accumbens shell and core or the dorsal and ventral hippocampus will be highly important for the integration of epigenetics with existing knowledge of functional neuroanatomy and behavior. A few groups have shown the way in this regard, notably (Roth et al., 2011), who found that chronic psychosocial stress after predator exposure, a model of PTSD, produced a significant *increase* in DNA methylation of the BDNF gene in the dorsal hippocampus while a *decrease* was seen in the ventral sub-region. As these two regions are known to be not only functionally distinct, but distinct in terms of gene expression phenotype (Fanselow and Dong, 2010), this finding is of great interest, and points the way for future work on the functional epigenetics of stress.

While it is well established that perinatal insults and abuse and neglect during childhood have an impact on susceptibility to neuropsychiatric disease, and higher order (i.e., non-molecular) epigenetic processes are implicated in that susceptibility. We have only just begun to understand how these influences operate at the level of molecular epigenetics. This is particularly true of the developmental period where many disorders first appear, that is adolescence (Veenema, 2009). To date, no studies of which this author is aware have examined molecular epigenetic mechanisms in the context of adolescent stress, and this is a situation which will hopefully soon be remedied. In addition, studies which examine the interaction of stress in earlier life with behavior during adolescence would be highly desirable.

While the usual suspects in stress research, GR, BDNF CRH, etc., have received justified attention as actors in the epigenetics of stress, it is evident that these cannot be the only players upon the stage. For example, the mineralocorticoid receptor, which has been implicated in altered histone methylation in the kidney (Zhang et al., 2009), has not been examined in the brain in any such context, despite its role in HPA feedback, hippocampal function and anxiety (Kolber et al., 2008). A benefit, in this regard, of emerging next-generation sequencing technologies is their genome wide reach, attentive researchers will be able to discover entirely new classes of genes mediating the response to stress in various brain regions. Further, we can now look beyond the genes themselves to the other 95% of the genome, 90% of which may be actively transcribed ENCODE Project Consortium (2004). This would suggest that the range of potential sites for epigenetic action is an order of magnitude greater than was foreseeable 10 years ago.

For epigenetic research to be translatable, epigenetic pharmacology will have to improve. Though a variety of histone deacetylase inhibitors are available and already approved as drugs for human use, drugs to alter histone methylation are fewer in number, though this situation appears to be changing for the better, e.g., (Spannhoff et al., 2009). As to other histone modifications, most of which have not been examined in the context of stress or mental disorders, still less is known. The various mechanisms and functions of ncRNA are not clear enough as yet for small molecule inhibitors to be examined systematically, though some work has begun with regard to microRNAs (Connelly et al., 2012). It is to be hoped that this foundation will continue to expand so that epigenetic science can fulfill in the clinic the promise it has thus far shown at the bench.

Many open questions remain, particularly with regard to cross talk between epigenetic actors and their interactions with neurotransmitter systems and intracellular signaling cascades. Precisely how epigenetic marks, which have been revealed to be quite dynamic in recent years, maintain stability and specificity over time is another question that deserves exploration. As next-generation sequencing technology improves and cellular resolution epigenetic analyses become more practicable our understanding will become more complex and the potential for novel therapeutic interventions in stress related diseases will be realized.

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# The reciprocal regulation of stress hormones and $GABA_A$ receptors

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Stress-derived steroid hormones regulate the expression and function of $GABA_A$ receptors ($GABA_A$Rs). Changes in $GABA_A$R subunit expression have been demonstrated under conditions of altered steroid hormone levels, such as stress, as well as following exogenous steroid hormone administration. In addition to the effects of stress-derived steroid hormones on $GABA_A$R subunit expression, stress hormones can also be metabolized to neuroactive derivatives which can alter the function of $GABA_A$Rs. Neurosteroids allosterically modulate $GABA_A$Rs at concentrations comparable to those during stress. In addition to the actions of stress-derived steroid hormones on $GABA_A$Rs, $GABA_A$Rs reciprocally regulate the production of stress hormones. The stress response is mediated by the hypothalamic-pituitary-adrenal (HPA) axis, the activity of which is governed by corticotropin releasing hormone (CRH) neurons. The activity of CRH neurons is largely controlled by robust GABAergic inhibition. Recently, it has been demonstrated that CRH neurons are regulated by neurosteroid-sensitive, $GABA_A$R δ subunit-containing receptors representing a novel feedback mechanism onto the HPA axis. Further, it has been demonstrated that neurosteroidogenesis and neurosteroid actions on $GABA_A$R δ subunit-containing receptors on CRH neurons are necessary to mount the physiological response to stress. Here we review the literature describing the effects of steroid hormones on $GABA_A$Rs as well as the importance of $GABA_A$Rs in regulating the production of steroid hormones. This review incorporates what we currently know about changes in $GABA_A$Rs following stress and the role in HPA axis regulation.

**Keywords: GABA, stress, inhibition, corticosterone, CRH**

$GABA_A$Rs are regulated by stress-derived steroid hormones and neurosteroids [for review see Belelli et al. (2009); Maguire and Mody (2009); Gunn et al. (2011)]. Conversely, the HPA axis, and thus the production of stress-derived steroid hormones and neurosteroids, is under robust GABAergic control [for review see Herman et al. (2004); Gunn et al. (2011)].

## GABAERGIC REGULATION OF THE HPA AXIS

Stress induces a physiological response which is mediated by the HPA axis. CRH is released from the hypothalamus and acts in the pituitary to signal the release of adrenocorticotropic hormone (ACTH), which triggers the release of cortisol from the adrenal gland in humans (corticosterone in mice). The HPA axis is regulated by inputs from numerous different brain regions, involving multiple neurotransmitter systems, as well as the feedback of steroid hormones acting on mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) [for review see Herman et al. (2003); Larsen et al. (2003); Ulrich-Lai and Herman (2009)]. These inputs impinge on CRH neurons in the paraventricular nucleus (PVN), which mediate the output of the HPA axis. Although CRH neurons receive a wide variety of inputs from diverse brain regions, their activity is ultimately regulated by GABAergic inhibition [for review see Decavel and van den Pol (1990); Herman et al. (2004)].

A role for GABA in HPA axis regulation has been well established. CRH neurons receive robust GABAergic inhibition (Decavel and van den Pol, 1990, 1992) [for review see Herman et al. (2004); Cullinan et al. (2008)]. It has been suggested that a third of the inputs onto CRH neurons are GABAergic and the density of GABAergic synapses in the parvocellular division of the PVN has been estimated to be above $20 \times 10^6$ synaptic contacts per $mm^3$ (Miklos and Kovacs, 2002), highlighting the importance of GABAergic inhibition in the regulation of CRH neurons. In addition, microinjection of GABA antagonists, such as bicuculline, into the PVN activates the HPA axis (Cullinan et al., 2008; Marques de and Franci, 2008) and microinfusion of GABA agonists, such as the stress-derived neurosteroid, THDOC, into the PVN decreases circulating levels of stress hormones (Sarkar et al., 2011).

GABA inputs onto CRH neurons originate primarily from local interneurons surrounding the PVN (peri-PVN) as well as from the subparaventricular zone, the anterior hypothalamic area, dorsomedial hypothalamic nucleus, the medial preoptic area, lateral hypothalamic area, and from multiple nuclei within the bed nucleus of the stria terminalis (BNST) (Cullinan et al., 1993; Roland and Sawchenko, 1993) [for review see Herman et al. (2004); Cullinan et al. (2008)]. In addition to the direct inhibitory connections from these brain regions, CRH neurons also receive

indirect inhibition from other regulatory brain regions including limbic and cortical regions which exert their influences on CRH neurons via interneuron mediators [for review see Herman et al. (2004); Cullinan et al. (2008)].

Despite the well-established role for GABAergic control of the HPA axis at the level of the PVN, very little is known about the $GABA_AR$ subtypes which mediate the GABAergic control over CRH neurons. $GABA_ARs$ are members of the large "Cys-loop" super-family of evolutionarily related and structurally similar ligand-gated ion channels. To-date, 19 different subunits; α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3 have been identified (Barnard et al., 1998; Whiting et al., 1999), which form heteropentameric receptors predominantly composed of 2 αs, 2 βs, and either the γ2 or the δ subunit. Depending on their subunit composition, $GABA_ARs$ have specific anatomical distributions (Pirker et al., 2000) including subcellular localization (Kittler et al., 2002), kinetics, and pharmacology (Hevers and Luddens, 1998; Mody and Pearce, 2004). $GABA_ARs$ mediate two distinct forms of GABAergic inhibition, tonic, and phasic, which are mediated by $GABA_ARs$ with unique subunit assemblies (Farrant and Nusser, 2005). Extrasynaptically localized δ subunit-containing receptors mediate tonic GABAergic inhibition in many brain regions and confer neurosteroid sensitivity (Mihalek et al., 1999; Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002; Spigelman et al., 2003). Only recently has it been demonstrated that these neurosteroid-sensitive, δ subunit-containing $GABA_ARs$ play a pivotal role in the regulation of stress reactivity (Sarkar et al., 2011).

Several $GABA_AR$ subunits have been identified within the PVN (Fritschy and Mohler, 1995). However, it has been historically difficult to conclusively determine which $GABA_AR$ subunits are expressed on the CRH neurons within the PVN due to the inability to specifically identify this subset of neurons within this heterogeneous nucleus. Dual hybridization histochemical studies have demonstrated mRNA expression of the $GABA_AR$ α1, α2, β1-3, and γ1-2 subunits in CRH neurons (Cullinan, 2000). Due to the sparse number of studies that have attempted to identify the specific $GABA_AR$ subtypes controlling CRH neurons, this list remains incomplete. Information regarding the $GABA_AR$ subtypes involved in regulation of CRH neurons will provide insight into pharmacological tools which may modulate HPA axis activity. It has recently been demonstrated that rostral ventrolateral medulla (RVLM)-projecting parvocellular neurons in the PVN are regulated by a THIP-sensitive tonic current (Park et al., 2007), indicating that neurosteroid-sensitive, extrasynaptic δ subunit-containing $GABA_ARs$ may play a role in the regulation of these neurons (Boehm et al., 2006; Mortensen et al., 2010). Further, recent studies have demonstrated $GABA_AR$ δ subunit expression in the PVN and $GABA_AR$ δ subunit-mediated tonic GABAergic control of CRH neurons (Sarkar et al., 2011). These findings demonstrate that $GABA_AR$ δ subunit-containing receptors on CRH neurons play a role in the regulation of the HPA axis.

Stress-derived steroid hormones can be metabolized to neuroactive derivatives, termed neurosteroids, such as the stress-derived neurosteroid, 3α, 21-dihydroxy-5α-pregnan-20-one (THDOC), and the ovarian-derived neurosteroid, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone). Neurosteroids are positive allosteric modulators of $GABA_ARs$ (Barker et al., 1986; Majewska et al., 1986; Puia et al., 1990; Purdy et al., 1991; Lambert et al., 1995; Morrow et al., 1995; Hosie et al., 2006; Smith et al., 2007), acting on a neurosteroid binding site identified on $GABA_ARs$ (Hosie et al., 2006). It has been demonstrated that neurosteroids act preferentially on $GABA_AR$ δ subunit-containing receptors (Mihalek et al., 1999; Belelli et al., 2002; Brown et al., 2002; Wohlfarth et al., 2002; Spigelman et al., 2003) at physiologically relevant concentrations (Stell et al., 2003). These data are consistent with previous findings demonstrating changes in $GABA_AR$ δ subunit expression in parvocellular neurons in the PVN following stress (Verkuyl et al., 2004), implicating these receptors in the regulation of the stress response. In response to stress, THDOC and allopregnanolone are released at levels which can potently modulate $GABA_ARs$ (Barker et al., 1986; Majewska et al., 1986; Puia et al., 1990; Purdy et al., 1991; Lambert et al., 1995; Morrow et al., 1995; Barbaccia et al., 1996a,b; Hosie et al., 2006; Smith et al., 2007). Under basal conditions, neurosteroids can exert a negative feedback onto the HPA axis, decreasing CRH and ACTH levels (Patchev et al., 1994, 1996) [for review see Morrow (2007)]. Recent data demonstrate a role for neurosteroid actions on $GABA_AR$ δ subunit-containing receptors on CRH neurons in the regulation of the HPA axis (Sarkar et al., 2011), and thus, production of stress hormones. This study demonstrates a decrease in the firing rate of CRH neurons upon the addition of a low concentration of THDOC (10 nM) under basal conditions (Sarkar et al., 2011). Further, the role of the $GABA_AR$ δ subunit in the neurosteroid regulation of CRH neurons was confirmed by demonstrating the loss of this regulation in mice lacking the $GABA_AR$ δ subunit (*Gabrd*$^{-/-}$ mice). Together, there is ample evidence that under normal conditions, there is a basal GABAergic inhibition of CRH neurons.

Interestingly, the effects of GABA on CRH neurons are dramatically altered following stress. Stress activates GABAergic neurons which project to the PVN (Cullinan et al., 1995; Campeau and Watson, 1997), which would intuitively suggest inhibition of the HPA axis rather than activation. However, GABA agonists have been shown to increase stress-induced corticosterone levels (Borycz et al., 1992; Sarkar et al., 2011) and blocking production with finasteride has been shown to blunt the corticosterone response to stress (Sarkar et al., 2011). However, due to the fact that both THDOC and allopregnanolone levels are elevated following stress, it isn't clear which of these neurosteroids are responsible for activation of the HPA axis. The role of neurosteroids on $GABA_AR$ δ subunit-containing receptors in the activation of the HPA axis following stress (Sarkar et al., 2011), implicates excitatory actions of GABA in regulation of the HPA axis. Recent evidence suggests that there are deficits in GABAergic control of CRH neurons following stress due to a depolarizing shift in the reversal potential for chloride ($Cl^-$) (Hewitt et al., 2009). The inhibitory effects of GABA require the maintenance of the $Cl^-$ gradient, which is primarily accomplished by the $K^+/Cl^-$ co-transporter, KCC2, in the adult brain (Rivera et al., 1999; Payne et al., 2003; Rivera et al., 2005). The surface expression and activity of KCC2 is regulated by phosphorylation of KCC2 residue Ser940 (Lee et al., 2007). Dephosphorylation of KCC2 residue Ser940 and downregulation

of KCC2 results in depolarizing and excitatory actions of GABA *in vitro* (Lee et al., 2011). Recently, it has been demonstrated that KCC2 plays a role in the regulation of the HPA axis (Sarkar et al., 2011). Following stress, there is a dephosphorylation of KCC2 residue Ser940 and downregulation of surface KCC2 expression in the PVN (Sarkar et al., 2011), resulting in excitatory actions of GABA on CRH neurons (Sarkar et al., 2011). Consistent with excitatory actions of GABA on CRH neurons following stress, recent data demonstrate that following acute restraint stress, THDOC increases the activity of CRH neurons and increases the corticosterone response to stress (Sarkar et al., 2011). The GABA-mediated activation of CRH neurons following acute stress is due to a collapse in the chloride gradient as previously demonstrated (Hewitt et al., 2009) and depolarizing and excitatory actions of GABA (Sarkar et al., 2011), overriding the inhibitory constraint of CRH neurons. These data demonstrate dramatic alterations in GABAergic control of CRH neurons following stress mediated by neurosteroids rather than the actions of steroid hormones on MRs or GRs. We propose a model in which rapid dephosphorylation and downregulation of KCC2 is the most efficient mechanism to overcome the robust GABAergic constraint of CRH neurons to mount a rapid, all-or-none stress response (**Figure 1**) (Sarkar et al., 2011). This model suggests that both downregulation of KCC2, resulting in excitatory actions of GABA and neurosteroid potentiation of $GABA_AR$ δ subunit-containing receptors is required to mount the full physiological response to stress.

## STRESS HORMONE REGULATION OF GABAERGIC INHIBITION

In addition to the well-established role of GABAergic transmission in the regulation of the HPA axis as outlined above, conversely, stress hormones can also alter GABAergic inhibition. This review will focus on changes that occur in adulthood and will not discuss the vast literature documenting changes in GABAergic inhibition resulting from early life stress. For a more in-depth review of the role of neurosteroids in stress, including prenatal stress, see (Gunn et al., 2011).

Acute and chronic stress has been shown to alter the expression of both GAD and GABA (Yoneda et al., 1983; Otero Losada, 1988; Maroulakou and Stylianopoulou, 1991; Acosta et al., 1993; Bowers et al., 1998) [for review see Cullinan et al. (2008)]. Increased GAD65 and GAD67 expression have been demonstrated following stress in brain regions associated with the regulation of the HPA axis, including the anterior hypothalamic area, dorsomedial nucleus, medial preoptic area, suprachiasmatic nucleus, anterior BST, perifornical nucleus, and peri-PVN region [Bowers et al., 1998; for review see Cullinan et al. (2008)].

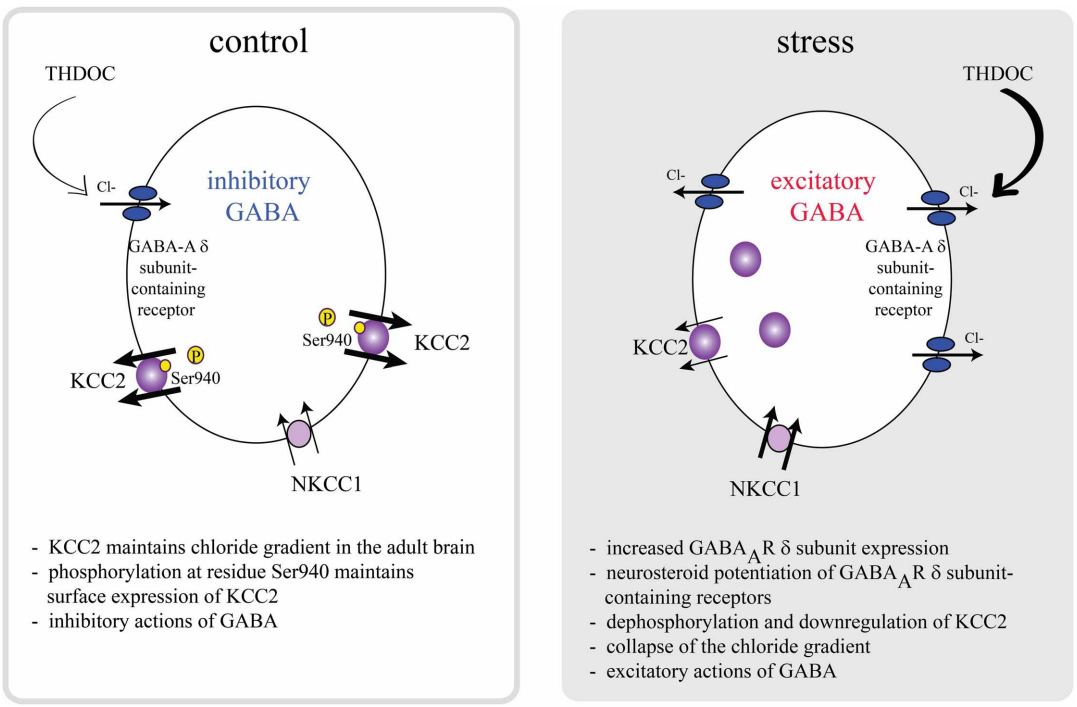


**FIGURE 1 | A model of HPA axis regulation.** The activity of the HPA axis is regulated by CRH neurons in the PVN, which are under robust GABAergic control. Under normal conditions, KCC2 is phosphorylated at residue Ser940, maintaining a low intracellular $Cl^-$ concentration and inhibitory effects of GABA. Further, these neurons are regulated by a neurosteroid-sensitive tonic GABAergic inhibition mediated by $GABA_AR$ δ subunit-containing receptors. Following stress, KCC2 residue Ser940 is dephosphorylated and surface KCC2 expression is downregulated, resulting in a collapse in the chloride gradient and excitatory actions of GABA on CRH neurons. Neurosteroid actions on $GABA_AR$ δ subunit-containing receptors following stress potentiate the excitatory actions of GABA on CRH neurons. Both the downregulation of KCC2 and excitatory effects of neurosteroids on $GABA_AR$ δ subunit-containing receptors are required to mount the full physiological response to stress in a rapid, all-or-none fashion [adapted from Sarkar et al. (2011)].

Despite the upregulation of enzymes responsible for GABA synthesis, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) has been shown to be decreased following stress (Verkuyl et al., 2004). Similarly, a high dose of exogenous corticosterone has been shown to decrease mIPSC frequency (Verkuyl et al., 2005) and adrenalectomy increases miniature inhibitory postsynaptic currents (mIPSC) frequency (Verkuyl and Joels, 2003) and the number of GABAergic synapses on CRH neurons (Miklos and Kovacs, 2002). Further, demonstrating presynaptic changes in GABAergic inhibition following stress, the expression of receptors for stress-derived steroid hormones (MRs and GRs) have been identified on GABAergic interneurons in the peri-PVN region and stress hormones have been shown to increase the burst firing of these neurons (Shin et al., 2011). These findings are in contrast with the decreased frequency of both mIPSCs and sIPSCs following stress (Verkuyl et al., 2004) and may represent a compensatory change to restore inhibition in this region following stress. In addition to potential changes in presynaptic GABAergic release suggested by changes in GAD expression and GABA levels, there is also abundant evidence of postsynaptic changes in $GABA_AR$ subunit expression associated with stress.

There is reduced [$^3$H]GABA and [$^{35}$S]TBPS binding following stress suggesting alterations in $GABA_A$ receptor ($GABA_AR$) expression (Skerritt et al., 1981; Schwartz et al., 1987; Akinci and Johnston, 1993; Serra et al., 2000) [for review see Skilbeck et al. (2010)]. One thing is for certain, the changes in binding to $GABA_ARs$ following stress is extremely variable and results differ according to gender, paradigm used, and laboratory where the experiments were conducted. These results leave little certainty regarding changes in radio-labeled ligand binding to $GABA_ARs$ following stress. Pharmacological changes more consistently point to alterations in $GABA_AR$ expression following stress. For example, stress and adrenalectomy have both been shown to alter benzodiazepine binding (Majewska et al., 1985; De Souza et al., 1986; Goeders et al., 1986; Miller et al., 1987, 1988; Weizman et al., 1990; Smith et al., 1992). However complex, these data suggest that there are changes in $GABA_AR$ expression associated with stress.

Studies investigating changes in $GABA_AR$ subunit expression following stress have demonstrated specific changes in $GABA_AR$ subtypes. There are brain region-specific alterations in $GABA_AR$ subunit expression following stress, including decreased $GABA_AR$ β1 and β2 subunit expression in the PVN following stress, with no change in $GABA_AR$ α1, α3, γ1, or γ2 expression (Verkuyl et al., 2004). Consistent with a role of extrasynaptic $GABA_ARs$ in the regulation of the HPA axis, a significant increase in $GABA_AR$ α5 subunit expression and a decrease in $GABA_AR$ δ subunit expression have been demonstrated in the PVN following stress (Verkuyl et al., 2004). In the hippocampus, $GABA_AR$ β1 and β2 subunit expression is increased (Cullinan and Wolfe, 2000) and $GABA_AR$ γ2 subunit expression is decreased (Maguire and Mody, 2007). Increased expression of the predominantly extrasynaptic $GABA_AR$ δ subunit was demonstrated in the hippocampus following stress (Maguire and Mody, 2007) [for review see Belelli et al. (2009); Maguire and Mody (2009)] and these changes can by mimicked by treatment with THDOC (Maguire and Mody, 2007). Although the exact mechanisms underlying alterations in $GABA_AR$ subunit expression associated with stress are not fully understood, it is thought that these changes are mediated by the actions of stress hormones and/or stress-derived neurosteroids.

Both steroid hormones and neurosteroids are elevated in response to acute stress (Majewska et al., 1985; Purdy et al., 1991; Barbaccia et al., 1996a,b). Acute stress induces an elevation in circulating levels of THDOC from 1–5 nM to 15–30 nM (Reddy and Rogawski, 2002) [for review see Reddy (2003)]. Stress can increase neurosteroid levels to concentrations which can act directly on $GABA_ARs$ to both potentiate the effects of GABA (Purdy et al., 1991; Barbaccia et al., 1996b) as well as alter $GABA_AR$ subunit expression (Maguire and Mody, 2007). Neurosteroids can potentiate the tonic component of GABAergic inhibition via action on $GABA_AR$ δ subunit-containing receptors at low concentrations (Stell et al., 2003), can potentiate the phasic component of GABAergic inhibition at higher concentrations, and at very high concentrations have even been shown to directly gate the receptor [for review see Lambert et al. (2009)]. In addition to the potentiation of GABAergic transmission by neurosteroids, steroid hormones themselves can alter synaptic GABAergic transmission (Maggio and Segal, 2009). Corticosterone alters the frequency of spontaneous sIPSCs in the hippocampus via actions on MRs (Maggio and Segal, 2009) and increases the amplitude of sIPSCs via actions on GRs (Maggio and Segal, 2009). Neurosteroidogenesis has been demonstrated to be essential for steroid hormone-linked alterations in $GABA_AR$ subunit expression (Maguire and Mody, 2007). These alterations in $GABA_AR$ subunit expression following stress are likely mediated by neurosteroid-mediated effects on $GABA_AR$ phosphorylation (Brussaard and Koksma, 2003), which controls $GABA_AR$ expression [for review see Kittler and Moss (2003)]. These data demonstrate the complex actions of both steroid hormones and neurosteroids on $GABA_ARs$ via direct modulation or by altering receptor expression.

The findings highlighted in this review demonstrate a reciprocal regulation of stress hormones and GABA receptors, in that GABAergic transmission plays a key role in the regulation of the HPA axis and the production of stress hormones and stress-derived neurosteroids can alter $GABA_AR$ subunit expression as well as directly modulate GABAergic transmission.

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# Synaptic regulation of the hypothalamic–pituitary–adrenal axis and its modulation by glucocorticoids and stress

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Dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis has been implicated in a range of affective and stress-related disorders. The regulatory systems that control HPA activity are subject to modulation by environmental influences, and stressful life events or circumstances can promote subsequent HPA dysregulation. The brain is a major regulator of the HPA axis, and stress-induced plasticity of the neural circuitry involved in HPA regulation might constitute an etiological link between stress and the development of HPA dysregulation. This review focuses on the synaptic regulation of neuroendocrine corticotropin-releasing hormone (CRH) neurons of the hypothalamic paraventricular nucleus, which are the cells through which the brain predominantly exerts its influence on the HPA axis. CRH neuronal activity is largely orchestrated by three neurotransmitters: GABA, glutamate, and norepinephrine. We discuss our current understanding of the neural circuitry through which these neurotransmitters regulate CRH cell activity, as well as the plastic changes in this circuitry induced by acute and chronic stress and the resultant changes in HPA function.

**Keywords: corticosteroid, glutamate, GABA, norepinephrine, neural circuits, depression, synaptic plasticity, paraventricular nucleus**

## INTRODUCTION

Glucocorticoids are released in response to physical, emotional, and/or metabolic stress, and many of the effects of glucocorticoids are thought to serve as adaptive responses to stressful events or circumstances. Physiological levels of glucocorticoids are regulated by the hypothalamic–pituitary–adrenal (HPA) axis, which is responsible for a cascade of hormone signals that begins in the brain and ends with glucocorticoid secretion from the adrenal cortex. An additional component of the HPA axis is a glucocorticoid-mediated negative feedback mechanism, wherein glucocorticoids act on the anterior pituitary and in the brain to suppress HPA activity. Given the association between glucocorticoids and stress, it is not surprising that abnormal regulation of the HPA axis is commonly associated with a range of affective and stress-related disorders. Research into these phenomena has dealt mostly with hypersecretion of glucocorticoid in patients with depressive illness (Gillespie and Nemeroff, 2005), although there has also been interest in the hyposecretion of glucocorticoids found in some cases of post-traumatic stress disorder (Yehuda, 2006) and depression (Morphy et al., 1985; Penninx et al., 2007; Ahrens et al., 2008). Dysregulation of the HPA axis has also been associated with panic disorder (Abelson et al., 2007), chronic fatigue syndrome (Van Houdenhove et al., 2009), obsessive-compulsive disorder (Gustafsson et al., 2008), fibromyalgia (Tanriverdi et al., 2007), generalized anxiety disorder (Lenze et al., 2011), and bipolar disorder (Daban et al., 2005). Importantly, stressful life events have been implicated in the onset of each of these disorders (Kendler et al., 1995; Hatcher and House, 2003; Gupta and Silman, 2004; Brawman-Mintzer et al., 2005; Garno et al., 2005; Goodwin et al., 2005; Sarkhel et al., 2011), and dysregulation of the HPA axis may be an etiological link between stress and the subsequent development of pathology.

The physiological origins of stress-induced variation in HPA regulation have remained largely unknown, although rodent models have shed some light on the matter, largely by providing insight into the ways that the brain regulates HPA activity. In fact, the neural circuitry that regulates the HPA axis has been found to be highly plastic, and that plasticity is induced by both stress and changes in glucocorticoid levels. It is possible that exposure to stress and/or the resultant fluctuations in circulating glucocorticoids results in pathology by evoking long-lasting alterations in the circuitry that regulates the HPA axis. Here, we will review what is known about the synaptic regulation of the HPA axis and the plasticity of the circuitry regulating the HPA axis induced under stress conditions. We will focus on the neuroendocrine corticotropin-releasing hormone (CRH) cells, which are located in the paraventricular nucleus (PVN) of the hypothalamus and are situated at the apex of the HPA axis. The CRH cells are of particular interest because it is principally through the regulation of CRH release into the portal circulation that the brain exerts its influence on the HPA axis. As with all neurons, CRH cells are stimulated by depolarization of their cell membrane, which stimulates action potentials and triggers the release of peptide from their axon terminals. However, unlike with classical neurons, these terminals do not form synapses with postsynaptic neurons; but rather, they are incorporated into a region at the base of the brain, the median eminence, where released neuropeptide accesses the pituitary portal circulation via fenestrated portal arterioles.

In addition to CRH, the CRH cells can synthesize and release vasopressin (VP). CRH and VP in the portal circulation bind to CRH and VP receptors on a subset of cells, the corticotropes, of the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH) into the systemic circulation. The ACTH then stimulates the synthesis and systemic secretion of glucocorticoids from the adrenal cortex. Hypothalamic CRH neuronal activity is largely orchestrated by three neurotransmitters: GABA, glutamate, and norepinephrine. In this review we discuss our current understanding of how these neurotransmitter systems regulate CRH neuron activity (and consequently HPA activity), and the modulation of these neurotransmitter systems by glucocorticoids and stress. One aim of this review is to provide a framework to help guide future investigations of the synaptic regulation of CRH cells that have the potential to advance our understanding of the neurological bases for HPA dysfunction. It is also an aim of this paper to inform those who study stress-induced abnormalities in HPA activity, but who may not be familiar with studies of synaptic transmission, in the hope of making these studies more accessible for the purpose of enhancing communication and promoting interdisciplinary investigations.

### METHODS OF IDENTIFYING CRH CELLS

Inasmuch as CRH cells play such a central role here, before discussing the synaptic regulation of the CRH neurons, we will briefly address the challenges in identifying the CRH cells in the course of physiological experiments. The PVN can be divided into three major cell types: the magnocellular neuroendocrine cells, which project their axons into the posterior lobe of the pituitary, the parvocellular neuroendocrine cells, which send their axons to the median eminence, and the parvocellular preautonomic neurons, which project to the brain stem and spinal cord. The CRH neurons fall into the parvocellular neuroendocrine cell type. The three cell types can be distinguished with reasonable reliability on the basis of their respective somatic sizes and shapes, dendritic morphologies, and positions in the PVN. Magnocellular neuroendocrine cells tend to have larger, more rounded somata and fewer dendritic branches, often bipolar in morphology, compared to the parvocellular neuroendocrine cells, which tend to be smaller, fusiform, and with multipolar dendritic arbors. The magnocellular neuroendocrine cells are concentrated in the lateral portion of the PVN, while the parvocellular neuroendocrine cells are located mainly in the medial region of the PVN. The dorsal-most and ventral-most regions of the PVN tend to be occupied by the parvocellular preautonomic neurons, which are intermediate in somatic size between the parvocellular neuroendocrine cells and the magnocellular neuroendocrine cells, and have multipolar dendritic arbors. While this morphological and topographical organization of the PVN can be used to identify enriched populations of the different subtypes of PVN neurons, there is fairly extensive overlap of the anatomical characteristics of the cell types within the PVN (Simmons and Swanson, 2009), such that it does not provide for a strict distillation of the three neuron subpopulations.

Distinct electrical properties have also been characterized in the three PVN cell types and have been used to assign electrical fingerprints for the reliable identification of individual cells in *in vitro* electrophysiological studies (**Figure 1**). Magnocellular neuroendocrine cells generate a robust A-type voltage-dependent $K^+$ current, which causes a prominent transient outward rectification that delays action potential generation (Tasker and Dudek, 1991). Most parvocellular preautonomic neurons generate a T-type $Ca^{2+}$ current, which causes a small low-threshold spike and clustering of $Na^+$-dependent action potentials (Stern, 2001; Luther et al., 2002). Parvocellular neuroendocrine cells can be distinguished from both magnocellular neuroendocrine cells and parvocellular preautonomic cells by the absence of both the transient outward rectification and the low-threshold $Ca^{2+}$ spike (Luther and Tasker, 2000; Luther et al., 2002; **Figure 1**).

While useful for narrowing down the field of the three cell subtypes, these anatomical and electrophysiological approaches do not allow investigators to specifically distinguish CRH neurons from the other PVN parvocellular neuroendocrine cells, such as thyrotropin releasing hormone and somatostatin cells. Electron and confocal microscopy immunohistochemical studies using selective antibodies for CRH and for neurotransmitters or vesicular neurotransmitter transporters have been useful for characterizing the anatomical innervation of CRH neurons and its plasticity in response to stress and adrenalectomy (Flak et al., 2009). Because basal CRH peptide expression is not robust, attempts to identify CRH neurons during electrophysiological recordings with a combination of intracellular dye injection and *post hoc* immunostaining for CRH have been largely unsuccessful. One method that has been useful for specifically targeting CRH cells for synaptic analysis is intracellular recording combined with single-cell, reverse transcription–PCR analysis (Di et al., 2003). This technique allows the correlation of molecular expression profiles, including CRH mRNA expression, with electrophysiological properties. Transgenic mice that express green fluorescent protein under the transcriptional control of the CRH promoter have recently become available (Alon et al., 2009) and offer the greatest promise for contributing significantly to the study of the synaptic regulation of identified CRH neurons and the synaptic plasticity of CRH neurons under conditions of stress.

## GABA INPUTS

Origins of GABA inputs to the PVN have been identified through the use of tract tracing in conjunction with immunohistochemical staining, as well as through *in vitro* electrophysiological analyses. A retrograde tracing-immunohistochemistry study revealed four discrete origins of significant GABAergic innervation of the PVN, which are: the area surrounding the supraoptic nucleus, the anterior perifornical region, the anterior hypothalamic area, and the anterior one-third of the PVN itself (Roland and Sawchenko, 1993). There is also evidence from tract tracing studies for a prominent GABAergic projection from the bed nucleus of the stria terminalis (BNST) to the PVN that relays inhibitory signaling to CRH cells from the prefrontal cortex (Radley et al., 2009) and the hippocampus (Radley and Sawchenko, 2011). Studies employing *in vivo* ibotenic acid lesions of subnuclei of the BNST have implicated specifically the posterior medial region of the bed nucleus as an inhibitory relay from limbic structures to the PVN (Choi et al., 2007, 2008). An *in vitro* brain

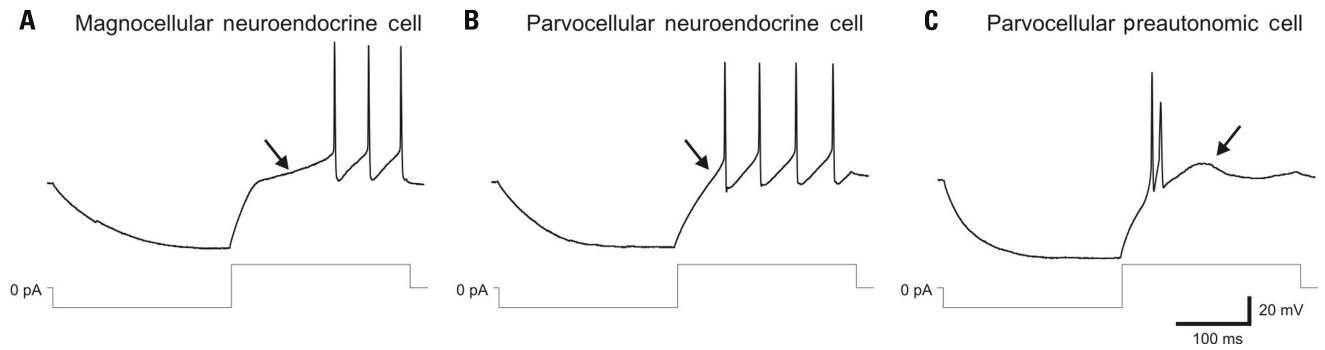


**FIGURE 1 | Distinct electrophysiological fingerprints of PVN magnocellular and parvocellular neurons. (A)** Magnocellular neuroendocrine cells recorded in current-clamp mode generate an A-type $K^+$ current-mediated transient outward rectification (arrow) that is generated by depolarization from a hyperpolarized holding membrane potential and delays the onset of spiking. Lower traces: current injection protocol. **(B)** Parvocellular neuroendocrine cells fail to generate a transient outward rectification and delay to spiking (arrow) in response to a similar current injection protocol. **(C)** Parvocellular preautonomic cells do not display a transient outward rectification, but generate a low-threshold spike (arrow), mediated by a T-type $Ca^{2+}$ current, and clustered action potentials in response to a similar current injection protocol. Modified from Luther and Tasker (2000).

slice electrophysiological study identified robust local GABAergic innervation of both parvocellular and magnocellular neurons of the PVN that originates in the perinuclear area surrounding the PVN (Boudaba et al., 1996). The perinuclear PVN area has also been postulated to serve as an inhibitory relay into the PVN from upstream limbic structures, such as the hippocampus and septum (Ziegler and Herman, 2002).

The inhibitory effect of GABA on CRH cells is mediated primarily by $GABA_A$ receptors. *In situ* hybridization assays detected the expression of the $GABA_A$ receptor α2, β1, and β3 subunits in nearly all the CRH cells of the PVN, and expression of α1 and β2 in about half of the CRH cells (Cullinan, 2000). Interestingly, microinjection of a $GABA_A$ receptor antagonist into the PVN is sufficient to activate CRH cells and elicit a surge in glucocorticoid secretion (Cole and Sawchenko, 2002), revealing a tonic inhibitory GABAergic input in the PVN that constrains CRH neuronal activity under basal conditions. The tonic inhibition of CRH neurons is preserved in organotypic hypothalamic slice cultures containing the PVN (Bali and Kovacs, 2003), which suggests that local GABA neuronal circuitry (i.e., GABA circuitry retained through the slicing procedure) plays a central role in this mechanism. The source of local GABAergic innervation of the PVN CRH neurons is predominantly extranuclear (**Figure 2**), including a robust input from the peri-PVN region (Boudaba et al., 1996).

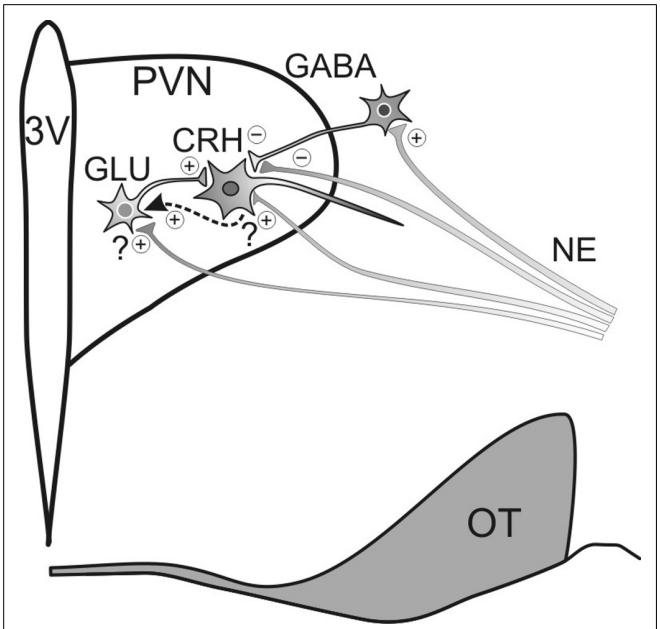


**FIGURE 2 | Model of noradrenergic regulation of PVN CRH neuron activity.** Noradrenergic inputs (NE) originate in the brainstem and regulate CRH neuronal activity by modulating glutamate and GABA release. Norepinephrine both suppresses and enhances GABAergic inhibition of CRH neuron activity, suppressing GABA release via $\alpha_2$-adrenoceptor activation at presynaptic terminals and promoting GABA release by activating $\alpha_1$-adrenoceptors on upstream GABAergic somata/dendrites. Noradrenergic facilitation of glutamate release onto CRH neurons is spike-dependent and is mediated by $\alpha_1$-adrenoceptor activation. Preliminary evidence suggests that the facilitatory effect on glutamate release may be mediated by the release of a retrograde messenger that stimulates upstream local glutamatergic circuits (dashed arrow). 3V, thrid ventricle; OT, optic tract.

## STRESS PLASTICITY OF GABA INPUTS

### *Acute stress plasticity of GABA inputs*

The GABAergic synaptic innervation of PVN neurons undergoes significant plastic changes in response to acute stress, which alters the excitability of the parvocellular neurons and activation of the HPA axis. A 30–60 min acute restraint stress is enough to cause a significant shift in the $Cl^-$ gradient across the PVN parvocellular neuron membrane via downregulation of the membrane $K^+$–$Cl^-$ co-transporter KCC2. This shift in the $Cl^-$ gradient causes a postsynaptic attenuation of the inhibitory GABAergic inputs to these cells and leads to the disinhibition of the HPA axis (Hewitt et al., 2009). It is not known whether glucocorticoids modulate KCC2 expression, although KCC2 expression has been found to be subject to steroidal modulation by sex hormones in substantia nigra neurons wherein, at postnatal day 15, $GABA_A$ receptors are hyperpolarizing in females and depolarizing in males (Galanopoulou

and Moshe, 2003). Interestingly, a similar phenomenon has been discovered in the magnocellular neurons of the PVN, in which the $GABA_A$ reversal potential shifts positive and the GABA signals are transformed into excitatory signals in oxytocinergic magnocellular neurons following dehydration, a chronic physiological stress (Kim et al., 2011); GABA was shown to be excitatory in VP cells, but not in oxytocin cells, under normal conditions (Haam et al., 2012).

Acute stress also appears to induce a long-term presynaptic plasticity in the GABAergic inhibitory innervation of PVN parvocellular neurons via a transient elevation in circulating glucocorticoids. Thus, a 30-min acute restraint stress was found to cause a persistent reduction in inhibitory synaptic inputs to putative parvocellular neurons in recordings performed in brain slices *in vitro* up to 5 h later, and this effect was mimicked by a previous *in vivo* subcutaneous injection of glucocorticoid (Verkuyl et al., 2005). These changes in inhibitory inputs were not spike-dependent, suggestive of plasticity in the GABA release probability, which was corroborated with paired-pulse analysis. An acute 20-min glucocorticoid application directly to brain slices had no rapid effect (<20 min) on GABAergic synaptic currents (Di et al., 2003), but resulted in a similar presynaptic suppression of GABA inputs to PVN parvocellular neurons 1–5 h later (Verkuyl et al., 2005), suggesting that these presynaptic glucocorticoid actions are transcriptional and occur directly in the hypothalamus.

GABAergic synaptic plasticity may also play a role in the HPA response to hypoglycemic stress via signaling through neuropeptide Y (NPY). There is a high density of NPY projections that terminate in the medial parvocellular PVN (Cowley et al., 1999), many of which originate in the arcuate nucleus, a hypothalamic region that provides access to the brain of circulating nutritional signals (Wang et al., 2004). Both insulin-induced hypoglycemia (Tuchelt et al., 2000) and intracerebroventricular injection of NPY (Dimitrov et al., 2007) activate the HPA axis and cause an increase in the circulating glucocorticoid level. *In vitro* electrophysiological studies reported that NPY caused a decrease in the probability of GABA release onto putative parvocellular PVN neurons via actions at multiple presynaptic NPY receptors (Cowley et al., 1999). The role of HPA axis activation in response to hypoglycemia may be related to the fact that glucocorticoids increase glucose bioavailability by shifting metabolic processes towards catabolism.

#### *Chronic stress plasticity of GABA inputs*

Chronic stress and sustained changes in systemic glucocorticoid levels also lead to long-term shifts in the GABAergic synaptic innervation of the PVN parvocellular neurons. Adrenalectomy, for example, which eliminates the main endogenous source of circulating glucocorticoids, leads to an increase in the number of GABAergic synapses and in the density of GABA receptors on CRH neurons, suggesting that the loss of circulating glucocorticoids induces a proliferation of afferent GABAergic synaptic inputs to CRH neurons. Thus, quantitative electron microscopic analysis of immunocytochemically labeled GABAergic synaptic profiles revealed a ∼55% increase in the number of GABAergic synapses on CRH cells in the PVN of adrenalectomized rats (Miklos and Kovacs, 2002). An *in vitro* electrophysiological study provided a physiological corroboration with the finding that putative parvocellular PVN neurons undergo a significant increase in inhibitory synaptic inputs in brain slices from adrenalectomized rats, and paired-pulse analysis suggested that this effect of adrenalectomy was due to an increase in the number of GABAergic synapses (Verkuyl and Joels, 2003). Glucocorticoid replacement in this study confirmed that the effect of adrenalectomy was due to the loss of endogenous glucocorticoids. Finally, binding of radiolabeled muscimol, a selective $GABA_A$ receptor agonist, was found to increase in the hypothalamus following adrenalectomy, suggesting an increase in postsynaptic expression or membrane localization of $GABA_A$ receptors following adrenalectomy (Majewska et al., 1985).

Whereas the loss of endogenous glucocorticoids by adrenalectomy was shown to increase the number of GABAergic synapses on CRH cells, chronic stress and chronically high glucocorticoid levels were reported to suppress GABAergic inhibitory synaptic inputs to PVN parvocellular neurons. *In vitro* electrophysiological recordings from putative parvocellular neurons in PVN brain slices taken after 3 weeks of a chronic variable stress treatment, which leads to sustained elevated glucocorticoids (Herman et al., 1995), revealed a decrease in GABAergic inhibitory synaptic inputs to parvocellular neurons without an accompanying reduction in the probability of release of GABA, suggesting a reduced number of GABAergic synapses (Joels et al., 2004; Verkuyl et al., 2004). A confocal immunofluorescence study, however, failed to detect a reduction in the density of GABAergic synaptic boutons on CRH neurons in the medial parvocellular PVN, although these measurements were taken following a shorter exposure (1 week) to the chronic variable stress treatment (Flak et al., 2009). The acute glucocorticoid-induced reduction in GABA release probability and the chronic glucocorticoid-induced decrease in GABA synapses, in conjunction with the adrenalectomy-induced increase in GABAergic synapses, suggest that there is an inverse causal relationship between glucocorticoid levels and the efficacy of GABAergic synaptic inhibition of CRH cell activity. Both acute and chronic stress, therefore, appear to downregulate GABAergic synaptic transmission in PVN parvocellular neurons, albeit in different ways. The progression from acute stress to chronic stress plasticity in the functional inhibitory synaptic regulation of parvocellular PVN neurons appears generally to involve a transition from a modulation of GABAergic synaptic strength to a structural modification of afferent GABA inputs.

While, to our knowledge, an analysis of the changes in the density of $GABA_A$ receptors in the PVN following chronic variable stress is still lacking, molecular studies on chronic stress-induced changes in $GABA_A$ receptor expression in the PVN have been performed, and have produced somewhat contradictory findings. An *in situ* hybridization study reported a decrease in the expression of the $GABA_A$ receptor β1 and β2 subunits (Cullinan and Wolfe, 2000). Another study using a single-cell RNA amplification approach reported no change in the β receptor subunit expression, but an elevated expression of the α5 subunit and decreased expression of the δ subunit in putative parvocellular PVN neurons following chronic variable stress (Verkuyl et al., 2004). However, the absence of a change in β-subunit expression in the latter study may have been a consequence of the small sample size, as the

investigators themselves concede. While the regulation of the α5 and δ $GABA_A$ receptor subunits may suggest a stress-induced change in extrasynaptic $GABA_A$ receptor signaling, the decrease in β1 and β2 subunits expression should lead to the downregulation of functional synaptic $GABA_A$ receptors and/or a change in synaptic $GABA_A$ receptor efficacy. Corresponding changes in postsynaptic $GABA_A$ receptor sensitivity have not been reported to date.

## GLUTAMATE INPUTS

Double immunohistochemical labeling for the vesicular glutamate transporter VGlut2 and for CRH has shown that PVN CRH neurons are densely innervated by glutamatergic fibers (Wittmann et al., 2005). Electrophysiological experiments have determined that functional local circuit glutamatergic innervation of putative parvocellular neurons originates in areas adjacent to the PVN, largely overlapping sources of peri-PVN GABAergic input, but at a much lower density (Boudaba et al., 1997; Tasker et al., 1998). There is also evidence that glutamatergic input to PVN neurons originates within the PVN itself (**Figure 2**). First, CRH-, oxytocin-, and VP-expressing neurons in the PVN have all been shown to co-express VGlut2 mRNA (Hrabovszky and Liposits, 2008). *In vitro* brain slice electrophysiological recordings from putative magnocellular neurons of the PVN revealed a norepinephrine-evoked stimulation of local glutamate circuits within the PVN that was spike-dependent, suggesting the presence of glutamatergic neurons in the PVN that mediate intra-PVN signaling (Daftary et al., 1998). Furthermore, intra-PVN glutamatergic circuits were also revealed in an autoradiographic study in which sources of glutamatergic input to the PVN were identified using the radiolabeled retrograde marker [$^3$H]D-aspartate, which selectively labels glutamate neurons (Csaki et al., 2000). In addition to the peri-PVN and intra-PVN sources of glutamatergic inputs to the PVN, significant glutamatergic afferents to the PVN have also been identified emanating from the ventromedial hypothalamic nucleus, the posterior hypothalamic nucleus, the dorsomedial hypothalamic nucleus, the anterior hypothalamic nucleus, the lateral hypothalamic nucleus, the paraventricular thalamic nucleus, and the medial nucleus of the amygdala (Csaki et al., 2000; Ulrich-Lai et al., 2011).

Microinjection of glutamate into the PVN caused a depletion of CRH in the median eminence and activation of the HPA axis (Feldman and Weidenfeld, 1997), and microinjection of an ionotropic glutamate receptor antagonist into the PVN attenuated the HPA hormonal response to acute restraint stress (Ziegler and Herman, 2000). The inconsistent activation of CRH neurons and the HPA axis by direct PVN glutamate microinjection reported in another study (Cole and Sawchenko, 2002) is likely due to the desensitization of the ionotropic glutamate receptors on the PVN neurons and/or stimulation of opposing inhibitory inputs via the activation of local circuit neurons upstream from the PVN neurons. Double-label immunohistochemical assays, for example, have shown that glutamate microinjection into the PVN increases Fos induction in peri-PVN GABAergic neurons, which may project to the CRH cells and oppose the direct excitatory actions of glutamate.

Molecular studies have reported the expression of multiple subtypes of ionotropic and metabotropic glutamate receptors (mGluRs) in the PVN. One *in situ* hybridization study that used riboprobes reported that mRNAs for the NMDA receptor subunits GluN1, GluN2A, and GluN2B are abundantly expressed in the medial parvocellular PVN, whereas there is significantly less expression of the GluN2C and GluN2D subunits (Herman et al., 2000). This study also reported substantial expression in the medial parvocellular PVN of the kainate receptor subunits GluK2 and GluK5 and of the AMPA receptor GluA1 subunit. Another *in situ* hybridization study that used oligonucleotide probes to test for the colocalization of ionotropic glutamate receptor subunits with CRH found mRNAs for the AMPA receptor subunits GluA1 and GluA2 to be present in 46 and 21% of CRH cells, respectively, and mRNA for the kainate receptor subunit GluK2 to be present in 31% of CRH cells (Aubry et al., 1996). This study also found that 70% of CRH cells expressed the NMDA receptor subunit GluN1 mRNA, but no colocalization with either GluN2A or GluN2B subunits was detected. The difference in GluN2 expression between these two studies may be due to the higher sensitivity of riboprobes relative to oligonucleotide probes. Thus, it appears that kainate, AMPA, and NMDA receptors all play a role in mediating glutamatergic input to CRH cells, with the AMPA receptor subunit GluA1 predominating over the GluA2 subunit, and the NMDA receptor subunits GluN2A and GluN2B predominating over the GluN2C and GluN2D subunits.

In addition to ionotropic glutamate receptors, mGluRs play a role in the regulation of CRH cell activity (Durand et al., 2008). Double-label immunocytochemistry revealed the presence of mGluR1a in a significant number of CRH cells (Kocsis et al., 1998). Intracerebroventricular injections of selective group I and group III mGluR agonists were found to elicit an increase in circulating glucocorticoids (Lang and Ajmal, 1995; Johnson et al., 2001). Unexpectedly, intracerebroventricular injections of antagonists of the group I mGluRs were also found to trigger the activation of the HPA axis (Bradbury et al., 2003). Based on previous findings of a pre- and postsynaptic modulation of magnocellular neuroendocrine cells by group I and III mGluRs (Schrader and Tasker, 1997; Tasker et al., 1998), a similar model for the regulation of CRH cell activity by group I and group III mGluRs was proposed (Johnson et al., 2001). According to this model, group III mGluRs disinhibit CRH cell activity by suppressing the release of GABA onto CRH cells, and group I mGluRs inhibit CRH cells through stimulation of afferent GABAergic neurons, while postsynaptic group I mGluRs stimulate CRH cells directly.

### STRESS PLASTICITY OF GLUTAMATE INPUTS

#### *Acute stress plasticity of glutamate inputs*

Glutamatergic synaptic inputs to CRH cells are suppressed by rapid glucocorticoid actions that appear to be involved in the glucocorticoid-mediated fast negative feedback of the HPA axis. Thus, corticosterone and dexamethasone cause a rapid (within 3–5 min) decrease in the frequency of miniature excitatory postsynaptic currents (mEPSCs) in each of the major neuroendocrine cell types of the PVN, including in CRH cells, recorded in hypothalamic slices (Di et al., 2003). This rapid glucocorticoid effect is mediated by a non-genomic mechanism via the activation of a membrane-associated receptor. Importantly, this glucocorticoid

effect was blocked by antagonists and mimicked and occluded by agonists of the cannabinoid type 1 receptor ($CB_1R$), suggesting the involvement of the endocannabinoid system. We recently corroborated this with the finding that the rapid glucocorticoid effect is absent in $CB_1R$ knockout mice (Nahar et al., submitted). This was supported by the finding that glucocorticoid rapidly (<10 min) triggers the synthesis of the major endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in slices of the PVN and immediate surround (Malcher-Lopes et al., 2006). Endocannabinoids signal in a retrograde fashion (i.e., from postsynaptic membrane to presynaptic membrane) and cause the inhibition of presynaptic neurotransmitter release (Freund et al., 2003). Consistent with the endocannabinoid acting as a retrograde messenger to inhibit glutamate release at glutamate synapses, the glucocorticoid effect was prevented by blockade of G protein and protein kinase activity and $Ca^{2+}$ signaling specifically in the postsynaptic cell (Malcher-Lopes et al., 2006; Harris and Tasker, 2011).

Interestingly, the glucocorticoid-induced rapid suppression of glutamate release in the PVN *in vitro* is not reversible in brain slice electrophysiological studies for over an hour after dexamethasone administration (Di et al., 2003), which indicates that glucocorticoids trigger a form of endocannabinoid-mediated long-term depression of synaptic excitation; long-term depression is recognized as a widespread form of synaptic plasticity in the brain that reduces the efficacy of synaptic interactions (Heifets and Castillo, 2009). The physiological significance of the extended duration of the glucocorticoid-induced suppression of excitatory inputs to CRH cells remains unknown; however, we have preliminary evidence for the desensitization to the rapid glucocorticoid-induced suppression of glutamate release in brain slices from animals that had been subjected to an acute 30-min restraint stress prior to sacrifice (Jiang and Tasker, unpublished observation). This effect is likely due to the long-term depression of glutamatergic synaptic inputs by a tonic activation of presynaptic CB1 receptors via glucocorticoid-induced retrograde endocannabinoid release, since a CB1 receptor-mediated inhibitory tone was observed in glutamate inputs to parvocellular neurons from acutely stressed rats, but not from unstressed rats. A downregulation of rapid glucocorticoid-induced endocannabinoid suppression of synaptic excitation was also reported in putative parvocellular neuroendocrine cells of juvenile rats subjected to a repeated immobilization stress, although the mechanism proposed was a chronic stress-induced desensitization of the cannabinoid signaling system (Wamsteeker et al., 2010), rather than the increased endocannabinoid inhibitory tone that we find after single exposure to an acute stress or stress level of glucocorticoid. It remains to be determined how long the glucocorticoid-induced long-term depression of synaptic excitation lasts, but it may be in place to provide the time necessary for the peptidergic stores in the somata and axon terminals to replenish after a rapid and robust secretion of CRH and VP into the portal circulation. Also, for those mechanisms that are sensitive to rates-of-change in glucocorticoid concentration (Jacobson and Sapolsky, 1993), long-term depression of the synaptic excitation of CRH neurons may reduce CRH release to allow for clearance of circulating glucocorticoids in order to ensure the sensitivity of glucocorticoid targets to the secretion of a subsequent bolus.

*N*-methyl-D-aspartate receptor expression in the medial parvocellular PVN has also been reported to be modulated by a single exposure to a stress stimulus. An *in situ* hybridization study found that acute immobilization stress caused a ∼35% increase in the expression of mRNA for the GluN1 subunit of the NMDA receptor in several brain regions, including in the medial parvocellular PVN (Bartanusz et al., 1995). The increased GluN1 expression suggests the possibility that acute stress may cause an overall increase in NMDA receptor function in the medial parvocellular PVN, since functional NMDA receptors are comprised of a requisite GluN1 subunit (Cull-Candy et al., 2001). However, a brain slice electrophysiological study demonstrated depressed NMDA receptor function in individual PVN parvocellular neurons following a 30-min acute immobilization stress (Kuzmiski et al., 2010). Paradoxically, the depression of NMDA receptor currents in the parvocellular neurons following acute stress placed the glutamate synapses in a permissive state that allowed them to undergo activity-dependent short-term potentiation in response to high frequency stimulation. Thus, the NMDA receptor mRNA expression and the functionality of the receptor do not appear to be regulated in parallel by stress, which suggests that there may be changes in the receptor protein expression or trafficking that may account for this discrepancy.

#### *Chronic stress plasticity of glutamate inputs*

In addition to inducing an apparent functional downregulation in the inhibitory synaptic inputs to PVN parvocellular neuroendocrine cells (Verkuyl et al., 2004), the chronic variable stress model of stress plasticity also gives rise to significant changes in the excitatory synaptic innervation of PVN parvocellular neurons. A double-labeling immunohistochemical study found that chronic variable stress significantly increased the density of synaptophysin-expressing synaptic boutons and VGlut2-expressing glutamatergic fibers in the medial parvocellular PVN, and increased the number of glutamatergic boutons directly contacting CRH-immunoreactive somata and dendrites (Flak et al., 2009). This suggests that chronic stress causes an increase in the glutamatergic synaptic innervation of the CRH neurons, which, along with the reduced GABAergic innervation of medial parvocellular neurons (Verkuyl et al., 2004), should lead to an increase in the excitability of these neurons following chronic stress exposure. Preliminary electrophysiological findings from our laboratory suggest that the increased density of immunolabeled glutamate synapses on the CRH neurons gives rise to an increase in the functional excitatory synaptic inputs to these cells (Franco et al., 2007).

Glutamate receptor expression in the PVN has also been reported to be modified by chronic stress exposure. An *in situ* hybridization study found that 2 weeks of chronic variable stress caused a decrease in the mRNA expression of the NMDA receptor subunit GluN2B in the medial parvocellular PVN, but found no changes in GluN1 or GluN2A subunit expression (Ziegler et al., 2005). This effect differed from the glutamate receptor plasticity induced by acute immobilization stress, which caused an increase in the expression of GluN1 reported in the aforementioned study (Bartanusz et al., 1995). The fact that the decrease in GluN2B induction caused by chronic stress was not accompanied by a change in GluN1 expression suggests that the downregulated

GluN2B does not reflect a decrease in the number of functional NMDA receptors, since the GluN1 subunit is an essential component of functional NMDA receptors (Cull-Candy et al., 2001). The decrease in NMDA receptor mRNA expression with chronic stress is surprising given the observation of an increase in the density of glutamate synapses with the same chronic stress paradigm (Flak et al., 2009). This suggests either that NMDA receptor mRNA and protein expression are differentially modulated, or that there is less of a contribution of NMDA receptors relative to AMPA receptors at glutamate synapses on CRH neurons following chronic variable stress. Follow-up electrophysiological studies will be necessary to distinguish between these possibilities. Interestingly, the stress-induced downregulation of GluN2B mRNA is apparently not mediated by glucocorticoids, as neither adrenalectomy nor high-dose glucocorticoid administration produced any change in GluN2B mRNA expression in the medial parvocellular PVN (Ziegler et al., 2005). Thus, this plasticity of NMDA receptor expression following chronic variable stress may result from repeated activation of glutamatergic receptors with the chronic activation of stress circuits.

Interestingly, glutamatergic synaptic plasticity resulting from the neonatal environment might be involved in lifelong changes in HPA responsivity. Pups that are handled daily during the first 10 days of life have been found to exhibit a suppressed HPA response to a novel stressor as adults (Liu et al., 1997). It is possible that this effect of handling is mediated by an increase in maternal attention, as handled pups receive more frequent bouts of maternal care in the form of licking, grooming, and arched-back nursing. One study using an early-life handling paradigm found that handled pups have lower VGlut2 protein content in the PVN, fewer VGlut2-immunoreactive synaptic boutons contacting CRH cells, and fewer asymmetrical, putative excitatory, synapses on CRH cells (Korosi et al., 2010). Electrophysiological recordings in putative parvocellular PVN neurons from these animals found that the frequency of miniature EPSCs also was decreased, suggesting a reduced excitatory synaptic input to the CRH cells. The reduced glutamatergic input in this study was found exclusively in the pups and not following maturation to adulthood, but the handled animals exhibited life-long reductions in PVN CRH expression, which suggests that reductions in glutamatergic input early in life may be involved in a cascade of events that leads to a life-long reduction in CRH expression and HPA stress responsivity.

## NOREPINEPHRINE INPUTS

Noradrenergic input to the medial parvocellular PVN originates in the brainstem, particularly in the nucleus of the solitary tract (NTS; Herman et al., 2003). Norepinephrine has long been known to play a prominent role in the regulation of the HPA axis and has been shown to be generally excitatory with regard to modulation of CRH neuron activity (Plotsky et al., 1987). Accordingly, microinjection of norepinephrine directly into the PVN increases CRH mRNA expression and elevates circulating ACTH in a dose-dependent manner (Itoi et al., 1994). There has been debate, however, regarding the role of the brainstem in driving HPA responses to stress. It has been proposed that the brainstem drives HPA responses to systemic stress, whereas HPA responses to psychological stress are driven by regions in the forebrain (Li et al., 1996). In support of this division, the glucocorticoid response to immune stress caused by intraperitoneal injection of interleukin-1α was found to be attenuated by depletion of noradrenergic inputs to the PVN, whereas no such effect of the depleted inputs was observed on the HPA responses to restraint stress (Chuluyan et al., 1992) or footshock stress (Li et al., 1996). However, later findings would suggest that ascending noradrenergic signaling plays a role in driving HPA responses to both systemic and psychological stress (Pacak et al., 1998; Dayas et al., 2001). More recently, Herman et al. (2003) presented an alternative model regarding the role of the brainstem in HPA responses to stress. According to this model, the extent to which the NTS promotes HPA activity results from an interaction between ascending sensory inputs to the NTS and descending inputs to the NTS from the forebrain. It was proposed that the ascending input to the NTS transmits information regarding the current homeostatic state, whereas descending input to the NTS transmits information regarding an anticipated homeostatic state. As a result of this convergence of signaling in the NTS, the HPA response to an anticipated homeostatic state would depend on the current homeostatic state, so that inhibitory signaling from the forebrain can be overridden by current somatic, visceral, or humoral disturbances; alternatively, excitatory signaling from the periphery to the medial parvocellular PVN could be modulated by descending contextual information (Herman et al., 2003).

Electrophysiological analyses have revealed mixed effects of norepinephrine on putative parvocellular neuroendocrine cells of the PVN; some cells are excited and other cells are inhibited by norepinephrine (Yang et al., 2007). The excitatory actions of norepinephrine on CRH cells appear largely to be mediated by modulation of glutamatergic synaptic inputs. Thus, the surge in circulating ACTH and glucocorticoids resulting from electrical stimulation of the ventral noradrenergic bundle was found to be inhibited by intra-PVN microinjection of ionotropic glutamate receptor antagonists in a dose-dependent manner (Feldman and Weidenfeld, 2004). An electrophysiological study found that about 36% of putative parvocellular neurons responded to norepinephrine with an increase in glutamatergic input, while another 14% responded with a hyperpolarization (Daftary et al., 2000). Post-recording biocytin labeling revealed that the cells that had exhibited a norepinephrine-induced hyperpolarization tended to be located close to the third ventricle in a region known to contain dopamine and/or somatostatin parvocellular neurons, so it is possible that none of the hyperpolarized neurons were CRH cells. The norepinephrine-induced increase in glutamatergic activity was mediated by $\alpha_1$-adrenoceptors and was spike-dependent. Perhaps the most surprising result from our electrophysiological studies is the absence of putative parvocellular neurons that are excited by norepinephrine directly (Daftary et al., 2000). Electron microscopy has revealed the presence of adrenergic boutons forming synaptic specializations with CRH neurons (Liposits et al., 1986) and dual *in situ* hybridization has demonstrated that the $\alpha_{1b}$-adrenoceptor is expressed by nearly all the CRH neurons of the PVN (Day et al., 1999). However, we have found no evidence, and there have been no electrophysiological reports, of norepinephrine directly activating an inward current or depolarization

in parvocellular neuroendocrine cells. As noradrenergic receptors are metabotropic, noradrenergic receptors on CRH cells may initiate signal transduction pathways without altering membrane conductance. On the other hand, postsynaptic noradrenergic receptors may stimulate the synthesis of a retrograde messenger that could trigger the activation of upstream local glutamatergic circuits. We have preliminary evidence for just such a retrograde mechanism, in which a messenger is released from CRH neurons in the PVN and stimulates a spike-dependent increase in glutamatergic inputs to the CRH neurons (Jiang and Tasker, unpublished observation; **Figure 2**).

In addition to regulating glutamate release, norepinephrine modulates GABA release onto parvocellular neurons in the PVN (**Figure 2**). An electrophysiological study found that about 58% of putative parvocellular neurons responded to norepinephrine with an increase in the frequency of spontaneous inhibitory postsynaptic currents (IPSCs) that was mediated by $\alpha_1$-adrenoceptors and was spike-dependent, suggestive of a locus of action upstream of the GABAergic axon (Han et al., 2002). In another 33% of the neurons tested, norepinephrine reduced the IPSC frequency, and this reduction was mediated by $\alpha_2$-adrenoceptors and was spike-independent, suggesting a presynaptic terminal locus of action (Han et al., 2002). In another study that specifically targeted parvocellular neuroendocrine cells with electrophysiological markers and retrograde staining from the median eminence, norepinephrine suppressed GABAergic input in 46% of the cells tested, and this was increased to 100% following blockade of either $\alpha_1$-adrenoceptors or spike generation (Yang et al., 2008). Consistent with $\alpha_2$-adrenoceptors located on GABAergic boutons inhibiting GABA release onto CRH cells, binding assays have reported substantial binding of radiolabeled clonidine, an $\alpha_2$-adrenoceptor agonist, in the CRH cell region of the medial parvocellular PVN (Cummings and Seybold, 1988). Moreover, electrophysiological analyses indicate that the $\alpha_2$-adrenoceptor-mediated decrease in GABA input is reflective of a reduced probability of GABA release (Yang et al., 2008). In keeping with its suppressive effect on neurotransmitter release, the $\alpha_2$-adrenoceptor is commonly found to suppress norepinephrine release as an autoreceptor (Raiteri et al., 1992). In support of a stimulatory effect of the $\alpha_2$-adrenoceptor on the HPA axis, clonidine has been reported to enhance CRH secretion from organotypic hypothalamic cultures (Calogero et al., 1988), and intracerebroventricular (Szafarczyk et al., 1990) and intraperitoneal (Shimizu, 1984) injections of clonidine *in vivo* induce a surge in ACTH release.

### PLASTICITY OF NOREPINEPHRINE INPUTS

Soon after the discovery that norepinephrine regulates HPA activity, it was found that ascending noradrenergic signaling plays a role in the glucocorticoid-induced suppression of the HPA axis, since depletion of noradrenergic inputs via knife cuts to the medulla attenuated the increases in hypothalamic CRH expression and CRH release caused by adrenalectomy (Sawchenko, 1988). Adrenalectomy was also found to reduce binding of radiolabeled clonidine in the CRH-expressing region of the medial parvocellular PVN (Cummings and Seybold, 1988), while $\alpha_{1B}$-adrenoceptor expression in the PVN was upregulated following adrenalectomy (Day et al., 1999). Microdialysis analyses revealed that adrenalectomy increases the synthesis, release, and turnover of norepinephrine in the PVN (Pacak et al., 1993), whereas 1 week of hypercortisolemia, induced by administration of exogenous glucocorticoid, reduced the synthesis, release, and turnover of norepinephrine in the PVN (Pacak et al., 1995). This last finding is surprising inasmuch as 1 week of chronic variable stress has been found to increase both the density of noradrenergic synaptic boutons in the PVN and the number of noradrenergic boutons abutting CRH-immunoreactive somata and dendrites (Flak et al., 2009). Additionally, 1 week of repeated intermittent cold stress was reported to enhance HPA responsivity to an acute immobilization stress via an increase in $\alpha_1$-adrenoceptor signaling within the PVN (Ma and Morilak, 2005).

In addition to the effects of adrenalectomy on norepinephrine release and adrenoceptor expression in the PVN, electrophysiological recordings revealed that adrenalectomy results in an increased fraction of putative parvocellular neuroendocrine cells that are excited by norepinephrine and a decreased fraction inhibited by norepinephrine (Yang et al., 2007). Additionally, it was found that the fraction of cells in which norepinephrine reduced GABA input was doubled following adrenalectomy, whereas there was an 80% decrease in the fraction of cells in which norepinephrine enhanced GABA input (Yang et al., 2008).

Although norepinephrine is thought to be generally excitatory with regard to CRH release, there is a body of evidence that suggests that norepinephrine suppresses CRH neuron activity in the absence of glucocorticoids; thus noradrenergic regulation of CRH cells may be more complex than previously thought. While norepinephrine normally enhances CRH secretion from organotypic hypothalamic cultures, it was found to reduce basal secretion of CRH from cultures maintained in a glucocorticoid-free medium (Szafarczyk et al., 1995). Also, while the $\alpha_2$-adrenoceptor agonist clonidine normally promotes CRH cell activity (Calogero et al., 1988), it was found to reduce basal CRH release from organotypic hypothalamic cultures maintained in a glucocorticoid-free medium (Feuvrier et al., 1998). If noradrenergic signaling from the brainstem does in fact play a more prominent role in driving HPA responses to systemic stress than to psychological stress, adrenalectomized rats might be expected to respond differently to certain stressors than do intact rats.

## PERSPECTIVES AND OUTLOOK

Plasticity of the neural circuitry regulating the HPA axis may constitute an etiological link between stress exposure and the subsequent development of HPA dysregulation and associated pathologies. Research into dysregulation of the HPA axis has focused mainly on hypersecretion of glucocorticoids, which is a condition that has been associated with depressive illness. Autopsies of depressed patients have reported higher than normal levels of CRH and VP mRNA in the PVN (Raadsheer et al., 1994, 1995), which is suggestive of elevated pre-mortem CRH cell activity. It is believed that diminished glucocorticoid-induced suppression of the HPA axis is an underlying factor in the HPA hyperactivity found in depressive illness (Gillespie and Nemeroff, 2005). Consistent with this, depressed patients exhibit an attenuated suppression of glucocorticoid secretion when given the

dexamethasone suppression test (i.e., the suppression of HPA activity by dexamethasone administration; Juruena et al., 2006). It has been suggested that this glucocorticoid resistance could result from a reduced expression of the glucocorticoid receptor (GR; Modell et al., 1997; Boyle et al., 2005), an idea supported by reports of reduced GR expression in post-mortem brain tissue of depressed patients (Webster et al., 2002). A similar downregulation of GR is seen in the rat brain following chronic stress (Kitraki et al., 1999). It is possible that a stress-induced downregulation of GR serves as a protective mechanism to limit allostatic load during periods of elevated glucocorticoid levels. Indeed, there is evidence that GR mediates the hippocampal deterioration that results from prolonged glucocorticoid exposure (Packan and Sapolsky, 1990). Stress could then result in hypercortisolemia by downregulating GR expression, thereby diminishing glucocorticoid-induced feedback inhibition of the HPA axis. However, we propose that a modified *central drive* underlies, at least in part, the link between exposure to stress and the subsequent development of HPA abnormalities. To be clear, we refer to the *central drive* as it pertains to the neural circuitry, namely the glutamatergic, GABAergic and noradrenergic circuitry that regulates the CRH neuronal activity. There is evidence that enhanced central drive of the HPA axis contributes to the hypercortisolemia associated with depressive illness. Specifically, dexamethasone-resistant depressed patients exhibit an enhanced ACTH response to metyrapone, a glucocorticoid synthesis inhibitor (Fava et al., 1984; Ur et al., 1992). This observation is consistent with a hyperactive drive of the HPA axis that is, in fact, suppressed by glucocorticoids. This glucocorticoid-independent *overdrive* of the HPA axis may reflect neural circuitry that favors CRH cell hyperactivity. Interestingly, an enhanced ACTH response to metyrapone in depressed patients has been correlated with a reduced efficacy of the selective serotonin reuptake inhibitor fluoxetine for the relief of depressive symptoms (Young et al., 2004), which suggests that resetting to a normal level of central drive to the CRH neurons may prove an effective therapy in cases of depression that are resistant to serotonin-based antidepressants.

Stress- and glucocorticoid-induced plasticity of neural circuitry regulating the HPA axis may constitute a means through which exposure to stress could play a role in a spectrum of HPA abnormalities, including aberrant HPA circadian rhythms, abnormalities in HPA responsivity to stress, and basal HPA dysregulation. We hypothesize that this synaptic plasticity constitutes an etiological link between exposure to stress and a range of affective and stress-related disorders. We frame this hypothesis on the basis of three premises: (1) exposure to stress increases the likelihood of developing subsequent HPA dysregulation and associated pathologies, (2) rodent models have demonstrated that the synaptic mechanisms that regulate the HPA axis are highly plastic, and that synaptic plasticity is induced by both glucocorticoids and stress, and (3) stress exposure and associated pathologies have, at times, been linked to hypoactivity of the HPA axis, and this phenomenon cannot be explained by diminished glucocorticoid-induced suppression of the HPA axis. With regard to this last premise, fibromyalgia (Tanriverdi et al., 2007) and chronic fatigue syndrome (Van Houdenhove et al., 2009) have both been linked to HPA hypoactivity, and stressful life events have been implicated in the onset of each of these disorders (Hatcher and House, 2003; Gupta and Silman, 2004). There is also evidence that HPA hypoactivity is involved in some cases of post-traumatic stress disorder (PTSD; Yehuda, 2006) and depression (Morphy et al., 1985; Penninx et al., 2007; Ahrens et al., 2008). Sexually abused girls (ages 5–7 years) have exhibited reduced salivary glucocorticoid levels within months following an abusive incident (King et al., 2001). Moreover, adult women who were victims of childhood sexual abuse have exhibited enhanced suppression of glucocorticoid release in response to the dexamethasone suppression test (Stein et al., 1997). Lastly, in rats, stress can result in HPA hypoactivity under certain circumstances. One study using female rats found that exposure to intense footshock, followed by 3 weekly reminders of the stimulus, resulted in lower glucocorticoid levels than those recorded previous to the footshock (Louvart et al., 2005). Reminders of the footshock involved placing the rat in a compartment adjacent to the compartment where the footshock had previously occurred, in what was intended as a rodent model of PTSD. Another study found that rats subjected to chronic variable stress, followed by several days recovery from the stress, exhibit attenuated pituitary–adrenal responses to acute novel environment and restraint stresses (Ostrander et al., 2006). It should be noted that there are some mechanisms through which glucocorticoids could actually enhance HPA activity. For example, glucocorticoids seem to attenuate GABAergic restraint on CRH cells, as described above; thus, in certain cell types, downregulated GR expression in response to stress could promote subsequent HPA hypoactivity. However, there is evidence that stress can, at times, result in upregulated GR. GR number was found to be larger in lymphocytes of combat veterans than in those of non-veterans (Yehuda et al., 1995). Also, increased GR binding capacity has been reported in the rat hippocampus following repeated inescapable footshock stress (van Dijken et al., 1993). Moreover, the enhanced response to the dexamethasone suppression test in adult victims of childhood sexual abuse (Stein et al., 1997) is suggestive of an increased sensitivity to glucocorticoids, at least at the anterior pituitary (Cole et al., 2000). Upregulated GR expression could be a protective response to hypocortisolemia, as some degree of GR activity may be critical for survival (Cole et al., 1995), and the initial hypocortisolemia could result from stress-induced synaptic plasticity that promotes CRH cell suppression. Indeed, GR expression generally increases following adrenalectomy (Olpe and McEwen, 1976; Svec et al., 1989; Isenovic et al., 2006).

With respect to future investigations of neural regulation of the HPA axis, recent advances in circuit tracing technology have seen the development of a Cre-dependent retrogradely transported marker derived from the rabies virus that will allow investigators to trace the precise sources of innervation to CRH cells (Wall et al., 2010). This tool will inevitably improve our understanding of how the brain integrates pertinent information into an HPA response. Also, one investigative team recently reported that perinatal stress, caused by an active construction site adjacent to the rodent vivarium, resulted in hypercortisolemia that persisted even after the construction had ended (O'Regan et al., 2010), which underscores the sensitivity of HPA regulatory mechanisms to environmental influences (at least perinatally), but also serves as a warning to investigators of the importance of documenting the

living conditions under which their subjects are kept. Lastly, it is important that assays of morphological plasticity and changes in ionotropic receptor expression are accompanied by electrophysiological recordings, as electrophysiology provides invaluable information regarding functional manifestations of synaptic activity. Dysregulation of the HPA axis may be a causal factor in a range of affective and physiological disorders, and there is increasing evidence that stress can result in persistent changes in HPA regulation. Rodent models of the past several years have provided significant insight into the etiology of stress-induced aberrations in HPA activity, but our understanding is still in its infancy. Studies of synaptic transmission will remain invaluable to this provocative and promising line of research, as we uncover future targets for pharmacological intervention.

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# Stimulus intensity-dependent modulations of hippocampal long-term potentiation by basolateral amygdala priming

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There is growing realization that the relationship between memory and stress/emotionality is complicated, and may include both memory enhancing and memory impairing aspects. It has been suggested that the underlying mechanisms involve amygdala modulation of hippocampal synaptic plasticity, such as long-term potentiation (LTP). We recently reported that while in CA1 basolateral amygdala (BLA) priming impaired theta stimulation induced LTP, it enhanced LTP in the dentate gyrus (DG). However, emotional and stressfull experiences were found to activate synaptic plasticity within the BLA, raising the possibility that BLA modulation of other brain regions may be altered as well, as it may depend on the way the BLA is activated or is responding. In previous studies BLA priming stimulation was relatively weak (1 V, 50 μs pulse duration). In the present study we assessed the effects of two stronger levels of BLA priming stimulation (1 V or 2 V, 100 μs pulse duration) on LTP induction in hippocampal DG and CA1, in anesthetized rats. Results show that 1V-BLA priming stimulation enhanced but 2V-BLA priming stimulation impaired DG LTP; however, both levels of BLA priming stimulation impaired CA1 LTP, suggesting that modulation of hippocampal synaptic plasticity by amygdala is dependent on the degree of amygdala activation. These findings suggest that plasticity-induced within the amygdala, by stressful experiences induces a form of metaplasticity that would alter the way the amygdala may modulate memory-related processes in other brain areas, such as the hippocampus.

**Keywords: emotional memory, plasticity, stress, limbic system, amygdala, rat**

## INTRODUCTION

It is generally accepted that memory is organized in multiple brain systems that can functionally interact with each other (Squire and Zola, 1996; Thompson and Kim, 1996). Two of these specialized systems are the hippocampus, which is crucial for associative type (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Morris, 2001; Day et al., 2003) and other types of learning and memory (Morris et al., 1982; Eichenbaum et al., 1996; Squire and Zola, 1996; Abrahams et al., 1999), and the amygdala, which plays a pivotal role in mediating many aspects of the stress response, fear-motivated learning, and memory for emotionally evocative events (LeDoux, 2000, 2003; McGaugh, 2004; Berretta, 2005). It has been suggested that emotional arousal/stress activates the amygdala and that this activation, specifically that of the basolateral amygdala (BLA), results in modulation of memory-related processes in the hippocampus (McGaugh, 2000; Richter-Levin and Akirav, 2000; Roozendaal, 2000; Packard and Cahill, 2001; Richter-Levin, 2004; LaBar and Cabeza, 2006). Thus, these two structures form a functional system relevant to the complicated effects of emotionality and stress on learning and memory (Kim and Diamond, 2002; Roozendaal, 2002; Prickaerts and Steckler, 2005; Lupien et al., 2007). On the one hand, considerable research shows that the interactions between amygdala and hippocampus are necessary for the enhanced encoding and consolidation of memory for emotionally arousing material and contexts (Cahill, 2000; Canli et al., 2000; Packard and Cahill, 2001; Phelps, 2004; Phelps and LeDoux, 2005). On the other hand, amygdala activation is suggested to mediate stress-induced impairment of hippocampus-dependent memory (Akirav and Richter-Levin, 2006; Hurlemann et al., 2007a,b; Maroun and Akirav, 2008).

Consistent with the complex relationship between stress and memory, stress can differently influence synaptic plasticity, such as long-term potentiation (LTP) (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Martin et al., 2000; Malenka and Bear, 2004). In the hippocampus, data so far suggest differential susceptibility to stressful events in its different subregions. Extensive observations from *in vitro* and *in vivo* electrophysiological studies indicate that variant stress paradigms, including unpredictable and inescapable restraint-tail shock (Foy et al., 1987; Kim et al., 1996; Garcia et al., 1997; Shors et al., 1997) or footshock (Shors et al., 1989; Li et al., 2005), forced exposure to brightly lit and unfamiliar chambers (Diamond et al., 1990; Xu et al., 1997), unavoidable exposure to a predator (Mesches et al., 1999), or platform stress (Maroun and Richter-Levin, 2003), discriminatory avoidance learning (Izaki and Arita, 1996), as well as contextual fear conditioning (Sacchetti et al., 2002; Hirata et al., 2009), all impaired LTP in the CA1. However, different stressors were reported to either impair (Diamond and Rose, 1994; Shors and

Dryver, 1994; Wang et al., 2000; Korz and Frey, 2005; Ahmed et al., 2006), enhance (Izaki and Arita, 1996; Gerges et al., 2001; Kavushansky et al., 2006; Spyrka and Hess, 2010), or have no effect (Bramham et al., 1998; Garcia, 2001; Gerges et al., 2001; Pavlides et al., 2002; Yamada et al., 2003; Vouimba et al., 2004; Yarom et al., 2008) on the ability to induce LTP in the DG. Furthermore, Korz and Frey (2003) reported a bidirectional effect of behavioral stress on the maintenance of DG-LTP, i.e., handling 15 min after the induction of early LTP resulted in an impairment of LTP, whereas a 2 min swim also 15 min after induction resulted in prolongation of LTP up to 24 h (Korz and Frey, 2003). Given critical role of amygdala in stress, one can suggest a possible way to explain complex effects of stress on hippocampus-dependent learning and memory that invokes differential effects of stressors on amygdala action, which inturn results in differential effects on LTP in different subregions of the hippocampus (Tsoory et al., 2008).

Indeed, the amygdala is critical in the modulation of hippocampal synaptic plasticity. Lesions of amygdala or pharmacological intervention of its function effectively prevented the effects of stress on hippocampal CA1 LTP, as well as on hippocampus-dependent spatial and fear memory (Kim et al., 2001, 2005; Goosens and Maren, 2004; Yang et al., 2008). Also, the behavioral or motivational reinforcement of hippocampal DG LTP (that can transform early LTP into late LTP) are reported to depend on the function of the amygdala (Almaguer-Melian et al., 2003; Korz and Frey, 2005). Consistently, hippocampus DG/CA1 LTP can be modulated by electrical stimulation of the amygdala (Akirav and Richter-Levin, 1999a,b; Abe, 2001; Frey et al., 2001; Akirav and Richter-Levin, 2002; Nakao et al., 2004; Vouimba and Richter-Levin, 2005; Vouimba et al., 2007). However, the profiles of amygdala modulation of hippocampal LTP are intriguing. Thus, while BLA lesions or its pharmacological suppression were reported to impair *in vivo* LTP of perforant path (PP)-DG (Abe, 2001), Korz and Frey (2005) reported facilitation of DG LTP in BLA lesioned animals (Korz and Frey, 2005). Similarly, BLA stimulation was reported to augment LTP in PP-DG pathway (Ikegaya et al., 1995, 1996; Akirav and Richter-Levin, 1999a; Frey et al., 2001), whereas later reports demonstrated that this effect vary considerably depending on the interplay of the strengths and timing of BLA and PP stimulation (Akirav and Richter-Levin, 1999b; Nakao et al., 2004; Vouimba and Richter-Levin, 2005). For example, although it was reported that BLA stimulation only affects weak and transient forms of LTP and LTP produced by a strong tetanus does not require the BLA for its induction nor its maintenance (Ikegaya et al., 1995; Frey et al., 2001), our previous series of results showed that BLA activation, applied 30s before or 1–2 h after strong tetanus application, could enhance or impair DG LTP induced by a strong tetanus, respectively, (Akirav and Richter-Levin, 1999a,b, 2002). Furthermore, consistent with our previous findings, we have recently showed that BLA activation (applied 30 s prior to or after the presentation of stimulation tetanus) impairs CA1 LTP in response to weak tetanus but enhances DG LTP in response to both weak and strong tetanus (Vouimba and Richter-Levin, 2005). Thus, we have suggested that, depending on how the amygdala is activated in terms of intensity, timing relationship, duration, and contextual input during information processing, the hippocampal outcome would involve the components of both/either enhancing and/or suppressing effects (Akirav and Richter-Levin, 2006; Tsoory et al., 2008), possibly providing synaptic plasticity mechanism underlying manifold/heterogeneous effects of stress on memory.

The present study was undertaken to further characterize the intriguing profiles of modulation of hippocampal synaptic plasticity by amygdala in order to elaborate on the mechanisms underlying the complex stress-memory relationship. We have previously demonstrated that relatively weak BLA activation 30 s prior to LTP induction in the hippocampus enhanced theta stimulation-induced LTP in DG, but decreased LTP in CA1 of the hippocampus (Vouimba and Richter-Levin, 2005). Here, we assessed the effects of two stronger levels of BLA priming stimulation on LTP, induced in hippocampal dentate gyrus (DG) and CA1 subregions in the anesthetized rat. The results demonstrate that BLA priming differently modulates subsequent LTP in the hippocampus depending on the degree of BLA activation.

## MATERIALS AND METHODS

### SUBJECTS

The experiments were performed using male Sprague–Dawley rats (Harlan Laboratories, Jerusalem, Israel) weighing 270–340 g. Rats were housed in Plexiglas cages (six rats per cage) and were maintained on a free-feeding regimen with a 12:12 h light/dark schedule. All electrophysiological testing was performed at least 1 week after their arrival, during the light phase of the cycle. During the course of the experiment, body temperature was monitored and maintained at $37 \pm 0.5$°C using a regulated heating pad.

All procedures and tests were approved by the Institutional Animal Care Committee and adhered to the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

### ELECTROPHYSIOLOGY

#### *Surgery*

Rats were anesthetized (40% urethane +5% chloral hydrate, 0.5 ml/100 g, i.p.) and mounted on a stereotaxic frame (Stolting, Wood Dale, IL). The scalp was incised and retracted, and the head position was adjusted to place bregma and lambda in the same horizontal plane. Small burr holes (1.5–2 mm diameter) were drilled in the skull for the placement of stimulating and recording electrodes. A reference electrode consisting of a 125 μm coated wire was affixed to the skull in the area overlapping the nasal sinus. A recording glass electrode (tip diameter, 2–5 μm; filled with 2 M NaCl) was stereotaxically positioned in the CA1 pyramidal cell layer [4.0 mm posterior to bregma (AP), 2.5 mm from midline (ML) and ∼2 mm dorsoventral (DV) from dura] or in the DG granular cell layer (4.0 mm AP, 2.5 mm ML and 2.7–3.0 mm DV from dura). Bipolar concentric stimulating electrodes (125 μm; Kopf, Tujunga, CA) were inserted in the ipsilateral BLA (2.8 mm AP, 4.8 mm ML, and 7.6 mm DV) and either in the contralateral ventral hippocampal commisure (vHC: 2 mm AP, 1.5 mm ML, and ∼3 mm DV from dura) for activating field potentials in the CA1 or in the ipsilateral perforant path (PP: 8 mm AP, 4.0 mm ML, and 2.5–3.0 mm DV from dura) for

activating field potentials in the DG. The DV location of the recording and stimulating electrodes was adjusted to maximize the amplitude of evoked field potentials.

#### *Stimulating and recording procedures*

CA1 and DG field potentials evoked by single pulses delivered to the vHC or PP, respectively, (0.1 ms rectangular monophasic pulses) were amplified (×1000) (AM-Systems amplifier), displayed on an oscilloscope, digitized at 10 kHz (CED) and stored to disk for off-line analysis (Spike-2 software). Baseline responses were established by means of a stimulation intensity sufficient to elicit a response representing 20–40% of the maximal amplitude of the evoked-field potentials. LTP was assessed by measuring both the increase in the population spike amplitude (PS) and the slope of the excitatory postsynaptic potential (EPSP) component, for the DG. However, in CA1, because of the early occurrence of the PS in some recordings, the slope was not measurable and, therefore, only the PS was analyzed and reported here. In our previous study (Vouimba and Richter-Levin, 2005) we have shown that both the PS and the slope of the EPSP (for both DG and CA1) follow the same pattern of changes responding to theta stimulation and amygdala modulation.

#### *Protocols*

Stable baseline recording of evoked-field potential in DG or CA1 was established for 30 min (1 pulse every 15 s) for all groups [For both DG and CA1: Control (electrode placed in the BLA but not stimulated), BLA Priming (1 V), and BLA Priming (2 V)]. Following baseline recording, LTP was induced by moderate theta burst stimulation (mTS: 10 trains, each consisting of 10 pulses at 100 Hz, with and intertrain interval of 200 ms; with trains delivered at test stimulus intensity) relative to our previously used strong TS (three sets of 10 trains; each train consisted of 10 pulses at 100 Hz, with an intertrain interval of 200 ms and an interset interval of 1 min) and weak TS (one set of five trains, each train consisting of five pulses at 100 Hz, with an inter-train interval of 200 ms) to the PP or vHC (Akirav and Richter-Levin, 1999a,b, 2002; Vouimba and Richter-Levin, 2005; Vouimba et al., 2007). After mTS stimulation, responses to test pulse stimuli were recorded every 15 s for 1 h. The BLA Priming groups received a priming stimulation of BLA (1 V or 2 V, 100 μs pulse duration, 10 trains of five pulses at 100 Hz; intertrain interval, 200 ms) 30 s before mTS to the PP or vHC was applied. The 1 V BLA priming stimulation (1 V, 50 μs pulse duration, 10 trains of five pulses at 100 Hz; intertrain interval, 200 ms) used in a previous study (Vouimba et al., 2007) had no significant effects on EPSP slope while having a small but significant effect on PS in DG when compared with the naive control; however, it had no effects on both EPSP slope and PS in CA1. In a pilot experiment for the current study, both BLA priming stimuli had no significant effects on EPSP slope in DG of the three tested groups (the two BLA priming groups and the naïve control group); the 1 V BLA priming stimulus resulted in a small but significant increase of PS in DG compared with the naïve control group, as was found in our previous study. The other stimulus intensity (2 V) had a small but not significant effect on DG PS and there was no significant difference between the two BLA priming groups. The two intensities of BLA priming stimuli had no effects on EPSP slope or PS in CA1. In addition, at both stimulus intensities, there were no visible responses in DG/CA1 in response to single stimulation pulses to the BLA. The Control animals were implanted in the BLA with stimulating electrodes through which no priming stimulation of BLA was applied.

#### HISTOLOGY

After completion of the electrophysiological examination of the recording in DG or CA1, electrical lesions of BLA were made by passing anodal current in stimulating electrodes in BLA group animals (5 mV DC for 20 s), not including the control animals with BLA stimulating electrodes through which no stimulation was given. Animals were then decapitated and their brains were frozen at –80°C for further analysis. Coronal sections (60 μm) were cut using LEICA cryostat, and mounted on glass microscope slides. Sections were stained with Cresyl violet. The stimulating electrodes placements were verified under microscopic examination according to the atlas (Paxinos and Watson, 1997).

#### STATISTICS

The amplitude of the PS and the slope of the EPSP were expressed as the mean percentage (± SEM) of the individual basal values of animals for each group. Only the animals that had correct positions of the stimulating electrodes in the BLA, according to the histology, were included in the analysis. Student's paired *t*-test was used to decide whether or not LTP was induced after TBS in each group. ANOVA for repeated measures with least significant difference test (LSD) as *post-hoc* test was used to compare LTP between groups. Statistical significance was set at $P < 0.05$.

## RESULTS

#### HISTOLOGY

**Figure 1** illustrates the location of stimulating electrode site in BLA. Histological analysis revealed that near 90% of rats recorded had correct positions of the stimulating electrodes in the BLA. Only data from these subjects was used in the analysis.

#### EFFECTS OF BLA ACTIVATION ON LTP-INDUCED BY MTS IN CA1 AND THE DG

Paired *t*-test revealed that in all the groups mTS reliably induced LTP of PS and slope in DG and LTP of PS in CA1 [*t*-tests for difference from baseline (100%) at 60 min after mTS. PS amplitude/EPSP slope in DG: Control group (201% of baseline, $t_8 = -6.3$, $P < 0.001$)/(123% of baseline, $t_8 = -8.8$, $P < 0.01$); 1V-BLA priming group (274% of baseline, $t_8 = -12.3$, $P < 0.001$)/(134% of baseline, $t_8 = -10.4$, $P < 0.001$); 2V-BLA priming group (157% of baseline, $t_9 = -3.7$, $P < 0.01$)/(114% of baseline, $t_9 = -6.0$, $P < 0.001$). PS amplitude in CA1: Control group (415.5% of baseline, $t_6 = -3.8$, $P < 0.01$); 1V-BLA priming group (243% of baseline, $t_6 = -5.7$, $P < 0.005$); 2V-BLA priming group (238% of baseline, $t_6 = 4.5$, $P < 0.005$). See **Figures 2A,C** and **3A**]. ANOVA analysis conducted on both DG LTP and CA1 LTP at different time points [groups × time


**FIGURE 1 | A diagram depicting a coronal section of the rat brain (2.8–3.0 mm posterior to Bregma) showing electrode placements in the BLA.** Filled circles indicate locations for all BLA groups ($n = 33$; B, basal amygdala; La, lateral amygdala; CeA, central amygdala).

$(3 \times 12)$] revealed significant difference between the groups (Control group, 1V-BLA priming group and 2V-BLA priming group) [PS amplitude/EPSPs slope in DG: $F_{(2,\ 25)} = 9.96$, $P < 0.005/F_{(2,\ 25)} = 5.41$, $P < 0.02$; PS amplitude in CA1: $F_{(2,\ 18)} = 3.858$, $P < 0.05$. See **Figures 2A,C** and **3A**].

In DG, 1V-BLA priming (30 s prior to application of mTS to PP-DG) enhanced LTP compared with Control, showing significantly greater PS-LTP at all time points pos-mTS ($Ps < 0.05$; See **Figure 2B**) and significantly greater slope-LTP at the last two 10 min-period blocks ($Ps < 0.05$; See **Figure 2D**). In contrast, 2V-BLA priming (30 s prior to application of mTS to PP-DG) impaired LTP of PS compared with Control, showing significantly smaller PS-LTP ($P < 0.05$; See **Figure 2B**) and significantly smaller slope-LTP ($P < 0.05$; see **Figure 2D**) at the last 10 min-period block post-mTS. Comparison between the two levels of BLA priming activation demonstrated that LTP in DG induced by mTS following 1V-BLA priming activation was significantly different from that induced by mTS following 2V-BLA priming activation for PS at time points post-mTS ($Ps < 0.01$; See **Figure 2B**) and for slope at all time points post-mTS except the first initial 10 min ($Ps < 0.05$; See **Figure 2D**).

In contrast, in CA1, the response to BLA priming activation was quite different. In comparison to Control, both levels of BLA priming (30 s prior to application of mTS to vHC-CA1) similarly impaired PS-LTP (1V-BLA priming vs. 2V-BLA priming, $Ps > 0.05$; see **Figure 3**), with 2V-BLA priming activation significantly inhibiting PS-LTP in CA1 at all time points post-mTS (compared with Control, $Ps < 0.05$. see **Figure 3B**) and 1V-BLA priming activation significantly inhibiting PS-LTP in CA1 at the last two 10 min-period blocks post-mTS (compared with Control, $Ps < 0.05$. see **Figure 3B**). Thus, BLA activation differentially modulated hippocampal plasticity, depending on both hippocampal subregions and the degree of BLA activation. **Figure 4** showed representative analog traces for baseline and the last 10 min-period block post-mTS from Control and BLA priming groups.

## DISCUSSION

We examined the effects of two levels of BLA priming activation on synaptic plasticity in the PP input to the granule cells in the DG, and the vHC input to the CA1 pyramidal cells of the hippocampus. The results show that 1V-BLA priming stimulation enhanced but 2V-BLA priming stimulation impaired DG LTP, whereas both levels of BLA priming stimulation impaired CA1 LTP. These results suggest that amygdala modulation of hippocampal synaptic plasticity is dependent both on the degree of amygdala activation and on hippocampus subregion examined.

We previously found that employing a similar pattern of amygdala priming but with a shorter pulse duration (50 μs) (weaker priming) enhanced PP-DG LTP induction (Akirav and Richter-Levin, 1999a,b, 2002; Vouimba and Richter-Levin, 2005; Vouimba et al., 2007). Our current results with 1V-BLA priming are consistent with those findings. However, further strengthing BLA priming intensity (2 V, 100 μs pulse duration) did not further enhance, but on the contrary inhibited DG LTP, suggesting that different degrees of BLA activation may exert bidirectional influence on synaptic plasticity in the DG. Nakao et al. (2004) reported that the effects of amygdala activation on DG synaptic plasticity could range from LTP to LTD depending on the degree and timing of neural activity of the BLA. They showed that strong BLA activation decreased the LTP-LTD crossover threshold to favor LTP, whereas weak BLA activation increased it. Beyond possible differences in the exact parameters/patterns of the BLA and PP stimulation between the two studies, there might exist an inverted U-shape function between the degree of BLA activation and its effects on the DG LTP, resembling that of glucocorticoid-hippocampal LTP relationship (De Kloet, 2004). In any case, the results demonstrate that BLA activation may result in enhancing or suppressing effects on DG-LTP, depending on the exact parameters of BLA activation.

In contrast, in the vHC-CA1 pathway, both 1V-BLA priming and 2V-BLA priming impaired vHC-CA1 LTP. These results are in agreement with previous findings demonstrating that relative weak BLA priming activation attenuated the LTP induction in this pathway (Vouimba and Richter-Levin, 2005; Vouimba et al., 2007). Together, the results suggest that LTP in CA1 is more susceptible to intereference by amygdala activation compared with DG.

Stress hormones, glucocorticoids (GC), and noradrenaline (NA) with their synergistic interaction in the BLA are suggested to mediate negative and positive influence on memory by emotion or stress (McGaugh, 2000; Lupien et al., 2007; Hurlemann et al., 2007a,b), and thus could be implicated in the underlying mechanism of our present findings. In support of this, we have previously demonstrated that BLA stimulation effects on DG-LTP involve both NA and GC (Akirav and Richter-Levin, 2002; Vouimba et al., 2007). Furthermore, despite that NA and β-adrenergic stimulation has been shown repeatedly to be involved

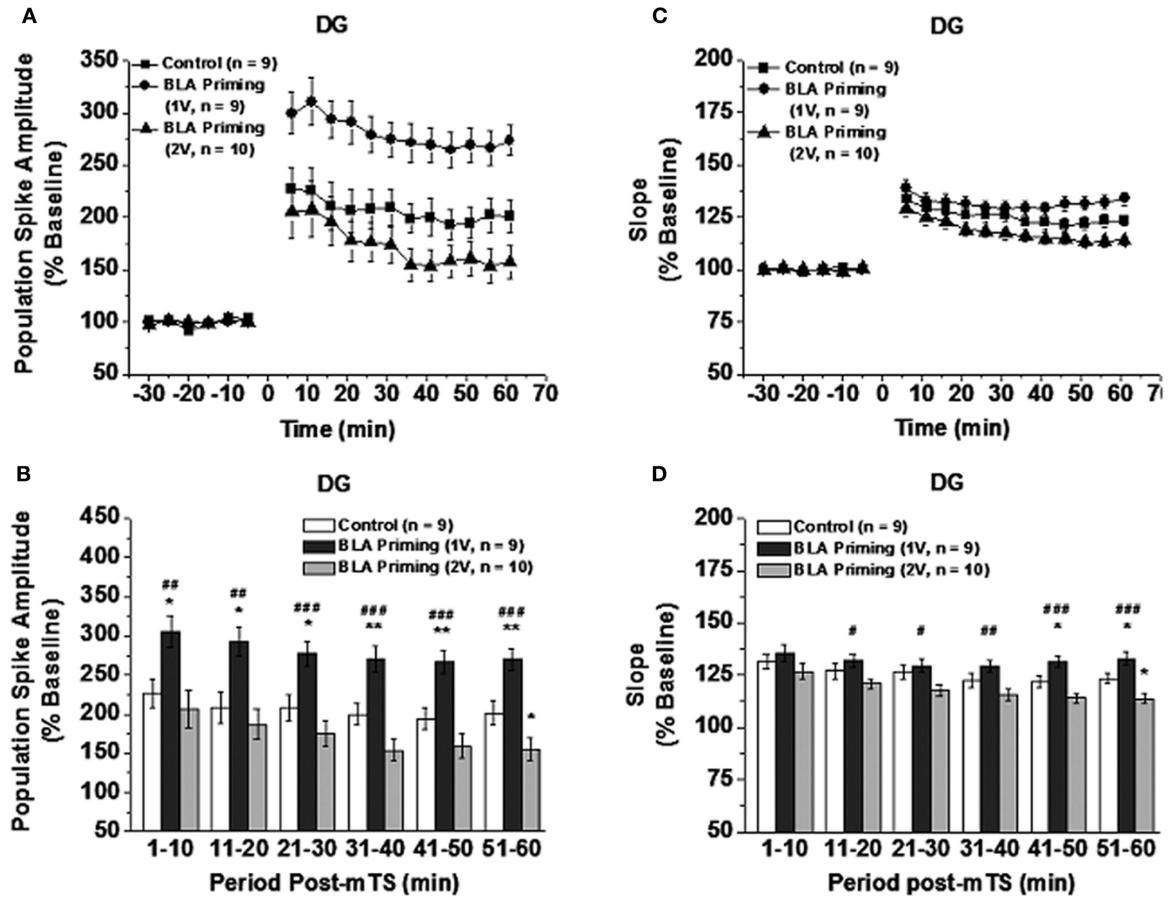


**FIGURE 2 | Effect of BLA activation [30 s before moderate theta burst stimulation (mTS: 10 trains, each consisting of 10 pulses at 100 Hz, with an intertrain interval of 200 ms)] on DG LTP induced by mTS: Mean (±SEM percentage of baseline).** The mTS reliably induced PS-LTP **(A)** and slope-LTP **(C)** with respect to the baseline in all the groups (Paired *t*-Test at 60 min after mTS, $t_s < -3.70$, $P_s < 0.01$). The 1V-BLA priming activation enhanced while the 2V-BLA priming activation impaired the PS amplitude **(**summarized in **B)** and the slope **(**summarized in **D)** of the EPSP component (Two-Way ANOVA with LSD as *post-hoc*). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the 2V-BLA priming group.

in the reinforcement of hippocampal LTP (Izquierdo and Medina, 1995; Thomas et al., 1996; Katsuki et al., 1997; Seidenbecher et al., 1997; Watabe et al., 2000; Gelinas and Nguyen, 2005), recent studies showed that low doses of NA (administered icv) effectively reinforced DG LTP while a higher dose was not effective (Almaguer-Melian et al., 2005). Systematically administered adrenaline enhanced DG LTP in an inverted-U dose-response function (Korol and Gold, 2008), possibly by causing NA release in many brain regions such as hippocampus and amygdala (Gold and van Buskirk, 1978; Williams et al., 1998; Miyashita and Williams, 2004). In contrast to DG LTP, BLA priming effects on CA1 LTP were not affected by blockade of NA or GC activation in BLA (Vouimba et al., 2007), suggesting that the mediating mechanisms of effects differ between CA1 and DG.

On the other hand, connected with stress and stress hormones such as NA showing to activate the amygdala in both humans and animals (Pelletier et al., 2005; Buffalari and Grace, 2009; Roozendaal et al., 2009; van Marle et al., 2009), different stressors may affect amygdala activity in different ways (Roozendaal et al., 2009). For example, Hand et al. (2002) have demonstrated differential release of corticotropin-releasing hormone (CRH) in the amygdala during different types of stressors. The level of activation of the MAP Kinase cascade in the BLA was also found to depend on the level of stress involved (Kogan and Richter-Levin, 2008; Ilin and Richter-Levin, 2009). Furthermore, activity in the BLA was found to be enhanced by the application of corticosterone in a dose-dependent manner (Kavushansky and Richter-Levin, 2006). These phenomena support the notion that different patterns of BLA stimulation mimic effects of different stressor-induced alterations in amygdala activation, and such alterations, as indeed demonstrated here, modulate the way the amygdala influences activity and plasticity in other brain regions, such as memory-related processes in hippocampus (Tsoory et al., 2008; Roozendaal et al., 2009).

The amygdala is a pivotal structure associated with stress. It has been suggested before that stress exerts its effects on brain regions, such as the hippocampus, at least partially by activating the BLA (for review: Richter-Levin, 2004 or Tsoory et al., 2008).

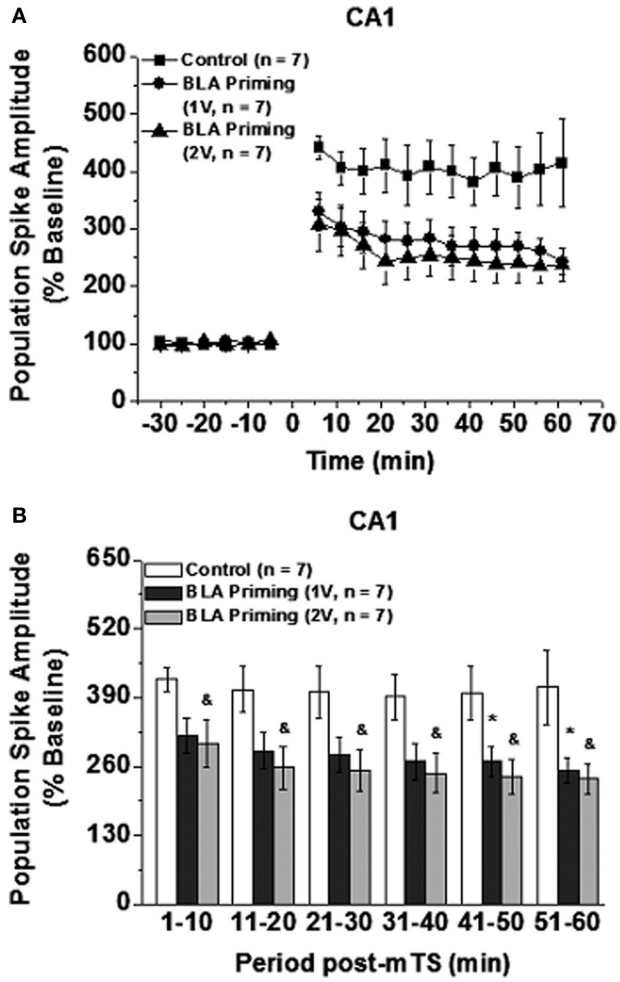


**FIGURE 3 | Effect of BLA activation [30 s before moderate theta burst stimulation (mTS: 10 trains, each consisting of 10 pulses at 100 Hz, with an intertrain interval of 200 ms)] on CA1 LTP induced by mTS: mean (± SEM percentage of baseline).** The mTS reliably induced PS-LTP **(A)** with respect to the baseline in all the groups (Paired *t*-Test at 60 min after mTS, $t_s < -3.80$, $P_s < 0.01$). Both levels of BLA priming activation impaired LTP of PS Amplitude **(**summarized in **B)**. $^*P/\&P < 0.05$ compared with the control (Two-Way ANOVA with LSD as *post-hoc*).

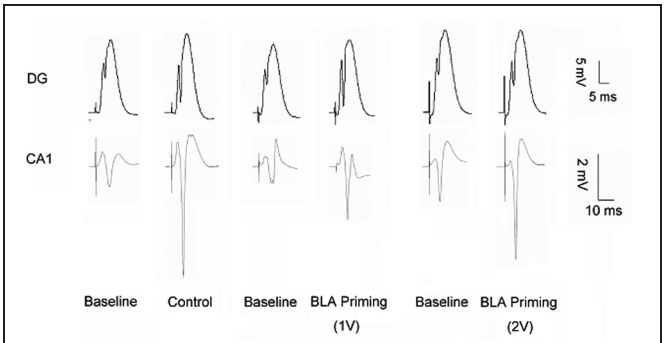


**FIGURE 4 | Representative analog traces recorded during baseline and during 51–60 min post the moderate theta burst stimulation from an individual of the Control and BLA priming groups.**

For example, BLA activation induces an increase in serum corticosterone (Rubin et al., 1966; Feldman et al., 1982; Dunn and Orr, 1984). In a recent study (Vouimba et al., 2007), we found that the 1V BLA priming stimulation protocol increased serum corticosterone levels to a lower level than that induced by swim stress in a platform-deprived water maze (Kavushansky et al., 2006), though both of these studies showed enhanced LTP in DG and impaired LTP in CA1. However, stronger BLA priming (2V) was found in the present study to impair both DG-LTP and CA1-LTP. This is in agreement with previous findings indicating that while water maze exposure stress enhances DG-LTP (Kavushansky et al., 2006), under-water trauma, which is considered to be a more intense stressor, blocked DG-LTP (Wang et al., 2000). Together, these results indicate that different patterns of BLA priming are likely to reflect specific amygdala activation states by different stressors.

Interestingly, it has been demonstrated that the exposure to a stressor may induce a form of metaplasticity that would affect the ability of a following stressor or of following BLA priming to modulate plasticity in other brain areas (Richter-Levin and Maroun, 2010). In relation to that Vouimba et al. (2004) found that while a single exposure to an elevated platform stress did not prevent LTP induction in the DG, it did induce a form of metaplasticity, since a repeated exposure to a similar stressor did suppress DG LTP. Thus, exposure to stress (Vouimba et al., 2004; Richter-Levin and Maroun, 2010) and amygdala priming (Richter-Levin and Maroun, 2010) were found to induce a form of metaplasticity that affects the ability of a following stress or following amygdala priming to modulate plasticity in other brain areas.

The current results further demonstrate that the BLA modulates synaptic plasticity in the hippocampus in a region-specific way (Vouimba and Richter-Levin, 2005; Vouimba et al., 2007). The nature of effects of BLA on synaptic plasticity differed between CA1 and the DG, such that in CA1 BLA activation impaired LTP, with stronger effects for stronger activation of the BLA, while in DG different stimulus intensities to the BLA resulted in differential effects on DG-LTP; a weak BLA stimulation enhanced DG-LTP but strong BLA stimulation impaired it. These results resemble those found following variant stressors, with a wide range of stressors found to suppress LTP in CA1 while different stressors found to affect DG-LTP on a spectrum from enhancement to impairment. Taken together, these findings lend further support to the notion that the BLA mediates some of the effects of stress and emotionality on hippocampal functioning (Richter-Levin, 2004).

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# Tuning synaptic transmission in the hippocampus by stress: the CRH system

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To enhance survival, an organism needs to remember—and learn from—threatening or stressful events. This fact necessitates the presence of mechanisms by which stress can influence synaptic transmission in brain regions, such as hippocampus, that subserve learning and memory. A major focus of this series of monographs is on the role and actions of adrenal-derived hormones, corticosteroids, and of brain-derived neurotransmitters, on synaptic function in the stressed hippocampus. Here we focus on the contribution of hippocampus-intrinsic, stress-activated CRH-CRH receptor signaling to the function and structure of hippocampal synapses. Corticotropin-releasing hormone (CRH) is expressed in interneurons of adult hippocampus, and is released from axon terminals during stress. The peptide exerts time- and dose-dependent effects on learning and memory via modulation of synaptic function and plasticity. Whereas physiological levels of CRH, acting over seconds to minutes, augment memory processes, exposure to presumed severe-stress levels of the peptide results in spine retraction and loss of synapses over more protracted time-frames. Loss of dendritic spines (and hence of synapses) takes place through actin cytoskeleton collapse downstream of $CRHR_1$ receptors that reside within excitatory synapses on spine heads. Chronic exposure to stress levels of CRH may promote dying-back (atrophy) of spine-carrying dendrites. Thus, the acute effects of CRH may contribute to stress-induced adaptive mechanisms, whereas chronic or excessive exposure to the peptide may promote learning problems and premature cognitive decline.

**Keywords: hippocampus, neurotransmission, corticotropin-releasing factor, long-term potentiation, volume transmission, CRF, CRH receptor, $CRFR_1$**

## LEARNING AND MEMORY MUST BE INFLUENCED BY STRESS, NECESSITATING MEANS TO INFLUENCE SYNAPTIC TRANSMISSION: WHAT DO WE KNOW AND WHAT ARE SOME OF THE REMAINING GAPS?

Stress is generally defined as a signal conveying threat or potential threat (López et al., 1999; Kim and Diamond, 2002; McEwen, 2004; de Kloet et al., 2005; Joëls and Baram, 2009; Lupien et al., 2009), and, operationally as a signal that activates a specific brain system (Pacák and Palkovits, 2001). Stress of different types is common and pervasive. In addition, there is a strong evolutionary advantage to remembering and learning from threatening situations (McEwen, 1999). In contrast, the continuation of normal life requires forgetting severely stressful events, because such haunting memories might interfere with emotional health and with carrying out life's daily tasks, as is found in post-traumatic stress disorder (Wingo et al., 2010; Yehuda et al., 2010). Therefore, it is not surprising that stress has been found to be a powerful modulator of synaptic plasticity and memory (Kim and Diamond, 2002; de Kloet et al., 2005; Joëls and Baram, 2009; Lupien et al., 2009; Sandi, 2011). In the context of this series, the overall focus is on the mechanisms by which stress affects the hippocampus in a time- and severity-dependent manner, with consequences that contribute to a cognitive and emotional health and disease. Thus, whereas acute stress (lasting seconds to minutes) may augment memory and related cellular processes, longer stress tends to impair hippocampus-dependent learning and memory (Kim and Diamond, 2002; de Kloet et al., 2005; Diamond et al., 2006; Joëls and Baram, 2009).

A remarkable body of work that focused on the basis of these effects of stress has centered on the roles of adrenal-derived corticoid stress hormones and on their signaling via glucocorticoid receptors (GRs) and, more recently, mineralocorticoid receptors (MRs) (Kim and Diamond, 2002; de Kloet et al., 2005; Lupien et al., 2009; Joëls and Baram, 2009; Krugers et al., 2010; Segal et al., 2010; Sandi, 2011; Yuen et al., 2011). In view of the fact that MR activation generally increases synaptic plasticity (Joëls and Baram, 2009; Krugers et al., 2010), and the relatively limited distribution of GR on hippocampal CA3 pyramidal cells that are highly vulnerable to stress (Magarinos and McEwen, 1995; Sanchez et al., 2000; de Kloet, 2004; Joëls and Baram, 2009), it is reasonable to consider potential additional factors that may contribute to the actions of stress on the cognitive functions taking place within the hippocampus.

Among the many factors that influence the effects of stress (e.g., type of stress, age, and gender of the involved brain), Time and Space are key. As mentioned, seconds-long stress improves learning whereas chronic, weeks-long stress perturbs both hippocampal function and structure. When and how does

the transition occur? Similarly, emerging evidence indicates that stress may affect the dorsal and the ventral hippocampus differentially (Maggio and Segal, 2009; Segal, 2010). In addition, stress may destroy dendritic spine within minutes and hours in hippocampus, yet increase complexity of dendrites in amygdala (Vyas et al., 2002). How is the spatial specificity take place? A large body of work, cited elsewhere in this monograph, has tackled these temporal and spatial issues. In the temporal domain, elegant studies have demonstrated rapid, non-genomic effects of MR and GR activation, followed by slower (hours to weeks) genomic actions. Rapid effects of neurotransmitters may translate to longer actions through influence on enzyme activity (reviewed in Joëls and Baram, 2009). Here we focus on an additional temporal solution: stress-provoked release of a neuropeptide within hippocampus. Neuropeptides classically function in the time-frame of seconds to a few hours depending, among other factors, on their degradation and reuptake (Koch et al., 1974). Similarly, peptides offer an attractive solution to the spatial conundrum of the actions of stress: unlike neurotransmitters, they are exuded into the neuropil that may bathe hundreds and thousands of synapses, providing a means to influence synaptic transmission of defined neuronal populations within a defined spatial domain (Fuxe et al., 1990; Agnati et al., 1995; Landgraf and Neumann, 2004; Nässel, 2009). In the current monograph, we describe the hippocampal corticotropin-releasing hormone (CRH) system and the action of this peptide on hippocampal structure and function. A key remaining challenge is to discover how the actions of neurotransmitters, corticosteroids and peptides interact to influence learning and memory in the stressed hippocampus.

## THE CRH SYSTEM OF ADULT HIPPOCAMPUS: CELLS, RECEPTORS, AND MIS-MATCHED SYNAPSES

### POTENTIAL SOURCES OF CRH EFFECTS IN THE HIPPOCAMPUS

CRH is expressed within adult hippocampus and is released locally during stress (**Figure 1**, and see below). However, the peptide is also released within the amygdala (Roozendaal et al., 2002), locus ceruleus (Valentino and Wehby, 1988; Snyder et al., 2012) and other brain regions. Because this peptide can travel long distances within the brain (Bittencourt and Sawchenko, 2000), an extra-hippocampal source and transport of the peptide from distal brain regions to act on hippocampal $CRFR_1$ receptors cannot be excluded. However, organotypic cultures of the hippocampus have helped clarify the source of endogenous CRH influencing hippocampal neuronal structure. Growing these cultures (where other brain regions are not included) in the presence of selective blockers of CRH receptor type 1 (the receptor most highly expressed in the hippocampal formation), has resulted in abnormal dendritic growth. Dendritic branching is exuberant and total dendritic length is increased under these conditions, suggesting a role for endogenous *hippocampal* CRH in selective pruning or sculpting of the dendritic tree of hippocampal pyramidal cells. Because dendrites may grow or die-back (atrophy), as a function of activity of excitatory synapses located on dendritic spines, a potential mechanism of the effects of CRH on dendritic structure is via influencing the integrity of such spines (see below). In addition, the structure of dendritic trees of mice lacking the $CRFR_1$ receptor is abnormal, with similar exuberant branching (Chen et al., 2004a, 2008). Interestingly, this is not found in mice lacking $CRFR_1$ only in principal forebrain neurons (Wang et al., 2011a), though the dendritic trees of these mice seem to be resistant to chronic stress induced atrophy. Taken together, available data largely support the idea that the main source of the CRH that activates $CRFR_1$ within hippocampus is the hippocampus itself.

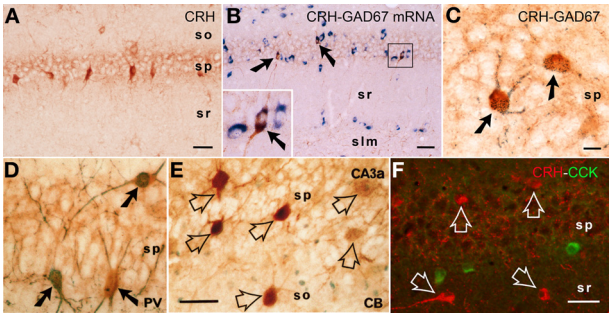


**FIGURE 1 | CRH is expressed in interneurons within the hippocampal pyramidal cell layer. (A)** CRH-immunoreactive (ir) neuronal somata in the pyramidal cell layer of area CA1. **(B,C)** All CRH-ir neurons in the pyramidal cell layer in CA1 area are GABAergic interneurons: they co-express the GABA synthesizing enzyme glutamic acid decarboxylase (GAD)-67 at both mRNA and protein levels, using immunocytochemistry coupled with *in situ* hybridization (GAD67, blue), and dual-labeled immunocytochemistry (CRH, brown; GAD67, black blue), respectively. Arrows indicate the dual-labeled neurons. **(D–F)** Many CRH-ir neurons in the hippocampus co-express parvalbumin (PV); none co-express calbindin D-28k (CB) or cholecystokinin (CCK). Solid arrows indicate the dual-labeled neurons, and empty arrows denote single-labeled CRH neurons. Abbreviations: so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; slm, stratum lacunosum-moleculare. Scale bars = 75 μm **(A,B,F)**, 25 μm **(C)**, and 50 μm **(D,E)**. Reproduced, with permission, from Chen et al. (2004b) **(B)** and Yan et al. (1998) **(D,E)**.

### WHO ARE THE HIPPOCAMPAL CRH-EXPRESSING CELLS, AND WHAT TYPE OF SYNAPSES DO THEY FORM?

CRH is produced in several populations of cells in the developing hippocampus (Yan et al., 1998; Chen et al., 2001), including Cajal-Retzius cells (Chen et al., 2001). In *adult* rodent hippocampus, the large majority of peptide is synthesized and contained within interneurons residing in the pyramidal cell layers of areas CA1 and CA3 (Sakanaka et al., 1987; Yan et al., 1998; Chen et al., 2001; Ivy et al., 2010) (**Figure 1**). CRH-expressing cells universally express the GABA synthetic enzyme GAD (**Figures 1B,C**), and include parvalbumin co-expressing basket cells (**Figure 1D**). Interestingly, there is no co-localization of CRH with calbindin D-28k (**Figure 1E**) or with cholecystokinin (**Figure 1F**).

The release site and mode of travel of CRH to target receptors are not fully understood. Light microscopy demonstrated a typical network of CRH-containing axon terminals surrounding the cell bodies of pyramidal cells (**Figure 2A**). In addition, both light and electron microscopy revealed that CRH is stored in axon terminals (**Figures 2B–F**) and released from axon terminal-vesicles surrounding the cell bodies and axon initial segments of pyramidal cells (Yan et al., 1998; Chen et al., 2001). These perisomatic release sites are >100 μm away from the location of the $CRFR_1$ receptors on dendritic spines in stratum

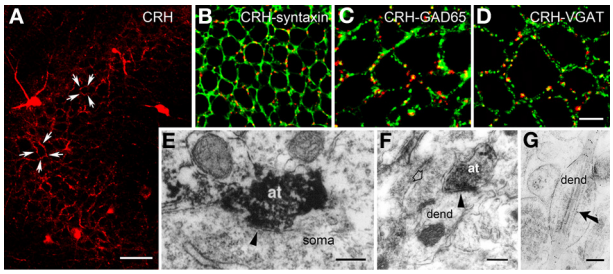


**FIGURE 2 | CRH is located in GABAergic axonal terminals. (A)** CRH-ir axon terminals form dense perisomatic baskets (arrows) around pyramidal cells in hippocampal area CA3. **(B)** CRH immunoreactivity localizes to pre-synaptic terminals, demonstrated by dual-labeled immunocytochemistry for CRH and for the pre-synaptic marker syntaxin. **(C,D)** CRH-containing terminals are GABAergic boutons as indicated by overlapping immunoreactivities of CRH and GAD65 **(C)** and of CRH and the GABA synaptic vesicular transporter VGAT **(D)** in axon terminals. **(E)** CRH-ir axon terminals (at) form axosomatic symmetric synapses (arrowhead) with pyramidal cell body (soma). **(F)** CRH-ir axon terminals form axodendritic asymmetric synapses (arrowhead) with dendrites (dend). Empty arrow indicates a bouton containing CRH-negative vesicles. **(G)** Electron micrograph demonstrating CRH-ir gold particles (arrow) within the dendrite, but not within vesicles. Scale bars = 50 μm **(A)**, 10 μm in **(D)** (25 μm for **B** and 12.5 μm for **C**), and 0.2 μm **(E–G)**. Reproduced, with permission, from Chen et al. (2004b) **(B–D)** and Yan et al. (1998) **(E)**.

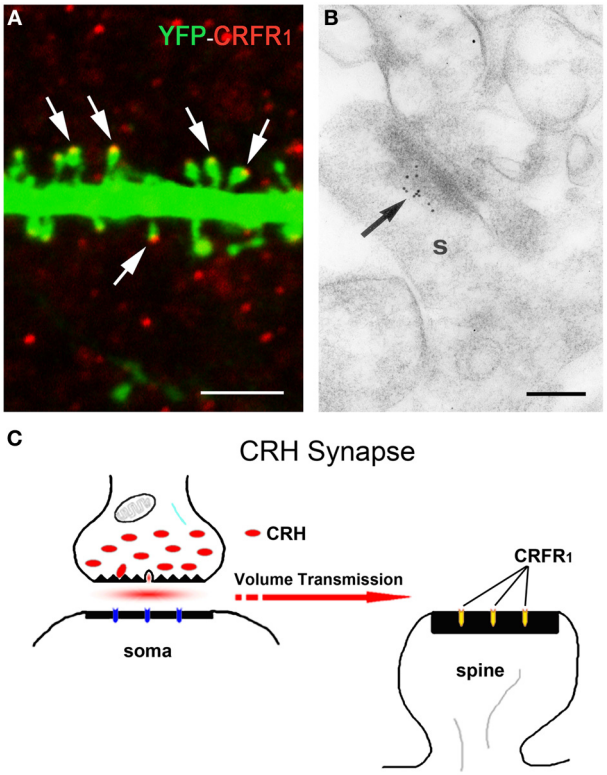


**FIGURE 3 | The post-synaptic location of the CRH receptor $CRFR_1$ on dendritic spines. (A)** The CRH receptor $CRFR_1$ (red) is located at the dendritic spine heads, as shown by confocal microscopy of CA3 pyramidal neuronal dendrite (green) from a YFP-expressing mouse. Arrows denote spine heads expressing the receptor. **(B)** Electron micrograph showing $CRFR_1$-immunogold particles (arrow) concentrated at the post-synaptic density (PSD) of an asymmetric (excitatory) synapse on a dendritic spine (s) in stratum oriens of hippocampal area CA3. Very few gold grains were observed elsewhere. **(C)** A cartoon depicting the concept of the mis-matched CRH synapse: pre-synaptic elements (axon terminal and boutons) are GABAergic (**Figure 1**), whereas post-synaptic elements on dendritic spines are consistent with excitatory synapses (**Figure 3A**). CRH that is released from inhibitory pre-synaptic elements may migrate via volume transmission and act on the $CRFR_1$ receptor at relatively distant excitatory post-synaptic sites. Scale bars = 3 μm **(A)**, 0.1 μm **(B)**. Reproduced, with permission, from Chen et al. (2010) **(A)**, (2004b) **(B)**.

radiatum (**Figures 3A,B**). The possibility that CRH is released from interneuronal dendrites closer to the receptors is not supported by electron microscopy studies, which show no evidence for vesicular localization of the peptide in dendrites (**Figure 2G**). Instead, these data support the idea that CRH released from interneurons in the pyramidal cell layer diffuses locally (via "volume transmission") (Agnati et al., 1995) to target receptors on dendritic spines (**Figure 3C**). Remarkably, these data indicate that the hippocampal CRH synapse is "mis-matched": the release site (pre-synaptic element) is an axon terminal of an interneuron (classically an element of inhibitory synapses), whereas the post-synaptic element resides on dendritic spines, and consists of dense post-synaptic elements, typical of excitatory synapses (**Figure 3C**).

## CRH-$CRFR_1$ SIGNALING CONTRIBUTES TO THE EFFECTS OF STRESS ON HIPPOCAMPAL SYNAPTIC STRUCTURE AND FUNCTION

CRH may signal through two identified G-protein coupled receptor family members: CRH receptor type 1 ($CRFR_1$) and type 2 ($CRFR_2$) (Perrin and Vale, 1999). The distribution of $CRFR_1$ and $CRFR_2$ in the brain is different (Chalmers et al., 1995; Chen et al., 2000; Van Pett et al., 2000). In general, $CRFR_1$ is primarily responsible for mediating the synaptic actions of CRH on hippocampal principal cells (Schierloh et al., 2007; Refojo et al., 2011; Stern et al., 2011). In accord, $CRFR_1$ is amply expressed in hippocampal pyramidal cells (Chen et al., 2000, 2004b; Van Pett et al., 2000; Refojo et al., 2011), whereas little $CRFR_2$ expression is observed (Van Pett et al., 2000). Notably, in addition to the cell body, $CRFR_1$ is found on dendrites and within dendritic spines, the location of post-synaptic portions of excitatory synapses (Chen et al., 2004b, 2010). Indeed, a short stress that combining physiological and psychological components activates $CRFR_1$-containing pyramidal cells, indicated by increases in immediate early gene expression. This activation requires CRH-receptor signaling, because selective local blockade of $CRFR_1$ prior to the stress prevents this activation (Chen et al., 2004b, 2006). Of note, $CRFR_1$ signaling may be required for hippocampal plasticity even in the absence of stress: synaptic potentiation is abnormal in hippocampal slices from mice lacking $CRFR_1$ (Schierloh et al., 2007), and these mice have learning deficits (Contarino et al., 1999).

## ACUTE (SECONDS TO MINUTES) EFFECTS OF CRH ON SYNAPTIC TRANSMISSION AND MEMORY

In line with the activating and memory-promoting effects of acute stress, the actions of CRH in the hippocampus are generally

excitatory (Baram and Hatalski, 1998). Application of CRH to hippocampal slices *in vitro* increases the firing rates of pyramidal cells by suppressing the after-hyperpolarization (Aldenhoff et al., 1983), and in the presence of an excitatory stimulus, CRH augments this input (Aldenhoff et al., 1983; Hollrigel et al., 1998). In a physiological context, brief application of CRH *in vitro* primes and augments LTP (Blank et al., 2002; Refojo et al., 2011) through $CRFR_1$ signaling. *In vivo*, a short treatment with CRH directly into the brain enhances memory (Wang et al., 1998, 2000; Blank et al., 2002; Joëls and Baram, 2009; Refojo et al., 2011). A significant additional body of work (e.g., Chen et al., 2004b, 2006, 2010) now demonstrates that stress induces rapid release of endogenous, hippocampal-origin CRH into the hippocampal intercellular space (**Figure 4**), as found also within the amygdala (Roozendaal et al., 2002), locus ceruleus (Van Bockstaele et al., 1996) and cortex (Behan et al., 1995). Taken together these facts suggest that CRH, rapidly released upon the onset of stress, excites synapses, and augments synaptic plasticity. This adaptive mechanism promotes learning and remembering during threatening situations, which might be teleologically advantageous.

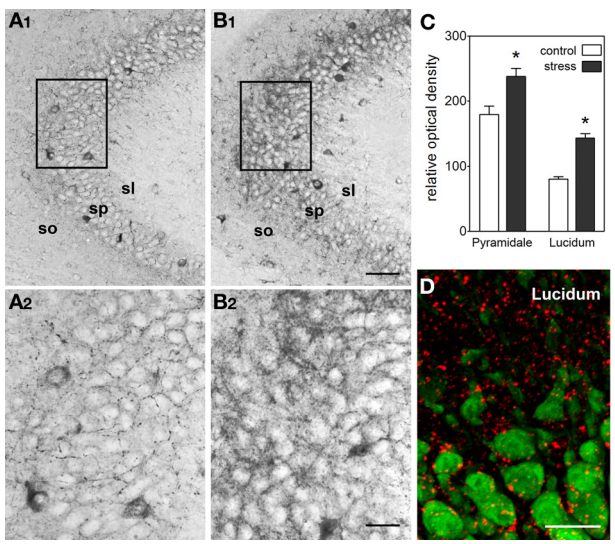


**FIGURE 4 | Stress induces rapid release of endogenous CRH into the extracellular space within hippocampus. (A,B)** Visualization of stress-induced release of endogenous CRH into the extracellular space (neuropil) in area CA3. Under control conditions **(A1,A2)**, there is little CRH-immunoreactivity outside of the cell bodies and terminals. In contrast, after a 30-min stress **(B1,B2)**, immunoreactive peptide is visible within the neuropil of the pyramidal cell layer and adjacent regions. See Chen et al. (2004b) for experimental details. Frames in **(A1,B1)** denote areas magnified in **(A2,B2)**, respectively, demonstrating the presence of CRH-immunoreactivity in the extracellular space. **(C)** Semi-quantitative analysis of CRH-immunoreactivity in presumed extracellular spaces of area CA3 ($n = 5$ animals, $^*P < 0.01$). **(D)** Stress-evoked release of endogenous CRH (red) into the extracellular space. NeuN (green) indicates CA3 pyramidal cells. Confocal image from a P18 rat sacrificed after a 30-min stress. Abbreviations: so, stratum oriens; sp, stratum pyramidale; sl, stratum lucidum. Scale bars = 60 μm in **(B1,A1)**, 20 μm in **(B2,A2,** and **D)**. Reproduced, with permission, from Chen et al. (2004b) **(D)**.

## SUBACUTE, HOURS-LONG EFFECTS OF STRESS LEVELS OF CRH ON SYNAPTIC FUNCTION AND STRUCTURE

The effects of stress on synaptic function vary with the duration of the stress (among other parameters, including the severity, type and context of the stress, and variables intrinsic to the age and gender of the hippocampus itself). Given that CRH is released during stress, it is not surprising that the consequences of stress levels of CRH on hippocampal synapse structure and function vary with the duration of exposure. From the electrophysiological perspective, application of the hormone onto adult hippocampal slices has major effects on synaptic physiology: field EPSPs begin to decline ∼75 min into the infusion period, and short- and long-term synaptic plasticity is obliterated (Chen et al., 2012).

The neuroanatomical basis for this loss of synaptic function involves a loss of synapses. Specifically, CRH at presumed stress levels (Tringali et al., 2009) leads to retraction of dendritic spines that harbor post-synaptic elements of hippocampal excitatory synapses (Chen et al., 2008). This hippocampal effect of CRH supports prior findings in the amygdala (Matys et al., 2004; Bennur et al., 2007). Interestingly, although only a minority of dendritic spines are lost upon hours-long CRH (or stress), their loss results in profound memory impairment and loss of LTP. The magnitude of the functional deficits derives from the fact that spine loss is fairly selective to the subpopulation of thin dendritic spines (Chen et al., 2012). Among the diverse populations of dendritic spines, thin spines are called "learning spines" (Bourne and Harris, 2008; Holtmaat and Svoboda, 2009). They are most influenced by patterned neuronal activity that promotes learning. As these thin spines begin to express more glutamate receptors of the AMPA-GluR1 type, they are converted to mushroom type spines associated with memory storage. Hence, a loss of thin spines will disproportionately hampers the potential for the spine-plasticity process associated with learning and memory (Bourne and Harris, 2008; Maras and Baram, 2012).

The molecular mechanisms by which CRH provokes spine retraction are not fully understood. Whereas CRH-$CRFR_1$ interaction activates several signaling cascades (Swinny and Valentino, 2006; Stern et al., 2011), the activation of an actin-regulating Rho-GTPAse, a specifically RhoA seems to underlie CRH-induced spine loss: blocking RhoA-mediated function rescued dendritic spines from CRH-provoked loss (Chen et al., 2012).

In summary, whereas a short exposure to CRH promotes synaptic function and plasticity, longer exposure, especially to presumed stress-levels of the peptide produces effects on hippocampal neurons that would contribute to stress-related learning and memory impairments. The precise transition from positive to harmful effects of the peptide, and the interaction between length of exposure and CRH levels, as well as the interaction of CRH with glucocorticoid and adrenergic mediators require future study.

## CHRONIC EFFECTS OF CRH ON HIPPOCAMPAL STRUCTURE AND FUNCTION

The effects of chronic stress on the integrity and neurotransmission of synapses have been extensively studied. Glucocorticoids, which are released peripherally in response to stress, can have

broad impacts on brain function (de Kloet, 2004; McEwen, 2004, 2011; Joëls, 2008; Lupien et al., 2009; Ulrich-Lai and Herman, 2009), whereas the local release of neurotransmitters and neuropeptides within the hippocampus itself provides for more spatially restricted modulation of specific synaptic populations (Joëls and Baram, 2009). The relative roles of glucocorticoids and CRH can be clarified in organotypic slice cultures or acute isolated hippocampal slices, which are not exposed to steroid hormones. Growing organotypic slice cultures chronically in the presence of exogenous CRH stunted dendritic growth (Chen et al., 2004a). These findings support a role for CRH in stress-related modulation of dendritic arborization and pruning.

A second approach to distinguish the requirement for CRH receptor signaling in effects of chronic stress on hippocampal synapses is via the use of transgenic mice, where the receptor is deleted in forebrain or hippocampus only. Adult mice lacking $CRFR_1$ in forebrain were relatively resistant to the deleterious effects of chronic social defeat stress (Wang et al., 2011a). Interestingly, the local deletion of this CRH receptor also protected adult mice from the adverse effects of chronic early life stress on learning and memory in adulthood (Wang et al., 2011b). Chronic early life stress, imposed by creating "simulated poverty" in the cage, results in cognitive problems and dendritic atrophy with loss of dendritic spines and synapses (Brunson et al., 2005). Infusion of $CRFR_1$ blocker immediately following this early life stress prevented the learning and memory defects, rescued LTP and restored the integrity of dendritic structure (Ivy et al., 2010). These findings provide direct evidence for a need for CRH-$CRFR_1$ signaling in the persistent effects of chronic early life stress on hippocampal synapses.

## SUMMARY

The body of work reviewed above suggests that, in addition to canonical stress hormones, hippocampal CRH tunes synaptic transmission during stress, influencing memory. In addition, stress is associated with high levels of CRH at hippocampal synapses, and long exposures to these levels result in neuroanatomical and functional defects of hippocampal function. Teleologically, rapid local release of the neuropeptide is adaptive, promoting excitation, and enhanced synaptic function during acute stress. The consequences of protracted elevation of CRH levels during chronic stress are likely maladaptive, providing a potentially treatable cause of stress-related cognitive problems. Challenges in the field include a better understanding of the effects of CRH during relatively short stresses that are common in modern life: namely, those lasting for several hours, with combined physiological and psychological components. A second topic requiring study is the degree and nature of the interaction among concurrently acting stress hormones: glucocorticoids, neurotransmitters, and CRH. How these mediators function, and how they interact in molecular and cellular terms, is a key enigma that impedes our full understanding of the effects of stress on synaptic neurotransmission within the hippocampus.

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# Differential changes of metabolic brain activity and interregional functional coupling in prefronto-limbic pathways during different stress conditions: functional imaging in freely behaving rodent pups

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The trumpet-tailed rat or degu (*Octodon degus)* is an established model to investigate the consequences of early stress on the development of emotional brain circuits and behavior. The aim of this study was to identify brain circuits, that respond to different stress conditions and to test if acute stress alters functional coupling of brain activity among prefrontal and limbic regions. Using functional imaging (2-Fluoro-deoxyglucose method) in 8-day-old male degu pups the following stress conditions were compared: (A) pups together with parents and siblings (control), (B) separation of the litter from the parents, (C) individual separation from parents and siblings, and (D) individual separation and presentation of maternal calls. Condition (B) significantly downregulated brain activity in the prefrontal cortex, hippocampus, nucleus accumbens (NAcc), and sensory areas compared to controls. Activity decrease was even more pronounced during condition (C), where, in contrast to all other regions, activity in the PAG was increased. Interestingly, brain activity in stress-associated brain regions such as the amygdala and habenula was not affected. In condition (D) maternal vocalizations "reactivated" brain activity in the cingulate and precentral medial cortex, NAcc, and striatum and in sensory areas. In contrast, reduced activity was measured in the prelimbic and infralimbic cortex (IL) and in the hippocampus and amygdala. Correlation analysis revealed complex, region- and situation-specific changes of interregional functional coupling among prefrontal and limbic brain regions during stress exposure. We show here for the first time that early life stress results in a widespread reduction of brain activity in the infant brain and changes interregional functional coupling. Moreover, maternal vocalizations can partly buffer stress-induced decrease in brain activity in some regions and evoked very different functional coupling patterns compared to the three other conditions.

**Keywords: limbic system, prefrontal cortex, functional imaging, functional coupling, stress, PAG, maternal separation**

## INTRODUCTION

The critical role of early adverse life experience, in addition to a genetic contribution, has been postulated to contribute to the susceptibility and pathogenesis of mood and anxiety disorders. There is clear evidence that early life stress, such as the separation of the newborn from its mother or parents as well as physical, sexual, emotional or verbal abuse all affect the maturation of endocrine and behavioral function in rodents, non-human primates, and humans (Korosi and Baram, 2009; Heim et al., 2010; Neumann et al., 2010; Pechtel and Pizzagalli, 2010; Bock and Braun, 2011; Pryce et al., 2011; Schmidt et al., 2011). The majority of studies on the neurobiological effects of stress exposure are focused on chronic stress in adulthood and its endocrine effects, its impact on the adult brain was mainly analyzed in the hippocampal formation (Sandi, 2004; Fuchs et al., 2006; Guterman and Richter-Levin, 2006; McEwen, 2010). Much less is known about the impact of acute, or repeated stress exposure on the immature developing brain, and on the functional maturation of prefrontal and limbic circuitries, which mediate perceptive, interpretative, and controlling aspects of emotionality. Due to the high vulnerability of the immature brain and the associated risks to develop dysfunctions we need to gain a more detailed understanding of the immediate and long-term neurobiological effects of early life stress, for example, child abuse and neglect or parental loss, in particular its long-term impact on the immature brain.

The trumpet-tailed rat *Octodon degus* is an established animal model to study the development of social behavior and emotional experience during postnatal and adolescent development (Colonnello et al., 2011) and to analyze the impact of early life stress on the development of prefronto-limbic brain circuits (Bock and Braun, 2011; Braun and Bock, 2011). This precocious,

diurnal South American rodent lives in complex social family structures, families are biparental and degu pups have been shown to develop a strong attachment to both parents (Fuchs et al., 2010). Similar to humans degus use an elaborated vocal communication system among family and colony members (Braun and Scheich, 1997).

So far, only little is known about the brain circuits and their networks, which are involved in information processing during or after exposure to positive or adverse emotional situations in the infant brain. One recent study, using cytochrome oxidase activity as a measure of long-term changes in brain metabolic capacity after 2 weeks of exposure to repeated separation stress, reports decreased activity in the medial prefrontal cortex and nucleus accumbens (NAcc) in 2-week-old mouse pups (Spivey et al., 2011). A PET study in young rhesus monkeys showed that acute maternal separation is associated with an activation in the right dorsolateral prefrontal cortex and ventral temporal/occipital lobes and decreased activity in the left dorsolateral prefrontal cortex (Rilling et al., 2001).

One aim of this functional imaging study was to systematically map metabolic activations/deactivations in the infant degu brain during different degrees of acute separation stress and thereby identify the underlying brain circuits in the infant brain. Since functionally correlated neuronal activity is essential for the activity-dependent maturation of neuronal networks, and deviations of these networks may contribute to the etiology of neurodevelopmental disorders (Uhlhaas et al., 2009; Stam and van Straaten, 2012), we also aimed to identify interregional functional coupling among the stress-responsive brain circuitries. In view of the recently emerging literature from human imaging studies exploring functional network connectivity to assess their involvement in different emotional states, the paucity of network analyses in animal models related to stress is surprising. We hypothesize that separation stress induces widespread alterations of metabolic brain activity and alters the interregional functional coupling among prefrontal and limbic brain regions. We also predict that the magnitude of the stress-induced metabolic changes should correlate to the degree of stress. Another aim was to test the hypothesis that a positive emotional stimulus, the maternal voice, which on the behavioral level has been shown to exert an "anxiolytic" effect (Braun et al., 2003; Ziabreva et al., 2003a,b) should ameliorate, restore or "normalize" stress-induced metabolic changes. Since functional MRI *per se* is a stressful procedure, and therefore requires sedation in the animals, we applied the 2-Fluoro-deoxy-glucose (FDG)-technique, which allows to quantify changes of regional metabolic activity in awake, freely behaving animals (Gonzalez-Lima and Scheich, 1986; Wallhäusser and Scheich, 1987; Gonzalez-Lima, 1992; Bock et al., 1996, 1997; Nair et al., 2001; Moriceau and Sullivan, 2006; Riedel et al., 2010).

## MATERIALS AND METHODS

### ANIMALS

The degus were bred in our colony at the Leibniz Institute for Neurobiology (Magdeburg, Germany). Family groups of an adult couple and their offspring were housed in large wire cages (100 cm × 84 cm × 40 cm) and exposed to a 12 h light/12 h dark cycle. Food and drinking water were available *ad libitum*. The rooms were maintained at an average temperature of 22°C. After the birth of the pups the home cages were not cleaned until the start of the experiments to avoid unspecific exposure to stressors (disturbing the cage) and handling.

All experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and according to the German guidelines for the care and use of animals in laboratory research. The experimental protocols were approved by the ethics committee of the government of the state of Saxony-Anhalt.

### EXPERIMENTAL GROUPS

All pups were reared in their natural family groups, i.e., together with their parents and siblings until the start of the experiments. All experiments were conducted between 11 am and 2 pm during the light phase (degus are diurnal, i.e., active during the day phase). For 2-FDG experiments an overall of 16 male degu pups from four families (litter size 7–8 animals) were analyzed at postnatal day (PND) 8. Due to the limited availability of degus (each breeding couple produces only 2 litters per year due to three months gestational period, and an extended weaning period) this experimental design does not completely exclude litter effects.

The pups were divided into four experimental groups of four siblings as follows:

*"parents" (control):* Pups of this unstressed control group were left undisturbed together with their parents and siblings in the home cage.

*"litter separation":* Pups of this group were exposed to separation stress by removing them from their home cage and their parents as a group of littermates, which were transferred to a paperboard box (25 × 25 cm), where they were left undisturbed for the duration of the 2-FDG experiment (1 h).

*"individual separation":* Pups of this group were exposed to separation stress as described for "litter separation", however, the pups of this group were also separated from their siblings and kept individually in paperboard boxes, where they were left undisturbed for the duration of the 2-FDG experiment (1 h). During the experiment no visual or tactile, but acoustic and olfactory contact among the siblings was possible.

*"individual separation + call":* Pups of this group were separated as described for "individual separation," but to ameliorate their stress levels maternal vocalizations were presented from a loudspeaker during the separation period (Braun and Poeggel, 2001; Ziabreva et al., 2003a,b). The presented vocalizations were "mothering calls", which the dams exclusively use when they have pups and which serves to attract the pups and stimulate their suckling behavior. The acoustic features of the maternal attraction calls and the metabolic activation patterns in the auditory cortex (AC) of degu pups were described in detail in Braun and Scheich (1997). It is important to note that all pups were stimulated with the same maternal calls, i.e., the calls were not from their own mother. The auditory stimulation was as follows: 10 s stimulation, 20 s break. This stimulation protocol was repeated during the entire imaging experiment.

### 2-FLUORODEOXYGLUCOSE AUTORADIOGRAPHY

The stimulus-evoked brain activation patterns were analyzed applying the 2-deoxyglucose method developed by Sokoloff et al. (1977). Due to its better penetration through the blood-brain barrier and the higher rate of phosphorylation we used 2-fluoro-2-deoxy-D-[U-14C]glucose (2-FDG) (Amersham) as originally described by Gonzalez-Lima (Gonzalez-Lima, 1992) in our work (Bock et al., 1996, 1997; Poeggel and Braun, 1997; Riedel et al., 2010). Pups of either experimental group were weight and immediately thereafter received intraperitoneal injections of 2-FDG, 6 μCi/animal in 0.2 ml sterile saline and were then exposed to the respective experimental condition for 1 h. After the experiment degu pups were decapitated, the brains removed from the skull and rapidly frozen on the freezing mount of a cryostat. Serial transverse 40 μm cryosections were collected on microscope slides, rapidly dried on a heating plate at 50°C, pressed on Kodak NMB1 X-ray film in Kodak-X-omatic cassettes, and exposed for 2 weeks.

2-FDG-uptake was analyzed using an image analysis system consisting of a computer equipped with the public domain software NIH Image/ImageJ and a high resolution CCD-camera (Grundig FA 85 I, Fürth, Germany). The following brain areas were analyzed (according to Paxinos and Watson, 1998):

1. Frontal cortical areas: anterior cingulate cortex (ACd, according to Cg1 rostral to the corpus callosum), prelimbic cortex (PL), infralimbic (IL), orbitofrontal cortex (OFC, consisting of the ventral and lateral OFC), posterior cingulate cortex (Cg, according to Cg1 and Cg2 dorsal to the corpus callosum), precentral medial cortex (PrCm, according to M2 rostral to the corpus callosum).
2. Limbic brain areas: dorsal hippocampus (hippo), lateral amygdala (LA), basomedial amygdala (BMA), Nucleus accumbens (NAcc).
3. Sensory and subcortical areas: dorsal striatum, somatosensory cortex (SSC), auditory cortex (AC), dorsal thalamus (thalamus), habenula, periaqueductal gray (PAG).

For each brain area five sections were measured and the values were averaged for statistical analysis. Since no significant differences of 2-FDG-incorporation between the left and right hemisphere were detectable the measurements of both hemispheres were pooled.

In order to compensate for metabolic differences between individual animals 2-FDG-incorporation was expressed as density ratio relative to the uptake in the corpus callosum (relative optical density, rOD). The corpus callosum was selected as reference area, because as a region of white matter it generally displays a uniform, stimulus-independent low glucose utilization. Optical density of the corpus callosum did not vary significantly across the experimental groups.

### STATISTICAL ANALYSIS

Data analysis and diagram compilation were performed with SigmaPlot 11 (Systat Software GmbH, Erkrath, Germany) and JMP, Release 7 (SAS Institute Inc.; Cary/NC, USA). Differences in the density ratios across the experimental groups were analyzed by using a Kruskal–Wallis One-Way ANOVA (degrees of freedom = 3), which was followed by a Mann–Whitney *U*-test for pair-wise comparisons.

In addition to the group comparisons, patterns of "correlated functional activity" were calculated for every single behavioral condition. This was achieved by calculating the correlation coefficients between the rODs (inter-regional correlations). The correlations were performed using Spearman's rank correlation coefficient, usually called "Spearman's rho (ρ)," which is a measure of statistical dependencies between non-parametric variables. A Spearman correlation of 1 results when the two variables are monotonically—not linearly—related (−1 indicates significant inverse relationship). To illustrate the strengths and direction of the inter-regional correlations, mosaic plots were compiled ($\alpha = 5\%$).

## RESULTS

### GROUP COMPARISONS OF METABOLIC BRAIN ACTIVITY

One-Way ANOVA revealed significant differences in the metabolic brain activity between the experimental animals in the frontal cortical areas ACd ($p \leq 0.001$, $H = 20.701$), Cg ($p \leq 0.001$, $H = 25.639$), IL ($p \leq 0.001$, $H = 22.037$), PL ($p \leq 0.001$, $H = 25.605$) PrCm ($p \leq 0.001$, $H = 23.006$), and OFC ($p \leq 0.05$, $H = 7853$), the limbic areas hippocampus ($p \leq 0.001$, $H = 22.748$), LA ($p = 0.032$, $H = 8.803$), and BMA ($p \leq 0.002$, $H = 14.707$) and the sensory and subcortical areas dorsal striatum ($p \leq 0.001$, $H = 24.692$), NAcc ($p \leq 0.001$, $H = 20.306$), SSC ($p \leq 0.001$, $H = 21.105$), AC ($p \leq 0.001$, $H = 17.344$), dorsal thalamus ($p \leq 0.001$, $H = 23.009$) and PAG ($p \leq 0.002$, $H = 14.888$). No significant alterations were found in the habenula.

Applying a Mann–Whitney *U*-test for post-hoc pair-wise statistical analysis we observed the following differences between the individual experimental groups:

### EFFECTS OF SEPARATION STRESS ON METABOLIC BRAIN ACTIVITY

#### *Frontal cortical areas*

In general, separation stress decreased metabolic activity in all analyzed prefrontal cortical areas. Compared to unstressed controls ("parents") a significantly ($p \leq 0.05$) reduced brain activity was measured in the ACd, Cg, PL, and PrCm of the "litter separation" and "individual separation" group (**Figures 1** and **4**). In the IL only the "individual separation" group showed a significant decrease compared to the "parents" group. No significant differences were found in the OFC. Compared to the pups from the "litter separation" group the "individual separation" group showed a more pronounced ($p \leq 0.05$) decrease of brain activity in the Cg, PL and PrCm but not in the ACd (**Figures 1** and **4**).

#### *Limbic brain areas*

Similar to the observations in the frontal cortical areas a significant ($p \leq 0.05$) reduction of brain activity was measured in the hippocampus and NAcc of the "litter separation" and "individual separation" group compared to the unstressed "parents" group (**Figures 2** and **4**). Again, the "individual separation" group showed a more pronounced ($p = 0.05$) reduction of brain activity in the hippocampus and NAcc compared to the "litter separation" group. No significant stress-induced differences were found in the amygdala (LA and BMA) and in the habenula (**Figure 2**).

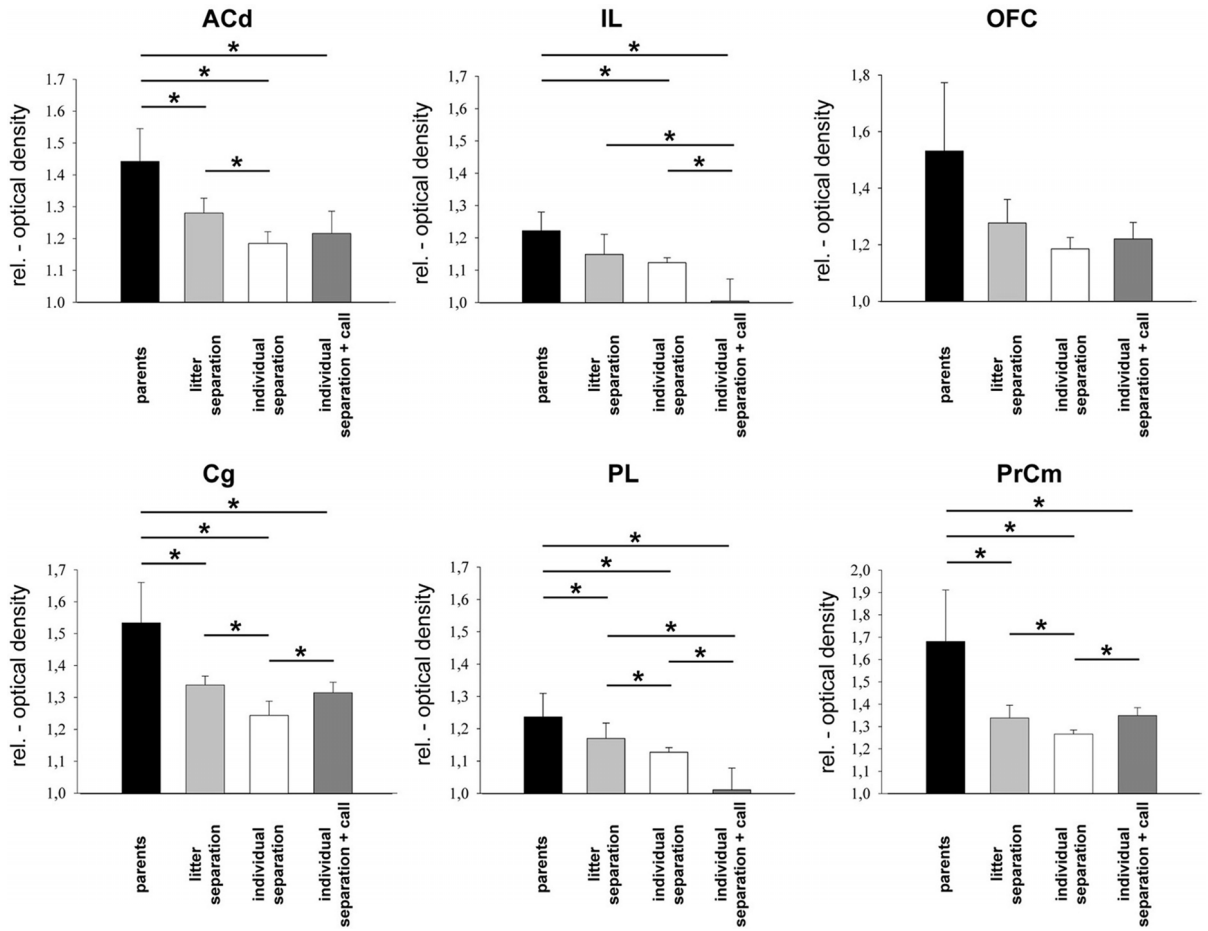


**FIGURE 1 | Relative optical density of frontal cortical areas in the four experimental groups.** *$p \leq 0.05$, Mann–Whitney *U*-test. ACd, anterior cingulate cortex; Cg, cingulate cortex; IL, infralimbic cortex; OFC, orbitofrontal cortex; PL, prelimbic cortex; PrCm, precentral medial cortex.

### *Sensory and subcortical areas*

In the dorsal striatum, somatosensory and AC and in the dorsal thalamus a significant ($p \leq 0.05$) reduction of brain activity was measured in the "litter separation" and "individual separation" group compared to the unstressed "parents" group (**Figures 3** and **4**). Also, in the dorsal striatum, SSC and thalamus the "individual separation" group showed a more pronounced reduction of brain activity compared to the "litter separation" group ($p \leq 0.05$). In contrast to all other brain areas the PAG showed a significant increase of brain activity in the "individual separation" group compared to the "parents" and "litter separation" group ($p \leq 0.05$) (**Figure 3**).

## MATERNAL VOCALIZATIONS ALTER STRESS-INDUCED CHANGES IN METABOLIC BRAIN ACTIVITY

### *Frontal cortical areas*

In the PrCm and Cg the presentation of maternal vocalizations during exposure to separation stress ("individual separation + call" group) resulted in a significant ($p \leq 0.05$) increase of brain activity compared to the "individual separation" group (**Figure 1**). In line with our prediction metabolic activity in the PrCm and Cg was upregulated in the "individual separation + call" group to similar levels as observed in the "litter separation group." The ACd of the "individual separation + call" group displayed reduced brain activity compared to the unstressed "parents" group ($p \leq 0.05$).

Opposite effects were observed in the IL and PL, where the presentation of maternal vocalizations induced a further downregulation of brain activity compared to the three other experimental groups ($p \leq 0.05$) (**Figure 1**). Again, no significant effects were observed in the OFC.

### *Limbic brain areas*

Similar to the findings for the prefrontal PL and IL presentation of maternal vocalizations during individual separation resulted in a significant ($p \leq 0.05$) decrease of brain activity in the hippocampus and in the BMA compared to the three other experimental groups (**Figure 2**). In the LA a significant decrease of brain activity was observed only compared to the "litter separation" and "parents group." In contrast, the NAcc of the "individual separation + call" group showed a significant ($p = 0.05$) increase of brain activity compared to the "individual separation" group. No significant effects were observed in the habenula.

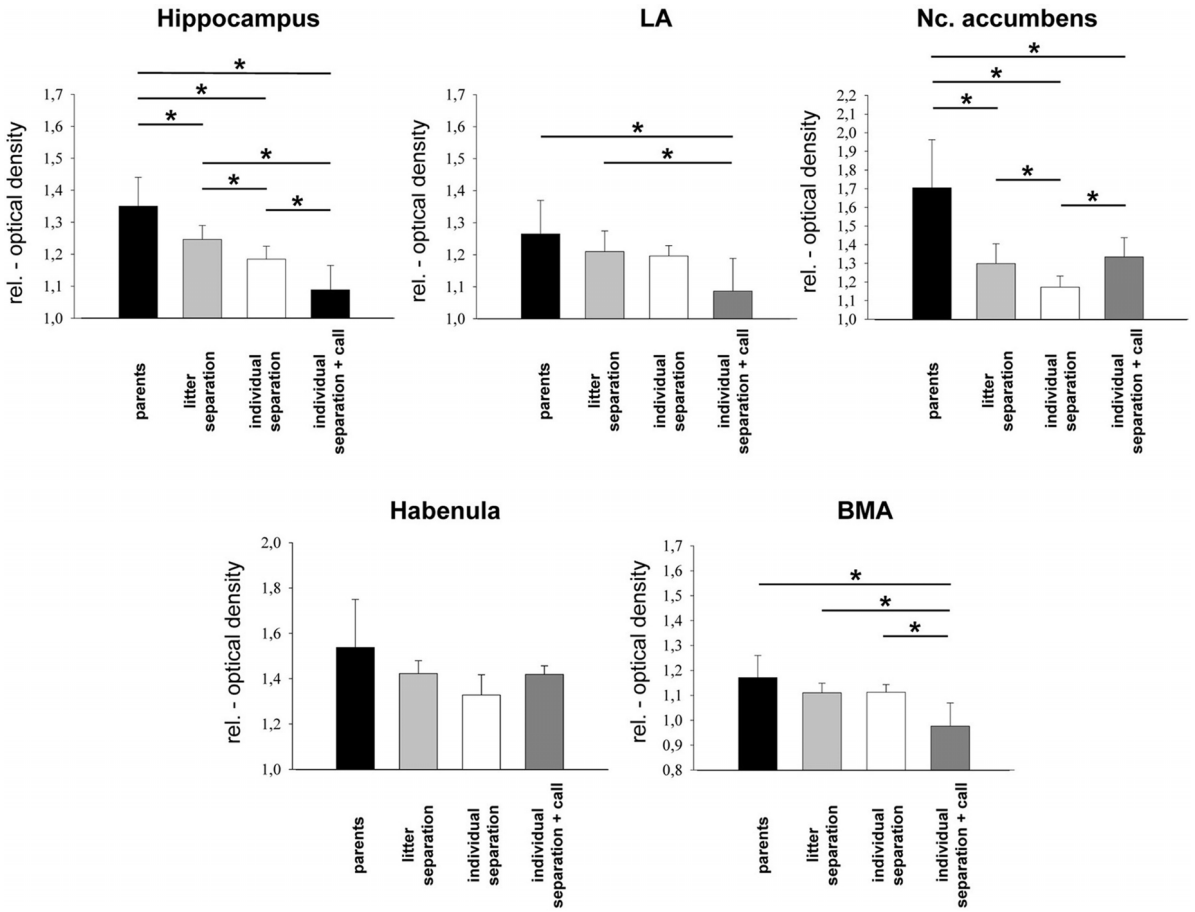


**FIGURE 2 | Relative optical density of limbic brain areas in the four experimental groups.** *$p \leq 0.05$, Mann–Whitney *U*-test. BMA, basomedial amygdala; LA, lateral amygdala.

### *Sensory and subcortical areas*

In the dorsal striatum and in the somatosensory and auditory cortex of the "individual separation-call" group the presentation of maternal vocalizations during the exposure to separation stress resulted in a significant ($p \leq 0.05$) increase of brain activity compared to the "individual separation" group (**Figure 3**). Brain activity in these brain areas of the "individual separation + call" group was upregulated to the levels measured in the "litter separation" group. In the PAG of the "individual separation-call" group the presentation of maternal vocalization induced a significant decrease of metabolic brain activity compared to the "individual separation" group ($p \leq 0.05$) and completely restored metabolic activity to the levels measured in the "litter separation" and "parents" groups (**Figure 3**).

## SEPARATION STRESS ALTERS THE FUNCTIONAL COUPLING OF METABOLIC BRAIN ACTIVITY

Calculation of the correlation coefficients (Spearman's rho, $\alpha = 5\%$) between the relative optical densities revealed a distinct pattern of inter-regional correlations for each experimental condition (**Figures 5–10**). Overall, in all experimental groups we found that the number of significant positive correlations between the analyzed brain regions was higher than the number of significant negative correlations. The highest degree of inter-regional correlation was found in the animals from the parents group (**Figure 5**). Among the frontal cortical areas the ACd, OFC, Cg, and PrCm displayed a high degree of positive correlations with the limbic areas, particularly hippocampus, BMA, and habenula and with the analyzed subcortical and sensory areas (**Figures 6–8**) A high number of positive correlations could also be observed among the limbic areas (**Figures 8–10**), with exception of the LA and also among the subcortical and sensory areas. The PAG showed positive correlations with the OFC, hippocampus, habenula, striatum, NAcc, and the thalamus (**Figure 10**). The prefrontal PL and IL displayed no positive correlations to the other brain areas but PL and IL were positively correlated with each other (**Figures 6** and **7**)

In comparison to the animals from the parents group the pattern of inter-regional correlations changed dramatically in the litter separated and individually separated animals (**Figure 5**). In general, the degree of correlated activity decreased in both separation groups. In the litter separation animals this effect was most prominent for the OFC (**Figure 7**), the BMA (**Figure 9**) and the analyzed subcortical and sensory areas. For the PAG the only

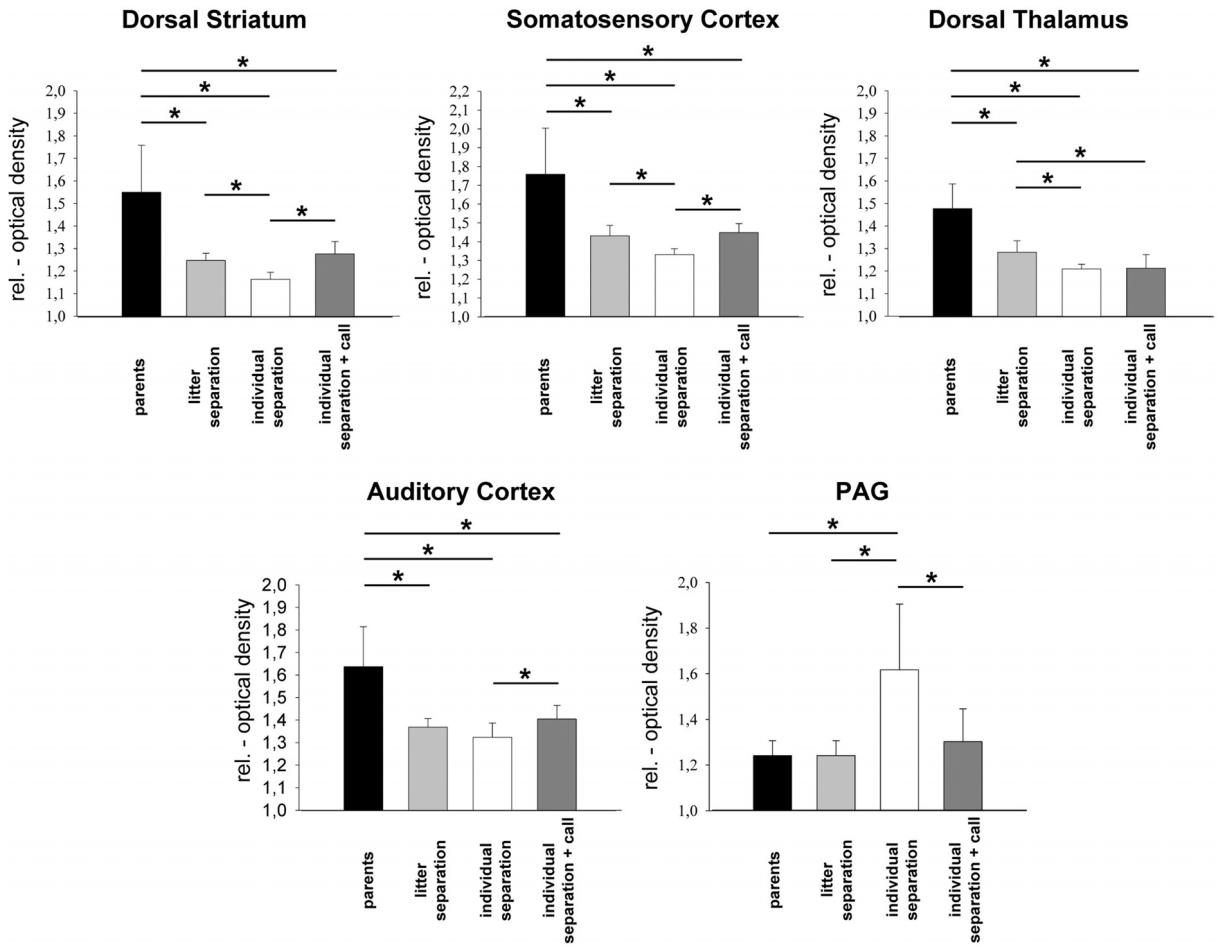


**FIGURE 3 | Relative optical density of sensory and subcortical brain areas in the four experimental groups.** $^*p \leq 0.05$, Mann–Whitney *U*-test. PAG, periaqueductal gray.

positive correlations were found with the hippocampus and thalamus (**Figure 10**). In the individually separated animals the degree of positive inter-regional correlation was relatively low (**Figure 5**), however a very distinct pattern was observed since there was a strong coupling among the hippocampus, LA, and BMA, which could not be observed in the other three experimental groups (**Figures 8** and **9**). Also, a number of positive correlations were found for the PAG, such as positive correlations with the frontal areas ACd and PrCm, which were absent in the other experimental groups (**Figure 10**). Interestingly, as opposed to all other experimental conditions, a high number of negative correlations were observed in the individually separated animals, particularly between the hippocampus, LA and BMA and the frontal IL and PrCm as well as with the striatum and NAcc (**Figures 5**, **7**–**10**). Also, the negative correlations of the PAG with the hippocampus and amygdala were only found in this experimental group (**Figure 10**).

A relatively low degree of inter-regional correlation was also found in the animals that were exposed to maternal vocalizations during separation (**Figure 5**). However, a coupling of activity was found for the frontal areas ACd, PL, IL, and OFC among each other, a pattern that was completely absent in the other experimental groups (**Figures 6** and **7**). Interestingly, in this group we found no correlations of the PAG with the other analyzed brain areas (**Figure 10**).

## DISCUSSION

Although, there is clear evidence that early traumatic experiences interfere with the maturation of endocrine and behavioral function in rodents, non-human primates, and humans and thereby are risk factors for the development of later psychopathologies (Heim and Nemeroff, 2001; Sanchez et al., 2001; Stevens et al., 2009; Loman and Gunnar, 2010; Bock and Braun, 2011), there is a paucity of information about the immediate, acute effects of these adverse situations on brain activity. It was proposed that emotions are part of an evolutionarily set neural mechanism, which serves to maintain an organism's homeostasis (Damasio et al., 2000), and there is evidence that emotion-evoked neural patterns constitute multidimensional maps of the organism's internal state.

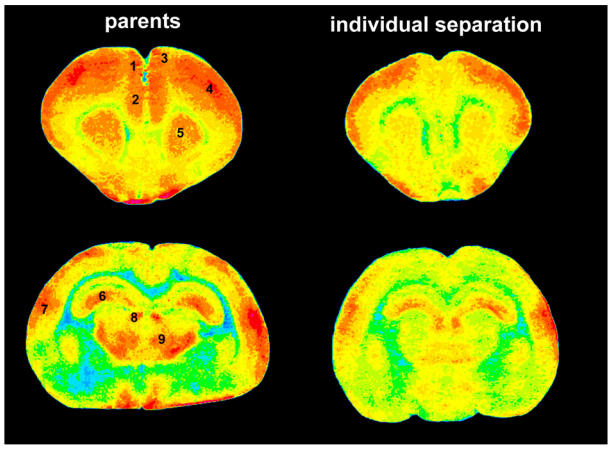


**FIGURE 4 | Pseudocolor images of anterior (top) and posterior (bottom) brain slices indicating 2-FDG uptake as measurement of brain activity.** *Red colors* indicate high levels of 2-FDG uptake (high brain activity) *green and blue colors* indicate low levels of 2-FDG uptake (low brain activity). Obviously, there is a dramatic decrease of brain activity in individually separated animals (*right panel*) compared to animals that stayed together with their parents (*left panel*). 1, ACd; 2, PL; 3, PrCm; 4, somatosensory cortex; 5, striatum; 6, hippocampus; 7, auditory cortex; 8, habenula; 9, thalamus.

Thus, the aim was to identify the activity within the affective brain circuits in the infant degu brain during different separation stress conditions, and to unveil interregional functional coupling within the stress-responsive brain circuitries. Our findings reveal for the first time that stress exposure induces widespread alterations of brain activity and changes in interregional coupling in the infant brain.

## METHODOLOGICAL CONSIDERATIONS

Functional imaging of emotional responses in awake, freely behaving animals is challenging since most functional imaging techniques either require the sedation of the animals (fMRI), which limits the spectrum of evocable emotional states and their detectability, or they do not provide sufficient spatial resolution (PET, SPECT) to distinguish small brain regions such as the amygdala and prefrontal cortical regions and their subdivisions. The 2-FDG autoradiography allows to image emotional brain responses while the animal is awake and actively interacts with its environment, and this technique provides sufficient spatial resolution to distinguish between subregions of the prefrontal cortex, hippocampus, the amygdala, and other areas. 2FDG is an analog of glucose, which is transported into cells by glucose transporters, phosphorylated by hexokinase and not further metabolized. The 2-FDG-phosphate accumulates intracellularly, which allows a quantitative assessment of regional glucose utilization as a measure of functional activity. Thus, the metabolic activity, which is measured, reflects both, glial and neuronal activity because neurons recruit their energy from direct glucose uptake as well as indirect glucose utilization via uptake of astrocytic lactate (Chih et al., 2001; Magistretti, 2006). One limitation of this method is that it only allows group comparisons of activity changes since it is not possible to test different time points in the same animal, and therefore the stress-induced changes cannot directly be correlated with baseline levels in the same individual.

The analysis of interregional correlations provides a sensitive tool for detecting subtle changes in functional coupling of activity in a region-specific manner (e.g., the differential correlation patterns of the two amygdala subregions in the "parent" situation). As cautionary note it should be pointed out that the large number of correlations and the small N per group may on one hand raise the possibility of spurious effects in some brain areas and on the other hand of insufficient N/power for detecting significant effects in other brain areas.

## DECREASED BRAIN ACTIVITY DURING SEPARATION STRESS

Separation of the entire litter from the home cage and both parents for 1 h induced a dramatic downregulation of brain activity in a number of brain areas, and this effect was much more pronounced in pups, which were individually separated from their siblings. Decreased activity was found in all analyzed frontal and sensory cortical areas, which supports the view that, similar to humans, emotionality in animals also includes cortico-perceptive aspects (Panksepp, 2003; Wright and Panksepp, 2011). In addition, decreased activity was detected in the hippocampus and in the striatum including NAcc, and in the thalamus. As stated by Panksepp (2003) emotional processes include motor-expressive (e.g., striatum), sensory-perceptual (e.g., somatosensory, AC, thalamus), autonomic-hormonal, cognitive-attentional (prefrontal regions), and affective-feeling (e.g., cingulate, n. accumbens, PAG and amygdala) aspects. The altered activity and functional coupling (see below) observed in the stressed animals shows that many of the brain regions, which mediate these different components of emotionality are "shut down" during stress, which may be indicative of a "panic-type" emotional status. This interpretation is supported by the observation that, in contrast to all other brain areas, metabolic activity was increased in the PAG in the most severely stressed group (individual separation) and also reflected by distress vocalizations (unpublished observations). The PAG is a key component of the basic emotional systems contributing to basic emotions in the mammalian brain such as negative emotions as rage, fear and panic, but also positive emotions as lust, care and play (Panksepp, 2011). In particular, the PAG is a mediator of different defensive responses such as freezing during fear and anxiety in adult as well as in young animals (Graeff, 2004; Brandão et al., 2008; Wiedenmayer, 2009).

Acute decreases of local cerebral glucose uptake, as a measure of neuronal activity, were also described in adult mice as an acute consequence of restraint stress (Warnock and Steckler, 2011). Similar to our findings in degu pups restraint stress reduced local cerebral glucose uptake in frontal cortical areas, the thalamus, and the hippocampus. In line with our findings, a recent study using cytochrome oxidase activity as a measure of long-term changes in brain metabolic capacity after 2 weeks of exposure to repeated separation stress, reports decreased activity in the medial prefrontal cortex and NAcc in two week old mouse pups (Spivey et al., 2011). In addition, fMRI and PET studies revealed deactivation of the hippocampus, medio-OFC and anterior cingulate cortex in human subjects during exposure to a psychosocial stressor (Pruessner et al., 2008). The deactivation of the hippocampus

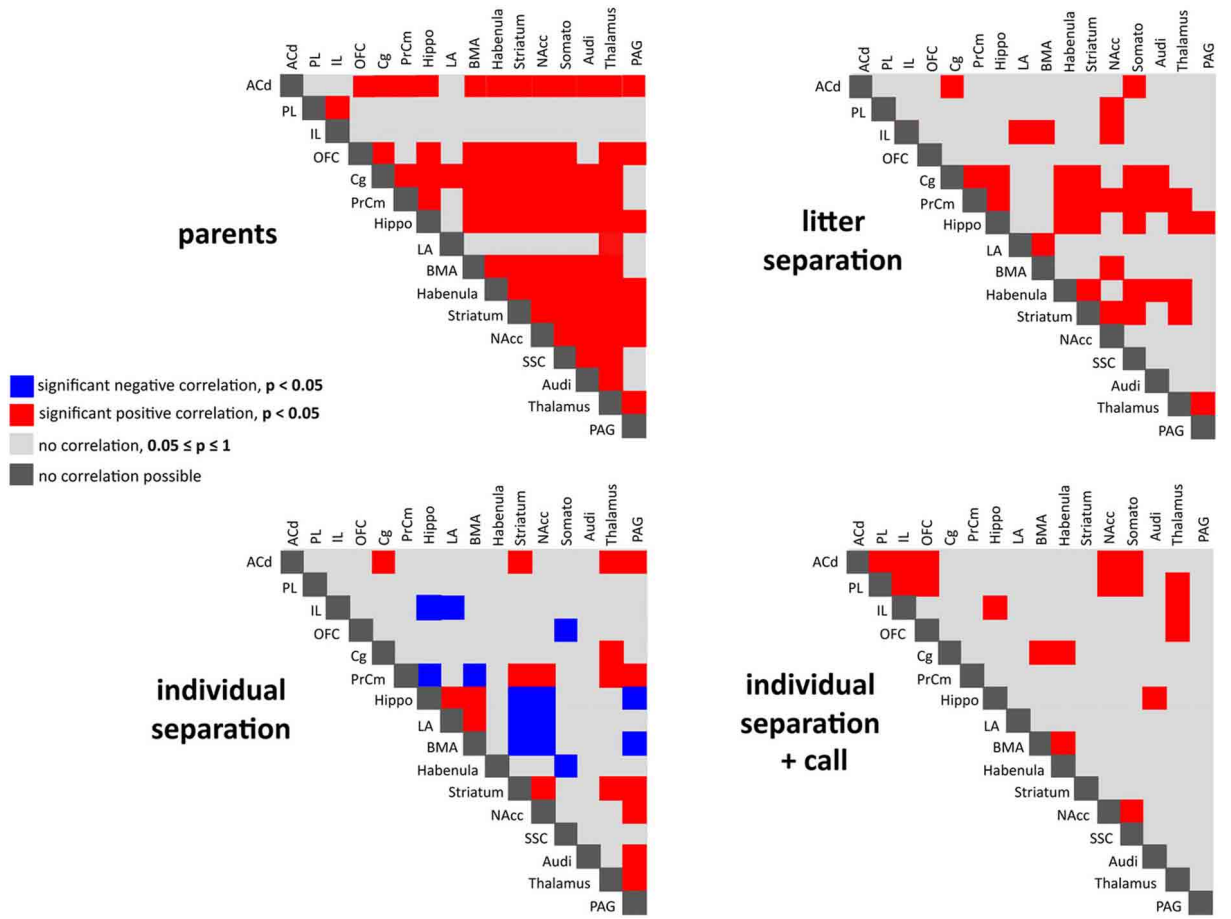


**FIGURE 5 | Interregional correlations of metabolic brain activity reflecting functional coupling of the analyzed brain areas in the four experimental groups.** Significant positive (*red*) and negative (*blue*) correlations analyzed using Spearman's rank correlation coefficient ($p < 0.05$).

in this study was directly correlated with the release of cortisol in response to the stress task. Therefore, it is tempting to speculate that the observed deactivations in our study may be caused by high levels of stress hormones such as cortisol. This hypothesis is supported by our findings that show dramatic increases of cortisol during separation stress in degu pups (Gruss et al., 2006). There is some evidence in hippocampal cell cultures and in peripheral tissues that glucocorticoids can inhibit glucose uptake into neurons and astrocytes (Munck, 1971; Horner et al., 1990; Virgin et al., 1991).

Surprisingly, some of the major emotional brain areas, including the amygdala and the habenula did not show metabolic changes in response to separation stress. This is in line with findings from 2-DG studies using swim stress in adult rodents and a PET study in infant monkeys using separation stress, where no changes of amygdala activity could be identified (Duncan et al., 1993; Rilling et al., 2001). Since there is evidence that fear-related amygdala activity attenuates with time (LaBar et al., 1998; Phelps et al., 1998), the stress-induced changes in the amygdala may either not be detectable with the PET and 2-DG techniques due to their limited time resolution (Rilling et al., 2001), or the amygdala just does not respond under these experimental stress conditions, perhaps also due to its immaturity in the infant brain (but see results of the correlation analyzes).

The higher variability of the animals in the "family" group compared to the other groups may be due to the different social interactions of the individual pup within the family setting during the 2-FDG experiment. While the behavioral options of the stressed animals were much more limited and focused on the stressful situation resulting in a more coherent activation pattern, the behavior of the pups in the "family" group may have been more variable, e.g., some pups might have been cuddling with a parent or siblings (low activity), others might have been playing (higher activity).

### MOTHER'S VOICE CHANGES BRAIN ACTIVITY DURING SEPARATION

The maternal voice is a positive emotional stimulus (Braun and Scheich, 1997; Poeggel and Braun, 1997; Braun and Poeggel, 2001), which on the behavioral level has been shown to exert an "anxiolytic" effect (Braun et al., 2003). Thus, another aim was to test the hypothesis that mother's vocalizations should "buffer," that is, ameliorate, or normalize stress-induced metabolic changes. As predicted we found that maternal vocalizations induced an increase in brain activity in the prefrontal

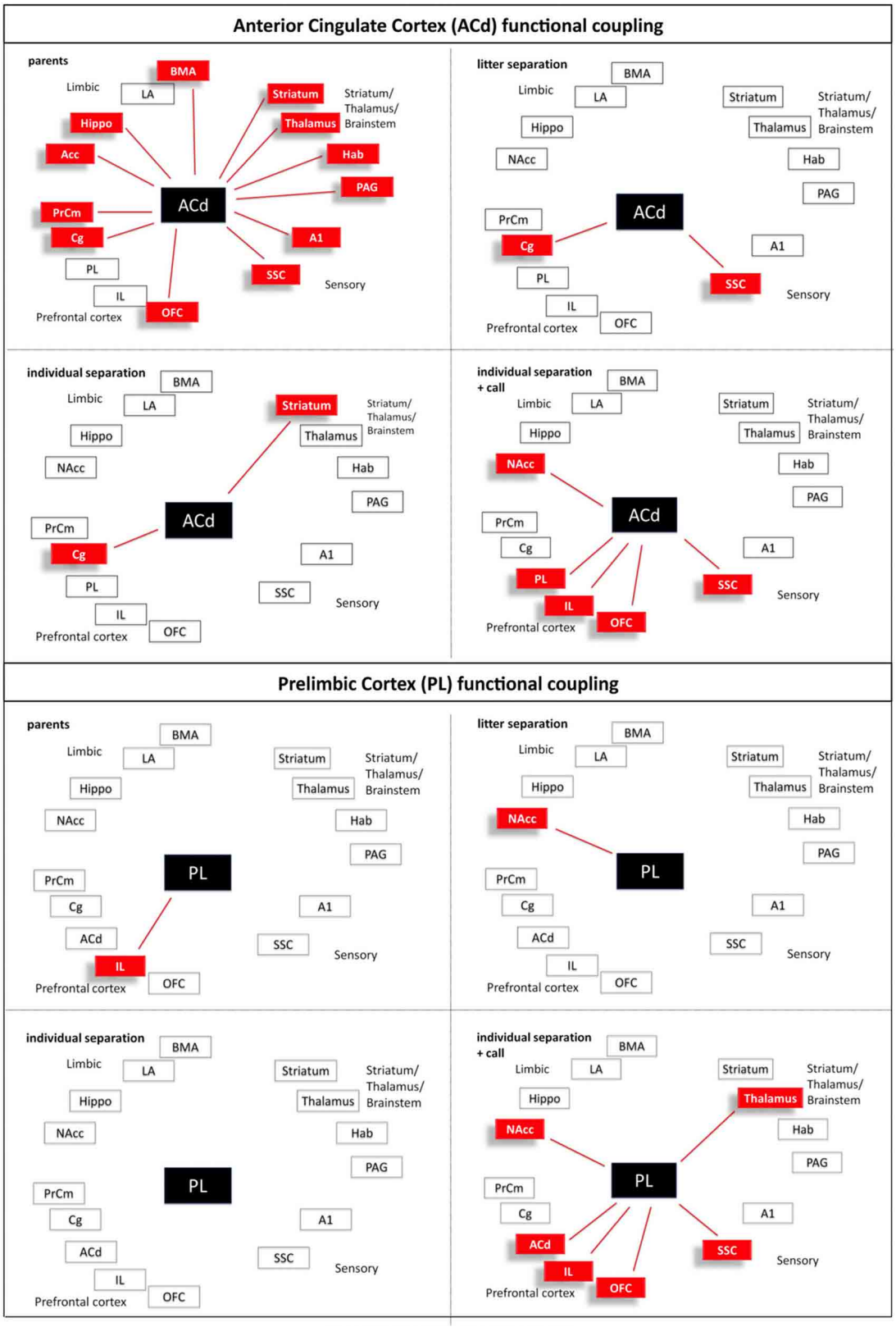


**FIGURE 6 | Interregional functional coupling of the anterior cingulate (ACd) and prelimbic (PL) cortex under different stress conditions.** *Red color* indicates positive correlations ($p < 0.05$).

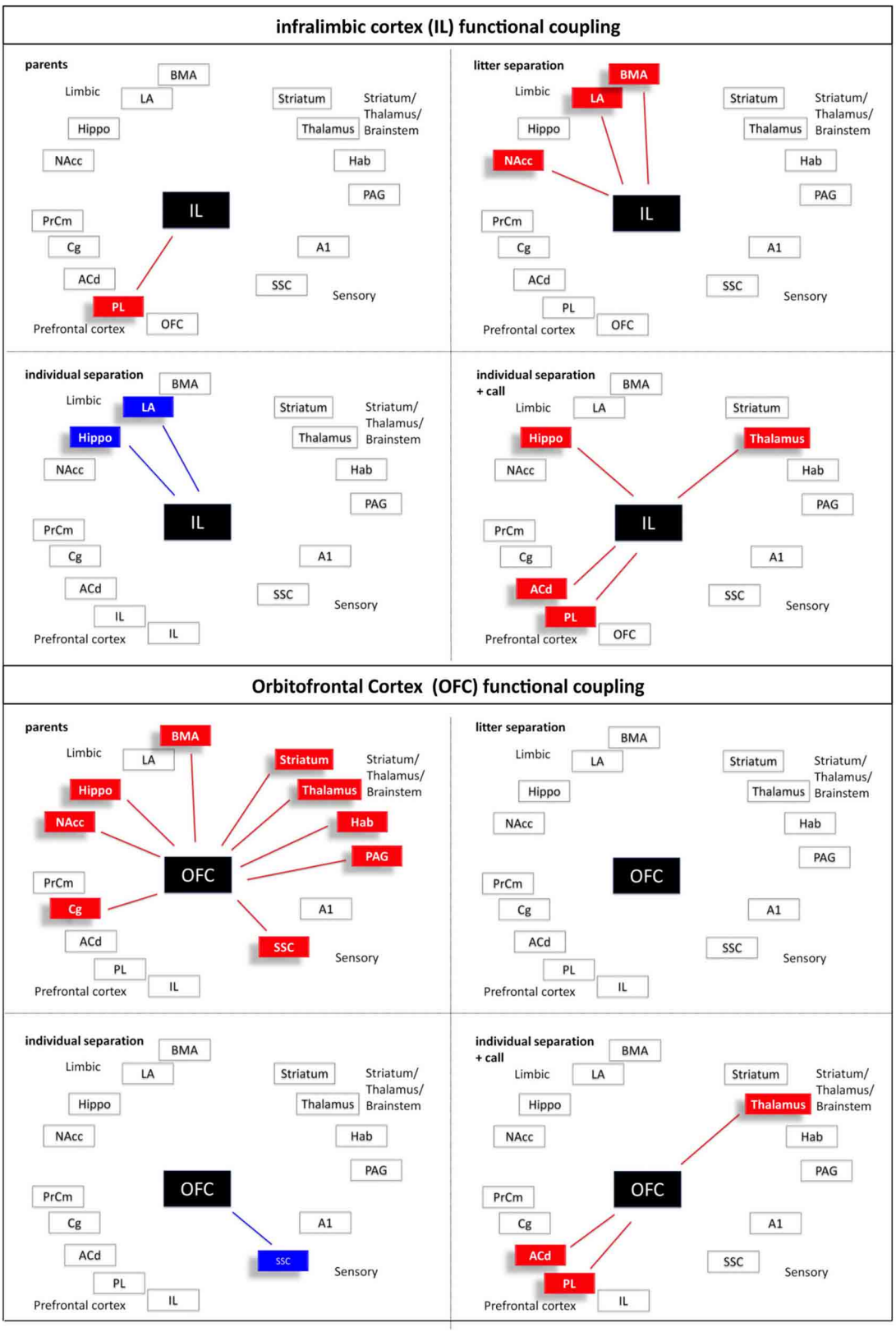


**FIGURE 7 | Interregional functional coupling of the infralimbic (IL) and orbitofrontal (OFC) cortex under different stress conditions.** *Red color* indicates positive, *blue color* indicates negative correlations ($p < 0.05$).

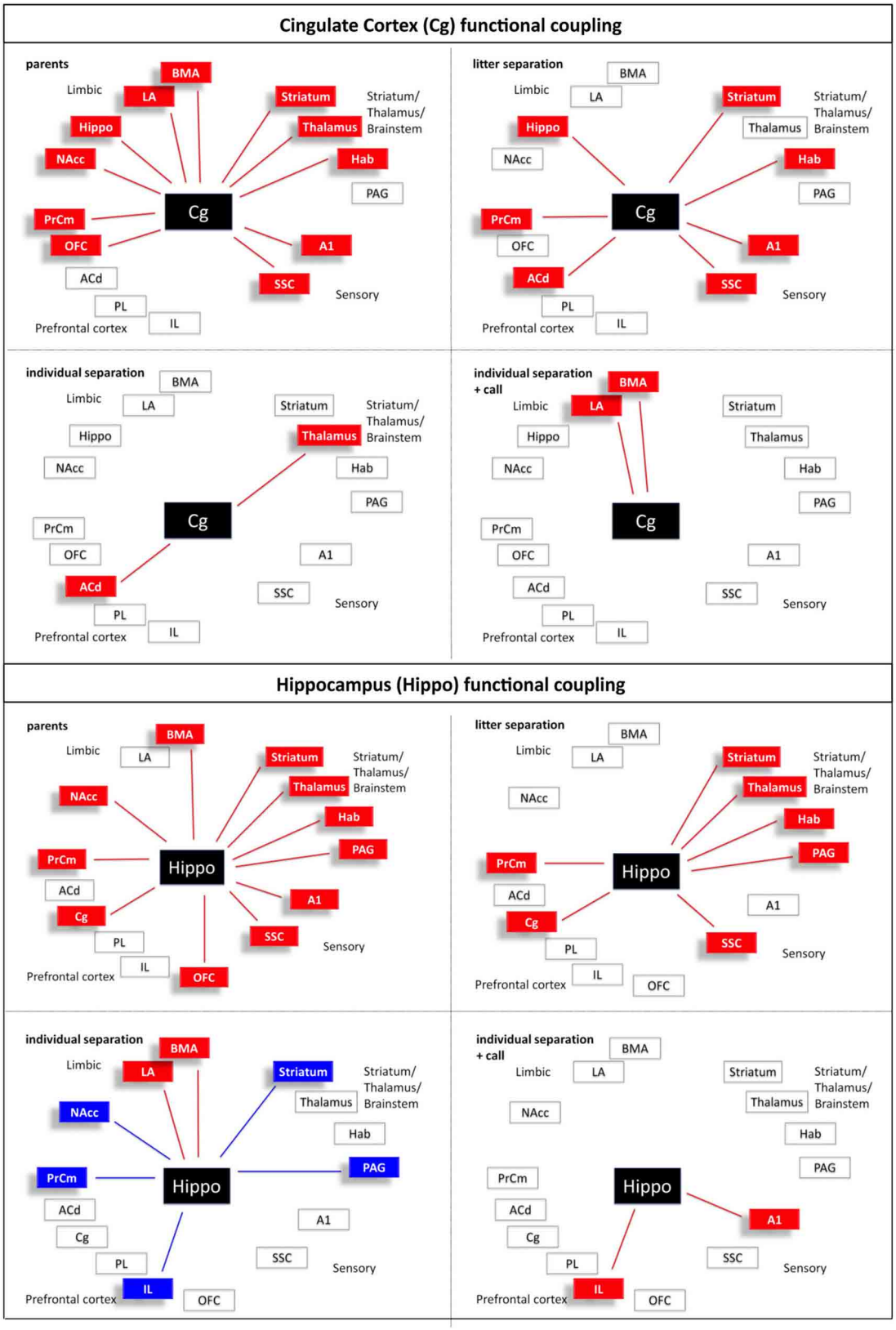


**FIGURE 8 | Interregional functional coupling of the cingulate cortex (Cg) and hippocampus (Hippo) under different stress conditions.** *Red color* indicates positive, *blue color* indicates negative correlations ($p < 0.05$).

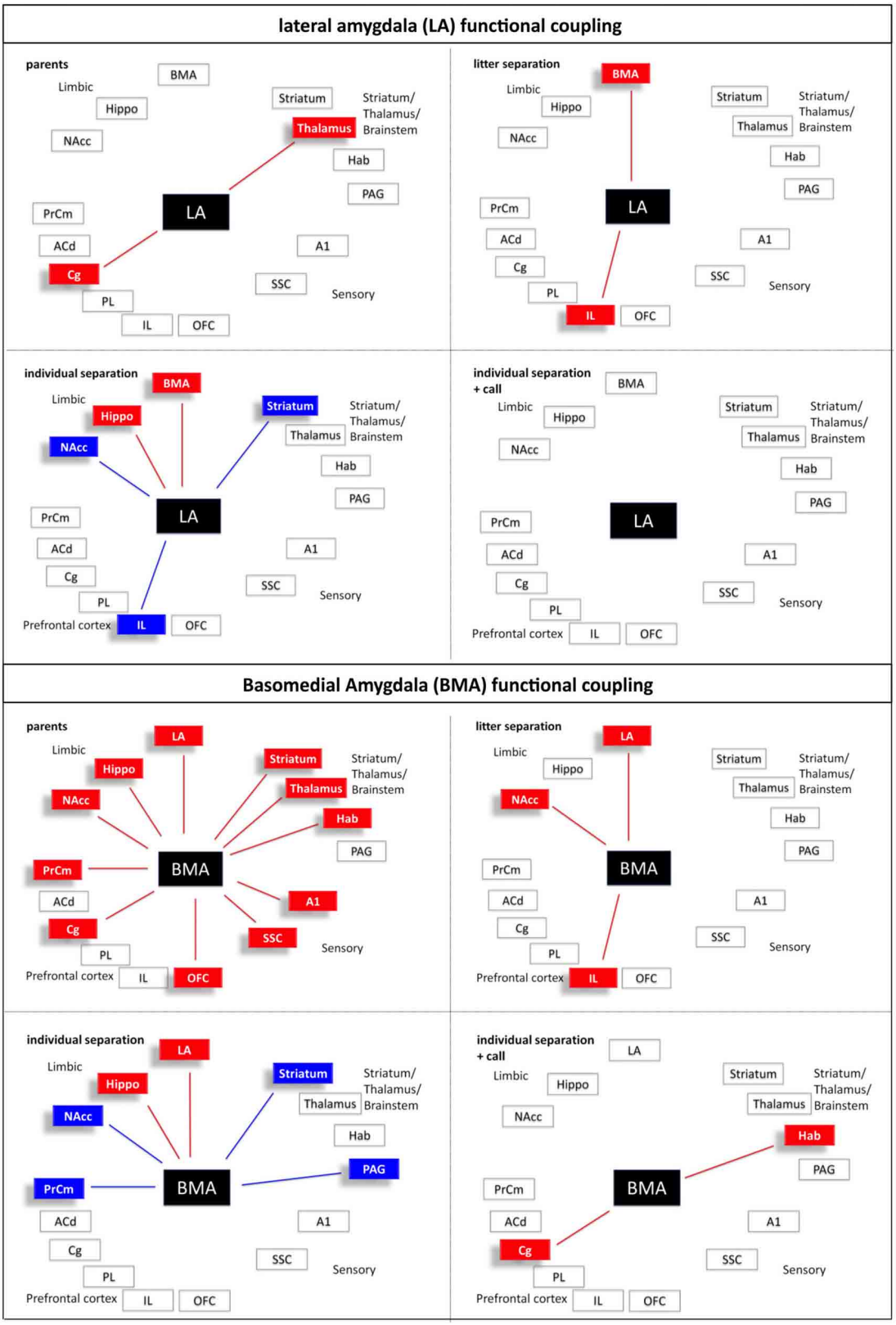


**FIGURE 9 | Interregional functional coupling of the lateral (LA) and basomedial (BMA) amygdala under different stress conditions.** *Red color* indicates positive, *blue color* indicates negative correlations ($p < 0.05$).

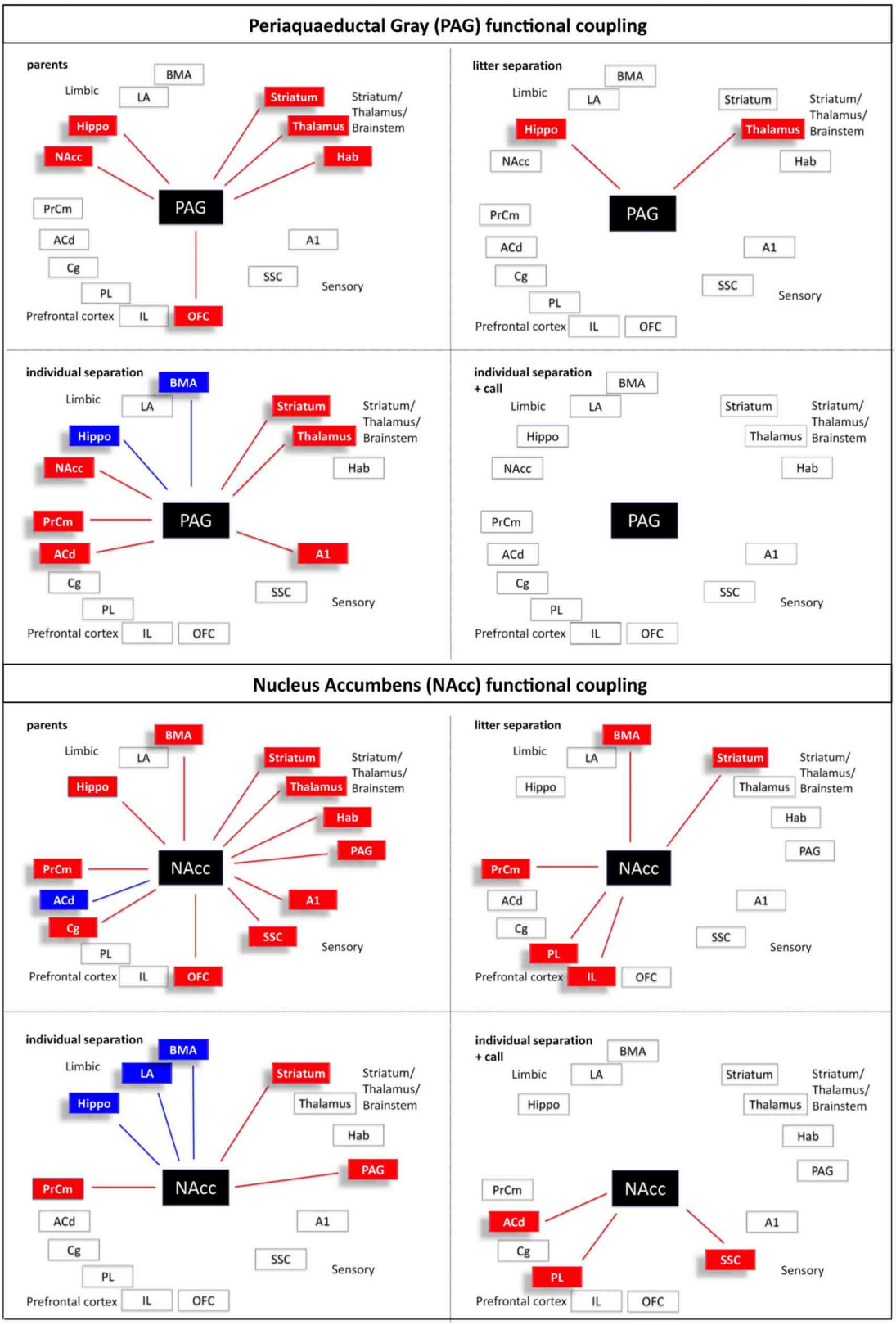


**FIGURE 10 | Interregional functional coupling of the periaqueductal gray (PAG) and nucleus accumbens (NAcc) under different stress conditions.** *Red color* indicates positive, *blue color* indicates negative correlations ($p < 0.05$).

Cg and PrCm, the striatum and NAcc, and in the somatosensory and auditory cortex compared to the individually separated animals without acoustic stimulation. In all these brain areas brain activity was restored back to the levels of the litter separation group, but did not reach activity levels seen in the parents (control) group. In contrast, brain activity in the prefrontal PL and IL and in the limbic hippocampus and amygdala was reduced upon presentation of maternal vocalizations. The stress-induced enhanced activation of the PAG, which specifically was observed in the individual separation group and most likely reflects a panic-type emotional status, was restored back to the activity levels of the litter separation and parents groups. The stress- and anxiety "buffering" effect of maternal vocalizations has also been found in other experiments where it seems to protect the infant brain from stress-induced changes. For example it has been shown in degu pups that short periods of separation stress increase the density of distinct neurotransmitter receptor subpopulations such as NMDA-, serotonin- and dopamine receptors in prefrontal ACd, PrCm, PL, and IL and hippocampus and amygdala, which were normalized by the presentation of maternal calls (Ziabreva et al., 2000, 2003a,b).

#### ALTERED INTERREGIONAL FUNCTIONAL COUPLING DURING SEPARATION STRESS AND IN RESPONSE TO MOTHER'S VOICE

Effective information processing, learning and also the execution of social or other complex behaviors is not restricted to the function or activity of single brain regions, but is the result of functional interactions between large-scale distributed neural systems (McIntosh and Gonzalez-Lima, 1998; Nair and Gonzalez-Lima, 1999; Nair et al., 2001). Moreover, there is considerable evidence that sadness in humans evokes a similar arousal pattern as the separation distress vocalization circuit in rodents (Damasio et al., 2000; Panksepp, 2003, 2011). Some aspects of these "emotional" maps, such as those in the brainstem, may not be directly accessible to consciousness, as opposed to the prefrontal and cingulate cortex, regions that receive regulatory signals from brainstem and hypothalamus as well as sensory signals (Damasio et al., 2000). Our correlation analysis in stressed infant degus revealed that similarly complex changes in activation/inactivation and functional coupling patterns occur within the same brain circuits as those in the human brain. Furthermore, we show for the first time that a positive, "comforting" emotional stimulus (mother's voice), which ameliorates the status of stress and panic in the animal, recruits very different functional coupling patterns than those seen in the unstressed parent control group.

In line with our hypothesis the patterns of correlated activity dramatically changed in the different stress situations compared to the parent's situation. In the parents group a high level of positive interregional correlations was observed especially between the prefrontal OFC, Cg, and PrCm and limbic areas. Also, the PAG showed positive correlations to OFC, hippocampus, habenula, striatum, NAcc, and thalamus. Overall, the number of positive correlations decreased in the two stressed (litter and individual separation) groups, indicating that the functional coupling within these circuits becomes increasingly disturbed. Particularly the OFC and the cingulate cortex, areas that are strongly related to executive function and decision-making, are almost completely uncoupled from the other brain regions. This uncoupling of prefrontal from limbic regions may indicate that during stress the prefrontal control of limbic structures such as the n. accumbens and the amygdala is lost. As a consequence, this may reduce the prefrontal cognitive control of fear- and anxiety-like behavior (distress vocalizations, freezing) and thereby change into a panic-like emotional state. This interpretation is supported by the finding that specifically during the most severe stress condition (individual separation) the PAG is functionally coupled to the amygdala (BMA subregion) in a negative manner, and in a positive manner with prefrontal subregions (ACd and PrCm) and the thalamus. This functional coupling may be mediated by direct connections of the PAG to the respective areas, since it receives afferent connections from the amygdala and medial prefrontal and cingulate cortex and sends efferent connections to parts of the thalamus (Linnman et al., 2012). Quite similar to our findings a fMRI human study provides evidence that an imminent threat elicits activity in the midbrain, including the PAG, that is paralleled by an increased coupling of the midbrain with the mid-dorsal anterior cingulate cortex, and decreased coupling with the amygdala and hippocampus (Mobbs et al., 2009).

It is hypothesized that the amygdala is a key structure for integrating cognitive and emotional neuronal networks (Pessoa, 2008). Interestingly, our imaging study revealed that the two amygdala subnuclei display very different functional coupling patterns. Similar to the OFC and ACd the BMA, which in the parent group shows a widespread coupling with almost all other regions, almost completely decouples under stress (litter separation group). With increasing stress (individual separation) a few "reconnections" occur compared to the parent group (e.g., with the hippocampus and the LA), but also some negative correlations emerge (e.g., with striatal regions including n. accumbens, the PrCm and the PAG). This coupling is "given up" when maternal calls are presented, when the BMA activity only correlates with the habenula and the cingulate cortex, the latter of which may indicate that conscious attentional and interpretative components are recruited. Some of these coupling/decoupling effects may be mediated by hormonal mechanisms. Studies in humans revealed that corticosteroid application decreased positive as well as negative functional coupling of the amygdala to brain regions involved in the initiation and maintenance of the stress response and to those, which exert executive control (Henckens et al., 2011).

In line with the view that the presentation of maternal calls as a positive emotional stimulus can ameliorate the stress level, we expected to see in the individual separation + maternal call group a partial return to the correlation patterns seen in the litter separation or parents groups. In contrast, the presentation of maternal vocalizations induced very different interregional correlations. The perception and behavioral response to the emotional acoustic stimulus recruits the positive functional coupling among the prefrontal and cingulate regions ACd, PL, IL, and OFC, which may reflect the enhanced attention and goal-directed orientation response toward the acoustic stimulus as observed in behavioral studies (Braun et al., 2003). Additional evidence for the anxiolytic effect of the maternal calls is the complete decoupling of the PAG

from the other brain areas. The PAG is part of emotional circuits mediating fear and panic (Panksepp, 2011). This is in line with the high degree of functional coupling of the PAG with prefrontal and limbic brain areas. Reaction to the maternal calls might reduce the feelings of fear and panic in the infant pups resulting in decoupling of the PAG from the respective systems.

It is important to note that the most severe stress situation (individual separation) is the condition where the animal most likely experiences panic, and that this is the only condition in which negative functional coupling occurred. The hippocampus, the LA, and BMA are positively coupled among each other, and under the most severe stress condition these three regions become negatively coupled to major regions of the fear circuitry (medial prefrontal cortex, n. accumbens, PAG) (Rodrigues et al., 2009) and the striatum. This stress/panic-induced negative coupling has not been previously described in human or animal studies. The negative coupling patterns might either map the functional circuitries while panic is experienced, or these maps rather reflect compensatory brain mechanisms to reduce anxiety/panic and regain emotional homeostasis.

## CONCLUSIONS

A concerted interregional activity is crucial for the activity-dependent maturation of cortical networks, and disturbances or deviations of these networks are related to neurodevelopmental disorders and neurological disease (Uhlhaas et al., 2009; Stam and van Straaten, 2012). Thus, deviations in functional coupling such as those occurring during separation stress, and most likely also under repeated or chronic stress conditions interfere with these developmental processes and eventually may result in dysfunctional neuronal networks and psychopathological behavioral disorders. Along this line dysfunctions of functional connectivity particularly of prefrontal and limbic regions are discussed in the context of a number of disorders in humans such as depression, obsessive-compulsive disorder, autism, schizophrenia, and Alzheimer's disease (Bassett and Bullmore, 2009; Minshew and Keller, 2010; Del Casale et al., 2011; Hulvershorn et al., 2011; Liston et al., 2011).

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# Interactions between noradrenaline and corticosteroids in the brain: from electrical activity to cognitive performance

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One of the core reactions in response to a stressful situation is the activation of the hypothalamus–pituitary–adrenal axis which increases the release of glucocorticoid hormones from the adrenal glands. In concert with other neuro-modulators, such as (nor)adrenaline, these hormones enable and promote cognitive adaptation to stressful events. Recent studies have demonstrated that glucocorticoid hormones and noradrenaline, via their receptors, can both rapidly and persistently regulate the function of excitatory synapses which are critical for storage of information. Here we will review how glucocorticoids and noradrenaline alone and in synergy dynamically tune these synapses in the hippocampus and amygdala, and discuss how these hormones interact to promote behavioral adaptation to stressful situations.

**Keywords: hippocampus, amygdala, mouse, electrophysiology, glutamate**

## INTRODUCTION

Situations which potentially disturb homeostatic processes in body and mind, and which are subjectively perceived as a threat, i.e., stress, initiate the activation of two systems aimed at helping the organism to adapt (de Kloet et al., 2005; Kvetnansky et al., 2009; McEwen and Gianaros, 2010). First, the autonomic nervous system is activated upon arousal, ultimately causing the rapid release of (nor)adrenaline from the adrenal medulla into the circulation which – as a hormone – can rapidly regulate the function of peripheral organs. Via indirect pathways involving the nucleus tractus solitarius as well as more directly via activation of noradrenergic cells in the locus coeruleus, noradrenaline is also abundantly released in the brain, including close to cells in limbic structures like the amygdala and hippocampus (McGaugh, 2004; Valentino and Von Bockstaele, 2008). In these areas, noradrenaline – as a neurotransmitter – regulates neuronal function via α- and β-adrenergic receptors (Gibbs and Summers, 2002; Roozendaal et al., 2009a).

Somewhat later, the hypothalamus–pituitary–adrenal (HPA) axis is activated, which causes the secretion of corticosteroid hormones from the adrenal cortex. The prevailing corticosteroid hormone in most rodents is corticosterone, while in humans cortisol is most prevalent. Corticosteroid hormones are very lipophilic and therefore easily pass the blood–brain barrier, in principle reaching all cells but acting only on those carrying receptors. Two types of corticosteroid receptors are expressed in the brain: (1) mineralocorticoid receptors (MRs), which bind corticosterone, cortisol, and aldosterone with high affinity; and (2) glucocorticoid receptors (GRs) with an approximately 10-fold lower affinity for corticosterone and cortisol (de Kloet et al., 2005). Due to this difference in affinity, MRs are already substantially occupied by the natural ligand under rest, whereas activation of GRs to a large extent only occurs when corticosteroid levels are high, e.g., after exposure to stressful experiences. Pyramidal cells in the hippocampal CA1 area and granule cells in the dentate gyrus abundantly express both MR and GR. In most other limbic areas, including in the basolateral amygdala (BLA), expression of GR is higher than that of MR; the exception is formed by CA3 pyramidal neurons, which highly express MR but have only low levels of GR.

Corticosteroid hormones interact with various neurotransmitter systems, e.g., serotonin, dopamine, and endocannabinoids (for reviews, see Czyrak et al., 2003; Joëls et al., 2007; Hill et al., 2010; Haj-Dahmane and Shen, 2011). In this review, the focus is on the interactive effects of the neurotransmitter noradrenaline and the hormone corticosterone, with respect to synaptic function and behavioral relevance. While most cells in limbic brain regions are exposed to both noradrenaline and corticosteroid hormones after stressful events, the kinetic properties of exposure differ for the two ligands (see **Figure 1**). *In vivo* microdialysis studies, e.g., in the amygdala have shown that noradrenaline levels quickly rise after stress, but are normalized within an hour (Quirarte et al., 1998). By contrast, corticosteroid hormone levels in the brain are raised with a delay of approximately 20 min (compared to the rise observed in plasma; Droste et al., 2008) and return to baseline after 1–2 h. Catecholamines such as noradrenaline primarily act through G-protein coupled receptors which, via second messengers, alter the functionality of ion channels. This causes rapid-onset changes in electrical properties of neurons, which

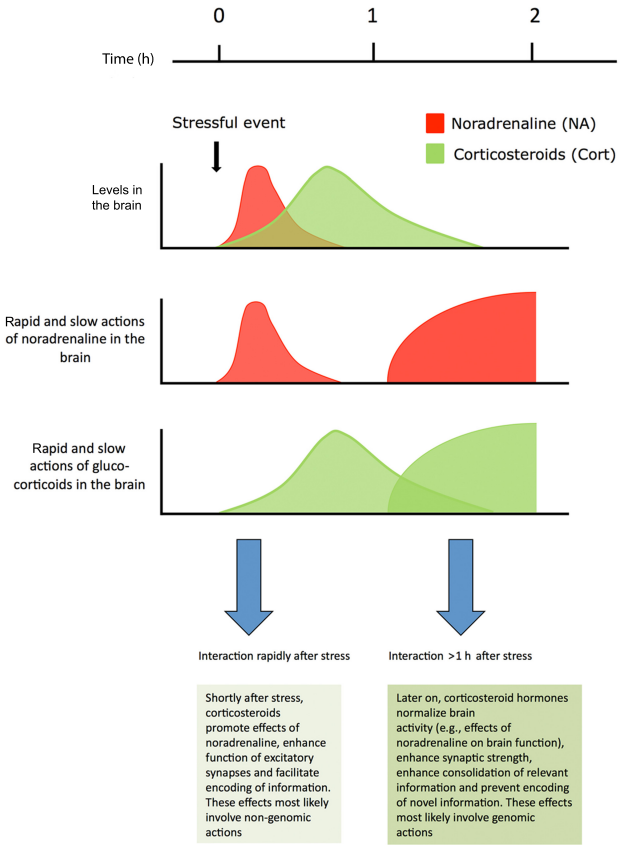


**FIGURE 1 | Shortly after stress noradrenaline levels in the brain are transiently elevated.** Corticosteroids reach the same brain areas later and remain elevated for approximately 2 h. For a restricted period of time neurons are exposed to high levels of both hormones. Noradrenaline primarily works through a rapid G-protein coupled pathway, but long-lasting secondary genomic effects requiring gene transcription may develop. Corticosteroid hormones exert rapid non-genomic actions via membrane receptors, and also slowly, but persistently, regulate neuronal function via nuclear receptors Corticosterone and noradrenaline regulate synaptic transmission and promote memory performance, both alone and in a synergistic fashion. For details, see text. Figure adapted from Joëls et al. (2011).

generally are also quickly reversible when noradrenaline levels in the synaptic cleft decline. Next to these rapid effects, gene-mediated events may develop, e.g., via CREB, which may lastingly affect synaptic function. This can induce a secondary, delayed response to noradrenaline, starting by the time that rapid effects have subsided, and last for hours.

In contrast to noradrenaline, corticosteroid hormones act through receptors – i.e., MRs and GRs – known to be transcriptional regulators (Duma et al., 2006). Upon binding of the hormone to the intracellular receptor, the latter dissociates from chaperone molecules and translocates to the nucleus, where it binds to response elements in an estimated 1–2% of the genes or, alternatively, changes gene transcription indirectly by interfering with the efficacy of other transcription factors. Both of these pathways were indeed demonstrated to take place in the brain (Morsink et al., 2006). Consequently, corticosteroid effects on neuronal activity are generally slow in onset, developing with a delay of approximately an hour, yet long-lasting. The most prominent of these corticosteroid effects after stress are caused by GR rather than MR activation (for review, see Joëls et al., 2007). Over the past decade, though, it has become increasingly evident that corticosteroid hormones also induce *rapid* effects on neuronal activity, which involve non-genomic signaling, via activation of MRs and GRs (Orchinik et al., 1991; Venero and Borrell, 1999; Di et al., 2003; Karst et al., 2005, 2010; Groc et al., 2008). Corticosteroid hormones can therefore rapidly and persistently regulate neuronal function, via activation of MRs and GRs.

Despite the different kinetics for adrenergic and steroid signaling, there is a window in time during which cells in limbic brain areas are simultaneously exposed to elevated levels of both catecholamines and corticosteroid hormones, allowing these neuromodulators to affect neuronal processes in concert (Joëls et al., 2011). Although, as mentioned, corticosteroid hormones are known to interact with various neurotransmitter systems, we discuss in this review only how noradrenaline (as a neurotransmitter) and corticosteroid hormones, alone or in interaction, regulate synaptic transmission (particularly via glutamate), synaptic plasticity, and memory formation.

## NORADRENALINE, CORTICOSTEROID HORMONES, AND EXCITATORY SYNAPSES IN LIMBIC REGIONS

One important function of stress is to induce long-term adaptive responses. Enhanced memory for stressful events is one of these well-known highly adaptive phenomena that help to remember relevant information. The current view of how memories are formed is that neurons are activated during the learning process, thereby changing the strength of synaptic connections between these cells. These changes in synaptic strength are generally believed to underlie storage of information, and learning and memory processes (Whitlock et al., 2006; Neves et al., 2008).

### NORADRENERGIC EFFECTS ON AMPA RECEPTORS

There is ample evidence that the dynamic regulation of AMPA-type glutamate receptors (AMPARs) – which mediate most of the fast excitatory synaptic transmission in brain cells – can change synaptic function and regulate storage of information (Kessels and Malinow, 2009). Recent studies have revealed that AMPARs are regulated by noradrenaline and glucocorticoid hormones. Thus, via activation of β-adrenergic receptors, noradrenaline and stress can rapidly – but reversibly – activate PKA and CaMKII, and increase the phosphorylation of GluA1 AMPAR subunits at Ser845 and Ser831 in the hippocampus, a critical step for synaptic insertion of these receptors (Wang et al., 2004; Hu et al., 2007). In addition, activation of β-adrenergic receptors facilitates the induction of hippocampal long-term potentiation (LTP; Thomas et al., 1996; Winder et al., 1999; Hu et al., 2007; Tenorio et al., 2010) and enhances activity-dependent synaptic insertion of AMPARs (Hu et al., 2007). Interestingly, activation of β-adrenergic receptors facilitates LTP in a time-dependent manner, i.e., only when receptors are phosphorylated by β-adrenergic activation, the induction of LTP is enhanced.

### CORTICOSTEROID EFFECTS ON AMPA RECEPTORS IN THE HIPPOCAMPUS AND PREFRONTAL CORTEX

Corticosteroid hormones can rapidly and reversibly change hippocampal synaptic transmission. Rapid corticosteroid effects on neuronal activity were first described in detail for parvocellular neurons in the hypothalamic paraventricular nucleus (PVN; reviewed in Tasker et al., 2006). In these cells, corticosterone rapidly decreases the mEPSC frequency, via a retrograde signaling pathway involving the endocannabinoid receptor 1. This potentially suppresses activity of PVN cells. In neurons located in the hippocampal CA1 area (Karst et al., 2005) and dentate gyrus (Pasricha et al., 2011) however, mEPSC frequency is rapidly and reversibly *enhanced* by corticosterone. Within minutes after application, glucocorticoids increase synaptic transmission in the hippocampus (Karst et al., 2005; Pasricha et al., 2011), via activation of low affinity MRs which are probably located in the cellular membrane. This rapid and reversible increase in synaptic transmission after glucocorticoid exposure most likely results from an increase in the presynaptic release of glutamate (Karst et al., 2005) in which the Erk pathway is critically involved (Olijslagers et al., 2008). Glucocorticoid exposure, via membrane MRs, also rapidly increases lateral diffusion of GluA1 and GluA2 subunits in primary hippocampal cultures, without altering the number of postsynaptic AMPARs (Groc et al., 2008) and promotes the activity-dependent synaptic insertion of GluA2-containing AMPARs (Groc et al., 2008). Furthermore, corticosterone shifts the voltage-dependent activation of a transient K-conductance ($I_A$) to the right, thus reducing its influence during depolarization and thereby its inhibitory action (Olijslagers et al., 2008). All of these actions potentially lead to a transiently raised hippocampal activity shortly after stress. Finally, glucocorticoids facilitate LTP in a time-dependent manner; LTP is only facilitated when elevated corticosteroid levels are present at the moment of high-frequency stimulation (Wiegert et al., 2006). These studies show that both noradrenaline and glucocorticoids can rapidly facilitate hippocampal synaptic plasticity and thereby increase the ability to encode information at the cellular level. The results are not entirely unequivocal, though. Recently, dexamethasone–BSA was reported to rapidly increase the frequency and amplitude of hippocampal spontaneous GABAergic currents within minutes (Hu et al., 2010). GABAergic transmission is also enhanced in the dorsal (but not ventral) hippocampus at a somewhat slower timescale, starting 25 min after treatment onset (Maggio and Segal, 2009). This would potentially decrease the activity of hippocampal cells.

After exposure to a stressful event, plasma corticosteroid levels slowly return to their pre-stress level, a process that requires about 2 h (de Kloet et al., 2005). Still, these hormones exert – via a slow, genomic mode of action – long-lasting effects on excitatory synapses. In hippocampal primary cultures – which contain cells from various hippocampal subregions – elevated glucocorticoid levels increase the membrane expression and synaptic insertion of GluA2-containing AMPARs (Groc et al., 2008; Martin et al., 2009). These effects are mediated via GRs, require more than an hour to develop as well as the synthesis of new proteins, and most likely result from increased lateral diffusion and/or altered ratio of endocytosis/exocytosis of GluA2-containing AMPARs (Groc et al., 2008; Martin et al., 2009). In hippocampal primary neurons as well as identified CA1 pyramidal cells, glucocorticoids slowly increase the amplitude of evoked as well as spontaneous AMPAR-mediated synaptic currents (Karst and Joëls, 2005; Martin et al., 2009), thereby enhancing AMPAR-mediated synaptic transmission in specific synapses. A similar effect has been observed >1 h after stress in prefrontal neurons, via the induction of serum- and glucocorticoid-inducible kinase and the activation of Rab4 (Yuen et al., 2009, 2011). While LTP is enabled when plasma corticosterone levels are low, elevated plasma hormone levels slowly suppress the ability to induce LTP (Diamond et al., 1992; Kim and Diamond, 2002). Elevated plasma corticosterone levels may hamper synaptic plasticity possibly because these hormones and synaptic plasticity make use of overlapping signaling pathways, which causes occlusion of one by the other (Groc et al., 2008). Corticosteroids facilitate long-term depression (LTD; Coussens et al., 1997; Xu et al., 1997) and increase endocytosis of synaptic AMPARs upon stimuli that weaken synaptic transmission (Martin et al., 2009). A vast amount of studies has seen this pattern of reduced LTP/enhanced LTD first reported for the hippocampus (see review in Kim and Diamond, 2002), but *enhanced* LTP was reported for the ventral-most part (20%) of the hippocampus (Maggio and Segal, 2007).

### CORTICOSTEROID EFFECTS ON AMPA RECEPTORS IN THE BASOLATERAL AMYGDALA

Activity of neurons in the BLA is mostly enhanced after corticosterone exposure in a slow GR-dependent way (Duvarci and Paré, 2007; Liebmann et al., 2008). BLA neurons respond rapidly to corticosterone in yet another manner. Thus, in slices prepared from animals under rest, having very low circulating corticosterone levels, exposure to corticosterone causes a non-genomic enhancement in mEPSC frequency via MRs, similar to the hippocampus (Karst et al., 2010). However, in BLA cells this enhancement is sustained, a phenomenon that requires not only the presence of MR but also of GR and protein synthesis. This alters the state of BLA neurons such that they respond differently to renewed exposure to corticosterone, now causing a *decrease* in mEPSC frequency, via a rapid GR-dependent mechanism involving the endocannabinoid receptor 1, reminiscent of the mechanism described in the PVN. BLA cells therefore seem – at least with respect to the non-genomic corticosteroid actions – more sensitive to the recent stress history of the animal than hippocampal CA1 and dentate cells.

Taken together, these studies indicate that exposure of limbic cells to either noradrenaline or corticosteroid hormones can rapidly but also slowly regulate limbic glutamatergic synapses (Krugers et al., 2010; Joëls et al., 2011).

### AMPA RECEPTORS, STRESS, AND BEHAVIOR

Behavioral studies indicate that regulation of AMPARs by noradrenaline and corticosterone is relevant for learning and memory. Studies using mice carrying mutations in the GluA1 phosphorylation sites indicate that noradrenaline-regulated phosphorylation of GluA1 facilitates emotional memory in a contextual fear conditioning task (Hu et al., 2007). Moreover, application of pep2m, which blocks trafficking of GluA2-containing AMPA

receptors prevents the memory-enhancing effects of stress in the Morris water-maze (Conboy and Sandi, 2010), and cued fearful memories (Migues et al., 2010). Also, stress-induced regulation of Rab4/SGK may underlie stress-effects on AMPA receptor function in the prefrontal cortex and working memory (Yuen et al., 2011).

## CROSSTALK BETWEEN GLUCOCORTICOIDS AND NORADRENALINE AT THE CELLULAR LEVEL

Noradrenaline and glucocorticoids not only change transmission independent from each other but also interact to regulate excitatory synapses (Joëls et al., 2011). This interaction may take place in two windows of time after exposure to a stressful experience. In the first window, shortly after exposure to stress, rapid non-genomic actions of corticosteroids coincide with initial effects of noradrenaline, so that interactions could take place while both stress mediators are present in substantial amounts within 1 h after stress and act on those cells carrying receptors for both types of modulators. In the second window, presumably hours after exposure to stress, corticosterone might also change noradrenergic actions via its slow genomic pathway, either when given in advance of stress and/or noradrenaline (a pharmacological approach) or in the aftermath of stress. In the following sections we will discuss the current evidence for interactions, at the cellular/circuit level, at the animal behavior level, and from human research.

### HIPPOCAMPUS

Recent data indicates that corticosterone and noradrenaline interact to rapidly regulate AMPA receptor function at the cellular level (Zhou et al., 2011). Thus, application of corticosterone for 15 min to hippocampal slices, at a dose that activates both MRs and GRs (30 nM), did not affect phosphorylation of the AMPAR GluA1 subunit at S845 or S831. Co-application of the β-adrenergic receptor agonist isoproterenol, however, largely increased S845 (but not S831) phosphorylation. Similarly, corticosterone alone did not rapidly change GluA1 and GluA2 surface expression in hippocampal primary cultures. However, combined administration of corticosterone and isoproterenol – which by itself was ineffective – enhanced surface expression. Interestingly, a high dosage of isoproterenol in the absence of corticosterone enhanced GluA1 surface expression, and this effect was *decreased* by corticosterone. Finally, in hippocampal primary cultures, mEPSC frequency was enhanced by the combination of isoproterenol and corticosterone (ineffective by themselves) while the same combination did not affect the amplitude. Although there are differences in dose-dependency of these various interactive effects of corticosterone and noradrenaline – which might result from differences in preparation –, the data indicates that there is an optimal combination at which noradrenaline and corticosterone interact to regulate AMPAR function and that beyond these concentrations the combined responses decline.

These rapid interactions aimed at glutamatergic transmission may bear relevance to observations at the circuit level in the dentate gyrus (Pu et al., 2007). For instance, perforant path stimulation in a theta-burst pattern – in slices from adult animals – by itself does not induce synaptic potentiation, but application of isoproterenol (1 μM) just prior to and during high-frequency stimulation causes robust synaptic potentiation. The onset of this potentiation is not instantaneous and was found to be accelerated when corticosterone (100 nM) was applied in addition to isoproterenol. One hour after high-frequency stimulation there was no difference between the signals recorded from slices exposed to isoproterenol alone or to the combination of the two hormones, suggesting that there were no interactions in the later time-domain. However, the 1-h delay may have been too short to reveal such interactions. This explanation is supported by the fact that in slices pretreated with a pulse of corticosterone >1 h before delivery of isoproterenol (and high-frequency stimulation), a significant attenuation of the isoproterenol effect was observed. Given that thus applied corticosterone by itself (i.e., without subsequent isoproterenol administration) did not change synaptic potentiation, these data support interactive rather than additive actions of the two hormones.

These observations in the dentate gyrus are in line with findings reported over two decades ago at the single cell level in the CA1 area (Joëls and de Kloet, 1989). In CA1 pyramidal neurons, noradrenaline (via β-adrenergic receptors) reduces a calcium-dependent K-conductance, causing cells to fire more action potentials during a depolarizing episode. The efficacy to do so was strongly attenuated by pretreatment with corticosterone, via a slow GR-dependent process. Thus, both in the hippocampal CA1 area and dentate gyrus, β-adrenergic facilitation of excitability is markedly attenuated by pretreatment with corticosterone via a slow and presumably gene-mediated pathway.

### BASOLATERAL AMYGDALA

In principal neurons of the BLA, isoproterenol causes a dose-dependent rapid enhancement of AMPAR-mediated synaptic responses, while the NMDAR mediated component is unaffected (Liebmann et al., 2009). This was not altered by simultaneous application of (100 nM) corticosterone. However, if corticosterone was applied >1 h in advance of isoproterenol, the facilitation of AMPAR-mediated synaptic responses by a moderate dose (0.4 μM) of the β-adrenoceptor agonist was strongly reduced.

This interaction was mirrored in recordings at the circuit level (Pu et al., 2009). Thus, isoproterenol was able to potentiate synaptic (field) responses for at least 60 min after delivery of a mild tetanic stimulation. In contrast to what was seen in the dentate gyrus, no acceleration of this effect by simultaneously applied corticosterone was observed. Instead, corticosterone gradually reversed the effect of isoproterenol; the corticosteroid hormone by itself did not affect synaptic responses after mild tetanic stimulation. The gradually developing attenuation by corticosterone was even more pronounced when the hormone was administered >1 h in advance of isoproterenol and high-frequency stimulation.

### SOME PRINCIPLES ABOUT HORMONAL INTERACTIONS AT THE SINGLE CELL/CIRCUIT LEVEL

Overall, these data at the single cell and circuit level shows that at the short-term corticosterone may accelerate or enhance the efficacy of noradrenaline to facilitate synaptic transmission and

potentiation in limbic cells (Joëls et al., 2011). These effects are relatively mild, though, and not always apparent. There is evidence that these interactions may only occur with intermediate levels of synaptic input and/or moderately high hormone concentrations; when input/hormone levels are too low, interactive effects remain sub-threshold, while too high levels of input/hormone levels seem to cause ceiling or even reversed effects. An important limitation of all studies so far is the fact that in actual life noradrenaline and corticosterone will not reach limbic cells at the exact same moment. None of the studies so far has addressed this issue (further discussed in Section "Conclusion").

The slow genomic effect by corticosterone seems rather consistent: attenuation of β-adrenergic actions by pretreatment with corticosterone was observed in the hippocampal CA1 area and dentate gyrus as well as the BLA. While an approach in which corticosterone is given >1 h in advance of noradrenaline is pharmacologically relevant and certainly has helped to delineate the slow corticosteroid actions, there is paucity in studies examining whether corticosterone co-administered with noradrenaline may reverse and normalize noradrenergic actions after approximately 1 h. At this moment we can only infer such effects from the experimental design using corticosteroid pretreatment. The exception is formed by a study on synaptic potentiation in the BLA which provides preliminary evidence that corticosterone can indeed exert such normalizing actions (Pu et al., 2009).

## INTERACTIONS AT THE BEHAVIORAL LEVEL IN RODENTS

Noradrenaline and corticosteroid hormones, via their receptors, mediate (at least in part) the memory-enhancing effects of stress and emotion (Roozendaal et al., 2009a; Joëls et al., 2011). Noradrenaline enhances memory formation of emotional events via brain β-adrenergic receptors: application of noradrenaline or β-adrenergic receptor agonists promotes memory consolidation in various aversive memory tasks such as inhibitory avoidance task, fear conditioning and in Morris water-maze learning (Hu et al., 2007; Roozendaal et al., 2009a; but see also Hatfield and McGaugh, 1999; Lee et al., 2001; Bush et al., 2010), and blocking β-adrenergic receptors reduces contextual fear memories (Ji et al., 2003). Activation of α-adrenergic receptors also enhances memory, presumably acting by enhancing β-adrenergic actions (Ferry et al., 1999). Noradrenaline has also been reported to enhance reconsolidation of information (e.g., Debiec and LeDoux, 2004).

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

In addition to the well-documented effects of stress and glucocorticoids on consolidation processes, these hormones also affect memory retrieval mechanisms (De Quervain et al., 1998). Exposure to stress and elevated corticosteroid levels hamper the retrieval of already stored information (De Quervain et al., 1998). Blocking MRs and GRs also hampers the reconsolidation of context and cue-conditioned fear respectively (Pitman et al., 2011; Zhou et al., 2011). Taken together, there is ample evidence that corticosteroid hormones, via activation of MRs and GRs, have a repertoire of behavioral effects that promote the consolidation and updating of relevant (fearful) information and ultimately favor behavioral adaptation (de Kloet et al., 1999).

Several recent reviews (e.g., Roozendaal et al., 2009a) have highlighted that particularly interactions between noradrenaline and corticosterone affect (emotional) memory formation, a process in which the hippocampus and amygdala play a crucial role. We will here only describe a few examples which nicely illustrate the principles. Thus, the presence of noradrenaline is crucial for facilitation of emotional memory in rodents (Quirarte et al., 1997). Moreover, post-training administration of noradrenaline or β-adrenergic receptor agonists into the BLA produces a dose-dependent enhancement of amygdala-dependent memory formation (Ferry et al., 1999). Corticosterone can modulate noradrenergic effects on memory formation but seems to be unable to enhance memory formation independent of noradrenaline. This is most clearly demonstrated by an experiment in which post-training corticosterone administration enhanced spatial and aversive memory formation, a process blocked by concurrent intra-BLA infusions of a β-adrenoceptor antagonist (Roozendaal et al., 2006). Similarly, corticosterone administered to naïve rats enhanced object recognition, an effect that was again blocked by the β-adrenoceptor antagonist propranolol. Corticosterone was ineffective in rats with reduced training-associated emotional arousal due to prior habituation to the experimental context (Okuda et al., 2004). Conversely, emotional arousal effects were mimicked in well-habituated rats by releasing endogenous noradrenaline via administration of the $\alpha_2$-adrenoceptor antagonist yohimbine (presumably causing higher noradrenaline levels) immediately after object recognition training (Roozendaal et al., 2006).

In contrast to the reduced preparations used for cellular studies, studies with intact animals should consider at least two other aspects of interactions between the two hormones. First, corticosteroids are known to increase the availability of noradrenaline in the BLA (McReynolds et al., 2010). Second, corticosteroid and noradrenergic actions in one region cannot be regarded independent from what happens in associated areas. For instance, interactions between noradrenaline and corticosterone in the BLA time-dependently influence the function of the dentate gyrus (Akirav and Richter-Levin, 1999). Corticosteroids and noradrenaline also interact in the prefrontal or insular cortices to enhance memory consolidation (Miranda et al., 2008; Roozendaal et al., 2009b). Thus, similar to what was described for the BLA, administration of a β-adrenoceptor antagonist into these brain regions prevents the memory enhancement by concurrently administered corticosteroids (Barsegyan et al., 2010). Due to reciprocal connections between the prefrontal cortex and BLA, interactions in one area will almost certainly influence the functionality in the other.

There is substantial evidence from behavioral studies that (of the two types of corticosteroid receptors) at least GRs play a role in the modulation of noradrenergic function (Roozendaal et al., 2009a). The relatively short delay between hormone application and behavioral effects seems to favor a non-genomic mode of action. Rapid and presumably non-genomic effects via *MRs*, however, are also involved in successful memory formation under arousing conditions (Zhou et al., 2010). To what extent genomic actions of corticosteroids interact with noradrenaline to change memory formation is more difficult to assess. Nevertheless, there is indirect evidence that such interactive effects do play a role. The most straightforward example comes from a study using mice that were genetically modified such that GRs do not homodimerize and thus cannot bind to the DNA (Oitzl et al., 2001). Training of these animals in a water-maze paradigm – which is sufficiently stressful to increase levels of both noradrenaline and corticosterone – resulted in a poor spatial performance compared to the wildtype controls. Calcium currents, and thus calcium-dependent attenuation of firing frequency, were not increased by corticosterone in these mutant mice (Karst et al., 2000). This may allow for more retrograde interference of stress-unrelated information, a possible explanation for the impaired behavioral performance. Interestingly, one behavioral study used a corticosterone-pretreatment paradigm which quite closely resembles that used in cellular investigations. In this study (Borrell et al., 1984), pretreatment with corticosteroids 1 h before adrenaline administration was demonstrated to dramatically reduce the efficacy of the latter to affect amygdala-dependent behavior. Both examples support the view that slow genomic GR effects reduce/normalize noradrenergic actions on behavior, which is in line with the observations at the cellular level.

## INTERACTIONS IN THE HUMAN BRAIN

Most studies in humans indicate that stressful and emotional events are remembered well. This most likely involves endogenous catecholamines like noradrenaline (Cahill et al., 1994; Strange and Dolan, 2004; Onur et al., 2009) but also corticosteroids (Lupien et al., 2002; Marin et al., 2011). Several studies have specifically investigated the interactions between noradrenaline and corticosteroid hormones. Some of these studies are discussed in the following paragraphs.

Smeets et al. (2009) examined if stress exposure prior to encoding of a list of words affected learning and memory performance. The stressor consisted of public speaking about one's personality in front of an unresponsive panel. The words to be learned were either related to personality or unrelated to personality but of comparable valence. Afterward each subject ranked all words on an arousal-scale, allowing – on an individual basis – a distinction between low-arousing and high-arousing words. It turned out that stress improved learning and long-term memory of high-arousing context-related words, at the cost of memorizing low-arousing context-related words, whereas no effect was seen with regard to the context-unrelated words. The improved memory for context-related high-arousing words was particularly evident in individuals who performed the task immediately after stress exposure, more so than in those who carried out the task 2 h later. If the order was reversed (learning prior to stress exposure), memory performance was unchanged. Interestingly, the memory for high- versus low-arousing context-related words in subjects stressed just prior to learning correlated significantly with a combined index for their salivary alpha-amylase and cortisol levels, which reflect the function of the autonomic and HPA systems respectively, but did not correlate with either of these parameters alone, underlining the potential relevance of interactions between the two systems.

Van Stegeren et al. (2010) used a pharmacological approach, specifically addressing the interactive effect of the two hormones on memory formation. Subjects received yohimbine and hydrocortisone, prior to encoding of arousing and neutral pictures. The timing of hydrocortisone administration (45 min before encoding) was slightly ambiguous, probably allowing the development of non-genomic as well as genomic effects. At the behavioral level, *combined* drug administration led to the best (surprise) recognition of the pictures, particularly of arousing material. Contrary to the observations in animals, hydrocortisone seemed more effective than yohimbine in improving memory (see also Maheu et al., 2005), but it cannot be excluded that the experimental setting already caused substantial release of endogenous catecholamines, so that exogenous administration of drugs tapping on the same system were less effective. Paradoxically, the very good memory performance in the group receiving both yohimbine and hydrocortisone was linked to reduced activity in the hippocampus, as revealed by simultaneously acquired fMRI data. Interestingly, this reduced hippocampal activity during encoding of later remembered material was also observed when subjects instead of receiving drugs were stressed during encoding (Henckens et al., 2009). At this time one can only speculate about this observation, but one explanation could be that under stressful conditions extensive filtering of the incoming information may take place, causing restricted but highly efficient functioning of the human hippocampus.

A third example illustrating that noradrenaline and corticosteroids interact at the level of the amygdala in the human brain was supplied by Kukolja et al. (2008). In this study subjects received either (i) the noradrenaline-reuptake inhibitor reboxetine,

(ii) hydrocortisone, (iii) a combination of these two drugs, or (iv) placebo, 105 min before they entered the scanner. They were exposed to film clips with actors who displayed either a happy, fearful or neutral facial expression. In control subjects happy or fearful (compared to neutral) expressions cause enhanced amygdala activity. Subjects receiving both reboxetine and hydrocortisone showed reduced amygdala activity to happy faces but enhanced activity to fearful facial expressions. This suggests that noradrenaline in interaction with cortisol gives rise to a negative bias of emotional functions.

More recently this was confirmed and extended in a study which specifically targeted the delayed and presumably genomic effects of cortisol administration under conditions that are probably sufficiently arousing to cause substantial release of endogenous noradrenaline (Henckens et al., 2010). Subjects ingested a placebo or a cortisol tablet (10 mg) either 75 or 285 min prior to exposure to a face-morphing task of both happy and fearful facial expressions while they were in a scanner. When given at 75 min prior to the test, cortisol suppressed amygdala activity to both happy and fearful faces. When cortisol was given 285 min in advance, normalized responses to fearful face stimuli but still reduced responses to happy face stimuli were observed. As seen in the study by Kukolja et al. (2008), this suggests a valence-specific slow corticosteroid effect, causing diminished amygdala processing preferentially for happy faces. Interestingly, normalization of the responses to fearful face stimuli after 285 min correlated with increased mPFC-amygdala connectivity, indicating augmented cognitive top-down control (Henckens et al., 2010). This suggests an important influence of delayed corticosteroid actions on executive functions, a field that is currently being explored.

Although the studies reported here represent only a selection of papers on the effect of stress on human cognition, they nicely illustrate the principle that noradrenaline and corticosteroid hormones interact to affect cognitive processing in the human brain.

## OPEN QUESTIONS

When an organism experiences a stressful event, its neurons in limbic brain structures are exposed to surges of both noradrenaline and corticosteroids. In addition to these two important stress mediators, there is a myriad of transmitters and hormones that join in the overall central response to a stressful situation, including catecholamines other than noradrenaline such as dopamine and peptides like CRH and vasopressin (Joëls and Baram, 2009). Here we discussed that corticosteroids and noradrenaline regulate the function of excitatory synapses and that this is thought to contribute to the memory-enhancing effects of these hormones (**Figure 1**). Neuromodulators such as noradrenaline and corticosteroids work in overlapping time-domains and most likely interact. Interactions between other stress mediators, though, have not yet been studied in great detail. There might be some redundancy in the activity of these stress mediators, but the regional distribution and sub-cellular localization of their receptors will confer quite some specificity to their contribution to the overall stress–response (Joëls and Baram, 2009).

In the rapid time-domain (i.e., within 30–60 min after stress, when both hormones are present in high levels in the vicinity of neurons in limbic areas) noradrenaline and corticosterone seem to act synergistically; current evidence supports that noradrenaline is indispensable in the rapid time-domain, whereas corticosterone seems to serve a more permissive role (Roozendaal et al., 2006). MRs are important in this phase, among other things for appraisal of the situation and selection of behavioral strategies (Schwabe et al., 2010). The behavioral consequences of corticosteroids in this time-domain however, particularly in humans, still need to be addressed in detail. Such investigations in human subjects are presently hampered by the fact that (1) there are no (oral) selective ligands available for membrane MRs mediating rapid effects and (2) peripherally administered drugs require some time to reach the brain, which hampers precise timing such as is possible *in vitro* or with intracerebroventricular administration in rodents. But even in reduced rodent brain preparations, the "natural" order of hormone exposure – i.e., first to noradrenaline and then, with an approximate delay of 20 min, to corticosteroids – has not been examined. This clearly requires dedicated experiments, aligning the experimental designs in the reduced cell preparations, animal behavior and human studies as much as possible.

The *cellular* studies in rodents and neuroimaging studies in humans regarding delayed effects of corticosteroid hormones on noradrenaline seem to be quite consistent, all finding a suppression of the latter by the former, probably via GRs. The evidence for this view in the human brain, however, is still limited. More importantly, support for this notion from behavioral studies in rodents is near-absent. Dedicated experiments, in which administration of corticosterone is precisely timed relative to mildly arousing learning situations, could resolve this issue. To what extent these experiments with corticosteroid treatment are indicative of what happens several hours after their release during stress also remains to be investigated. If this would be the case, one could postulate that the delayed effects of corticosteroid hormones primarily play a role in response normalization after stress and consolidation of the stress-related information, a notion that is indeed supported by behavioral investigations in humans and experimental animals. Whether the interactive effects of noradrenaline and corticosteroids on excitatory synapses are crucial for the memory-enhancing effects of these neuromodulators needs to be verified.

A final consideration regards the effect of multiple surges of corticosteroid hormones. Recent cellular investigations in the rodent basolateral nucleus of the amygdala suggest that exposure to a single surge of corticosterone changes cellular properties such that these cells respond in the complete opposite way to a second exposure to corticosteroids (Karst et al., 2010); this "flip" in response depends on protein synthesis and activation of GRs. The behavioral relevance of these metaplastic responses needs further investigation, but given the pulsatile release pattern of corticosteroid hormones throughout the day (Lightman and Conway-Campbell, 2010), metaplasticity is likely to change the background excitability of these amygdala cells, even in the absence of stress. How this affects the responsivity of amygdala versus hippocampal cells to stress over the day is one of the challenging questions for the next years.

## CONCLUSION

Shortly after stress, cells in limbic brain areas are exposed to a wave of catecholamines including noradrenaline and, slightly later,

to corticosteroid hormones. These two stress mediators regulate synapses and memory performance. They interact in multiple time-domains: (1) up to approximately 1 h after stress via rapid non-genomic actions, i.e., while levels of stress mediators are high; and (2) several hours after stress exposure via genomic effects, i.e., at a time when concentrations of noradrenaline and corticosteroids have returned to pre-stress levels. Cellular studies over the past decade have shown that the two stress mediators act synergistically in the initial time-window, particularly with intermediate concentrations. Animal behavior and human studies indicate that these rapid actions may promote the encoding of stress-context related and relevant information. The latter actions, primarily exerted by corticosteroid hormones, may serve to normalize earlier effects of catecholamines and protect the encoded stress-related information. Corticosteroid hormones given out of the stress context (e.g., 1–4 h in advance of stress) generally suppress noradrenergic facilitation of neuronal activity, as shown in rodents at the cellular level and in the human brain. In the rodent amygdala, corticosterone administration *after* stress quickly resets neuronal activity. These out-of-context effects of corticosterone could be of relevance for pharmacotreatment of stress-related disorders.

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# Steroid modulation of hippocampal plasticity: switching between cognitive and emotional memories

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Several new observations have shifted the view of the hippocampus from a structure in charge of cognitive processes to a brain area that participates in the formation of emotional memories, in addition to its role in cognition. Specifically, while the dorsal hippocampus is involved in the processing of cognitive memories; the ventral sector is mainly associated with the control of behavioral inhibition, stress, and emotional memory. Stress is likely to cause this switch in control of hippocampal functions by modulating synaptic plasticity in the dorsal and ventral sectors of the hippocampus through the differential activation of mineralocorticosteroid or glucocorticosteroid receptors. Herein, we will review the effects of stress hormones on synaptic plasticity in the hippocampus and outline the outcomes on stress-related global functions of this structure. We propose that steroid hormones act as molecular switches: by changing the strength of synaptic connectivity in the hippocampus following stress, they regulate the routes by which the hippocampus is functionally linked to the rest of the brain. This hypothesis has profound implications for the pathophysiology of psychiatric disorders.

**Keywords: hippocampus, synaptic plasticity, stress, LTP, corticosterone receptors**

## INTRODUCTION

Steroid hormones have been traditionally associated with regulation of peripheral organs, associated with stress (corticosterone) or with gonadal function (estrogen and androgens). Over the years, it became evident that these hormones also act within the hypothalamus, in a feedback regulatory loop, to affect the release of the neural factors that modulate production of the steroid hormones. More recently, several observations have elucidated new roles of steroid hormones in modulating higher CNS functions. Specifically, both stress and steroid hormones have been shown to affect synaptic receptors and ion channels and therefore regulate in several different ways synaptic transmission and neuronal plasticity. Consequently, stress hormones have been implicated in processes ranging from homeostatic to cognitive functions. Furthermore, in some disorders of the nervous system, hormones have been shown to play critical roles: favoring or halting the disease process. Thus, the interaction between peripheral hormones and central networks seem to be more intense than ever imagined before.

In the present study we review current knowledge on the effects of steroid hormones on synaptic plasticity and define their influence on hippocampal cognitive and emotional functions.

## THE DIFFERENT FAMILIES OF CORTICOSTEROID RECEPTORS IN THE BRAIN

Following the exposure to stressful stimuli, the steroid hormone corticosterone (cortisol in humans) is released from the adrenal glands in order to set up the best response to the challenge by acting on steroid receptors (de Kloet et al., 2005). These are distributed throughout the body and have a particularly dense distribution in the CNS (de Kloet et al., 2005). In the brain, the cellular and molecular targets for the action of corticosterone include, in addition to basic metabolic processes, an effect on excitatory (Karst and Joels, 2005) and inhibitory (Maggio and Segal, 2009a) synaptic transmission, as well as an effect on voltage-gated calcium channels (VGCC; Karst et al., 2000; Chameau et al., 2007). These effects are mediated by the activation of mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs; Joels, 1999, 2008; de Kloet et al., 2005). Initially, it was suggested that both receptors act as nuclear transcription factors that modify protein synthesis and produce a slow, persistent change in the function of the cell (de Kloet et al., 1993; Joels, 2001, 2008). More recently, the existence of a new family of membrane-bound MR and GR (mMR and mGR, respectively), which act through novel non-genomic pathways, has been reported (Karst et al., 2005; de Kloet et al., 2008). In this route, mMR and mGR can rapidly affect ionic conductances and thereby modify cell excitability and function (Karst et al., 2005; de Kloet et al., 2008). These membrane-bound receptors appear to differ from their intracellular cognates, not only in their location on the cell membrane, but also in their molecular structures (Joels et al., 2008), in their affinities for corticosterone, and in their downstream mechanisms of action which involve activation of G proteins (Joels et al., 2008). Specifically, intracellular MR (iMR) have a very high affinity for corticosterone and are highly expressed in all hippocampal subfields, as well as in cells of the central amygdala, lateral septum, and some motor nuclei in the brainstem (Joels, 2006). Intracellular GR (iGR) have a relatively low affinity, are widely distributed throughout the brain, and are expressed both in neurons and in glia (Joels, 2006). Consequently, it has been proposed that iMR hardly participate, if at all, in the fast response to stressful stimuli, due to their characteristic of being

already saturated by the low ambient levels of corticosterone at rest (Joels, 2006, 2008). Conversely, iGR have been reported to become gradually activated by rising levels of corticosterone following a stressful event (Joels, 2006, 2008; **Figure 1A**). Therefore, under physiological conditions, cells that coexpress both receptor types, such as principal cells in the CA1 region, the dentate gyrus (DG), and the central amygdala, will shift between predominant iMR activation and concurrent mMR and iGR activation (Joels and Krugers, 2007).

## THE ROLES OF CORTICOSTEROID RECEPTORS IN THE REGULATION OF HIPPOCAMPAL LTP

The identification of the molecular cascades linked to the effects of corticosteroids in the brain resulted in a series of studies examining the role of corticosterone in neuronal plasticity as well as in the cellular mechanisms underlying learning and memory such as long-term potentiation (LTP) and long-term depression (LTD; Bliss and Collingridge, 1993). Initial studies indicated that induction of LTP in the hippocampal area CA1 is impaired in a rat exposed to behavioral stress, such as inescapable shock (Foy et al., 1987; Shors et al., 1989). Administration of high doses of corticosterone either *in vivo* (Diamond et al., 1992) or *in vitro* (Pavlides et al., 1996; Alfarez et al., 2002) produced the same effects, indicating that corticosterone is likely to mediate this action of stress. Specifically, corticosterone-induced impairment of LTP seems to be due to the activation of iGR, which depresses NMDA receptor-dependent LTP (Krugers et al., 2005; **Figure 1B**). Conversely, it was also shown that LTP could be enhanced in the presence of low to moderate concentrations of corticosterone, while in absence of corticosterone LTP induction was impaired (Diamond et al., 1992). These studies show that the effects of corticosteroids on LTP induction are dose-dependent and follow an inverted U-shaped relationship (Diamond et al., 1992; Joels, 2006).

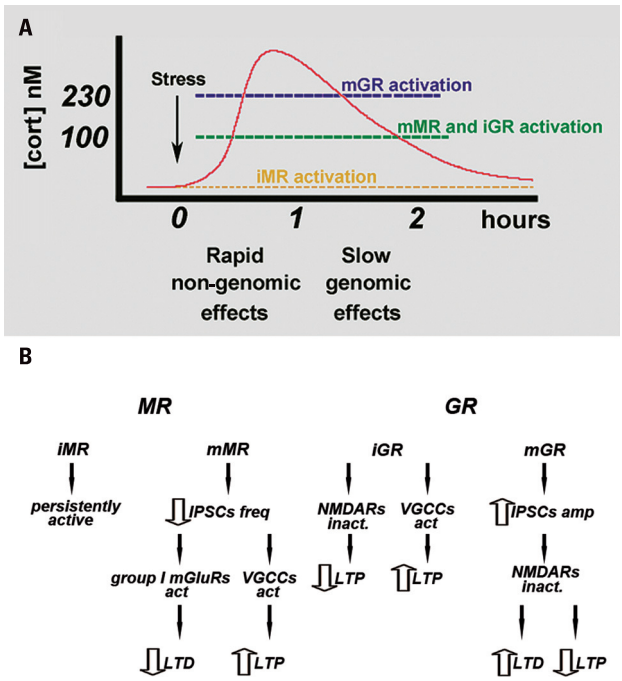


**FIGURE 1 | (A)** Time course of MR and GR activation following stressful stimuli. At a resting level, iMR are already saturated by the baseline levels of corticosterone. Rising concentration of corticosterone activates both mMR and iGR, whereas an additional increase in corticosterone levels also activates mGR. mMR- and mGR-mediated effects appear in a faster time course than those mediated by the intracellular receptors. Modified from Maggio and Segal (2010). **(B)** Proposed mechanism by which corticosteroid receptors differently regulate LTP and LTD in the hippocampus. iMR are believed to be fully occupied at baseline level of corticosterone, therefore they might play a marginal role in synaptic plasticity. mMR might play a fundamental role in synaptic plasticity especially in VH: mMR activation reduces IPSC frequency. This determines an increase in the excitability of the pyramidal cells and raises the possibility of VGCC activation, thus enhancing LTP. In addition, a decrease in GABAergic inhibition can impair LTD through a group I mGluR-mediated mechanism. iGR are thought to both decrease NMDA-mediated LTP and increase VGCCs mediated LTP both in the hippocampus and amygdale. Their effects may occur at longer time scale due to their lower affinity to corticosterone. mGR might be involved in the regulation of synaptic plasticity mainly in DH: mGR activation increases IPSC amplitude and following hyperpolarization of the pyramidal cell membrane and inactivation of NMDA receptors, might impair LTP and enhance LTD. Modified from Maggio and Segal (2010).

Further studies, however, have presented a more complex picture of the effects of steroids on synaptic plasticity. Specifically, it seems that the same dosage of corticosterone that impairs NMDA-dependent LTP can indeed enhance VGCC-dependent LTP (Krugers et al., 2005). This species of LTP is found in the amygdale where it is believed to underlie the formation of fear memories (Blair et al., 2001; Bauer et al., 2002) and can be evoked in the hippocampus as well (Borroni et al., 2000; **Figure 1B**). Interestingly, in the hippocampus, corticosterone appears to enhance VGCC LTP through an iGR-dependent mechanism (Krugers et al., 2005). It has been proposed that this effect requires a genomic pathway, as it occurs after a long delay between the exposure to stress and/or corticosterone and the recordings (Krugers et al., 2005), thus probably depending on the binding of GR homodimers to DNA that causes an increase in calcium currents (Karst and Joels, 2005; Chameau et al., 2007). Recent data from our group have shown that MRs are also able to enhance VGCC LTP (Maggio and Segal, 2007b): either stress or physiological concentrations of corticosterone can enhance LTP in the ventral hippocampus (VH), while inhibiting it in the dorsal hippocampus (DH; Maggio and Segal, 2007b). In particular, corticosterone enhances LTP through MRs since a selective MR agonist, aldosterone, shares the same effect in the VH (Maggio and Segal, 2007b). The proposed mechanism excludes an interaction between MR and NMDA receptors, as aldosterone by itself does not increase NMDA-dependent synaptic potentials (Maggio and Segal, 2007b). Conversely, MR-induced LTP can be blocked by nifedipine, suggesting that VGCCs are likely responsible for this effect (Maggio and Segal, 2007b; **Figure 1B**). It is likely that MR activates VGCC by modulating ionic conductances or changing VGCC activation kinetics. *In vivo* experiments have shown that MR activation is able to increase LTP in the DH as well (Avital et al., 2006). Specifically, animals which were injected with a GR antagonist prior to the stressful exposure, such that only MR could be activated by stress, show a much larger LTP than controls. In contrast, those animals previously injected with an MR antagonist and then exposed to stress, allowing only GR activation, show a much lower LTP than controls (Avital et al., 2006).

These recordings were performed in the DG and even though there could be differences in the effects of stress and steroids between the DG and CA1 (Joels and Krugers, 2007), MRs were still shown to mediate an enhancement of LTP.

These experiments raise several issues. It could be argued that the experiments in the VH were conducted using an *in vitro* preparation where ambient corticosterone maintained normally through the circulation is washed out. Consequently, MRs are not occupied, and are ready to be activated by the superfused drug and produce LTP enhancement in the VH. This might not reflect the situation in the intact animal, where the brain is constantly exposed to fluctuating concentrations of corticosterone. In fact, MR should be already saturated by the resting concentration of corticosterone and should not respond to the stress-induced rise of corticosterone in the presence of a GR blockade. This, however, does not seem to be the case (Avital et al., 2006). Furthermore, even though both MR and GR are expressed in the VH, corticosterone action is mediated by activation of MR rather than GR. This reflects the observation that in the VH, MR concentration is double that of GR (Robertson et al., 2005). If so, according to the U-shaped curve model of corticosterone effects, MR should be saturated rapidly by the rising concentration of corticosterone and their effect should fade away faster in favor of the slower GR activation. This is in contrast with the experimental evidence. Altogether, it seems that the simple, dose-dependent, inverted, U-shaped curve does not fully explain the modulatory functions of MR and GR on LTP in the different sectors of the hippocampus, therefore calling for the involvement of other factors.

A possible mechanism that may clarify the MR-dependent enhancement of LTP should take into consideration the activation of mMR. These receptors act through a faster mechanism (de Kloet et al., 2008) and have lower affinities for corticosterone compared to their intracellular cognates (Joels, 2008) and similar to that of the iGR (Joels, 2008). In addition, MR activation enhances LTP in the VH within 1 h, too short time window to be accounted for by activation of genomic mechanisms (Joels and Krugers, 2007; Joels, 2008), but compatible with the faster time course of the non-genomic routes. Thus, mMR could be the preferential target for rising concentrations of corticosterone in the VH if one takes into account the similar affinities for corticosterone between mMR and iGR, and the denser distribution of the former over the latter (Robertson et al., 2005; **Figure 1**).

Mineralocorticoid receptors are likely to enhance LTP through activation of VGCC. In our experiments, we could not detect any effect of iGR on VGCC LTP. This could most likely be due to the shorter time window of observation in our experiments compared to those done by others (Krugers et al., 2005). In any case, both MR and GR were reported to increase VGCC LTP (Krugers et al., 2005; Maggio and Segal, 2007b). This apparent contrast could probably be explained by considering the different time courses of MR and GR enhancement of VGCC LTP. Specifically, MR has an earlier effect than GR and it could be that in the VH stress mediates a fast enhancement of LTP by MR followed by a second, slow increase in LTP due to GR activation. This proposal is compatible with the proposed role of the VH as a key player in the pathway that conveys stressful information to the hypothalamus and the amygdale so as to organize the stress response (Moser and Moser, 1998; Maggio and Segal, 2010; Segal et al., 2010).

## CORTICOSTEROID REGULATION OF SYNAPTIC PLASTICITY REGULATES HIPPOCAMPAL FUNCTIONS

The regulation of LTP by corticosterone in the hippocampus has profound system implications. Following stress, the quick MR-mediated increase in LTP facilitates the flow of the information related to stress from the VH to the ventral hypothalamus and other lower brain centers, so that the autonomic response to stress can be organized. Later on, the MR-mediated response fades away and the effect of GR dominates. As previously mentioned, GR enhancement of VGCC LTP has been shown to have a role in the formation of fear memories in the amygdale (Blair et al., 2001; Bauer et al., 2002). In this respect, GR could play the same function in the VH: the formation of the memory for the stressful event at the VH–amygdala pathway. Indeed, the evidence that MR and GR act on the same mechanism can have different purposes due to the time window of the respective outcomes that take place. Considering this, it could be interesting to study the relationship between the MR and GR responses in the VH.

In the DH, the reduction of LTP is mediated by GR (Maggio and Segal, 2007b). This effect seems to occur in less than 1 h, a relatively quick response that is unlikely to be mediated by a genomic mechanism. GR could reduce NMDA-mediated LTP either by a direct or an indirect mechanism. As far as it concerns the indirect mechanism hypothesis, we have demonstrated that a GR agonist, dexamethasone, increases IPSCs and mIPSCs amplitude in the DH within 10 min (Maggio and Segal, 2009a, 2012), consistent with the possible activation of mGR. Therefore, the increase in $GABA_A$ conductance could hyperpolarize the membrane, thus preventing the cell from reaching the threshold of depolarization that unlocks NMDA receptors from the $Mg^{2+}$ block (**Figure 1B**). All in all, our experiments indicate that GR affect LTP through a fast, probably non-genomic mechanism. Even though this hypothesis needs to be explored further, the fast suppression of LTP in the DH can underlie the switch in the weight between the DH and VH; by reducing DH LTP and simultaneously enhancing LTP in the VH, the stressful stimuli could temporarily suppress the cognitive route of the hippocampus to cortical structures and enable the transmission of the emotional information through the VH to the amygdala.

Conversely, LTD induction is facilitated by behavioral stress, through a mechanism that requires GR (Pavlides et al., 1995; Xu et al., 1997, 1998) and their effect on NMDA receptors (Kim et al., 1996; Yang et al., 2005). We replicated previous experiments where both stress and corticosterone facilitate LTD through a GR-dependent mechanism in the DH, but we have also shown that LTD is impaired in the VH through a MR-dependent mechanism (Maggio and Segal, 2009b). Specifically in the latter case, LTD is transformed into a slow-onset LTP following the exposure to stressful stimulation (Maggio and Segal, 2009b). As is the case for LTP, changes in LTD either in the DH or VH were observed at approximately 1 h after the exposure to the stress, a time window that could be compatible with non-genomic mechanisms. The MR-induced conversion of LTD to LTP in the VH could be due

to the activation of VGCC, which will further facilitate the ventral route to the amygdale (**Figure 1B**). Group I mGluR have been shown to enhance LTD in CA1 (Fitzjohn et al., 2001; Rammes et al., 2003), but, interestingly, they have been reported to induce a slow-onset potentiation in the DG (Manahan-Vaughan and Reymann, 1996). In a previous study, we showed that, in the VH, application of DHPG, a group I mGluR agonist, increases the population spike amplitude in response to a baseline stimulation (Maggio and Segal, 2007a). Taken together, these observations suggest that in the VH, a decrease in GABAergic inhibition can shift LTD to a slow-onset LTP through a group I mGluR-mediated mechanism (**Figure 1B**).

## CONCLUSION

In conclusion, corticosteroid regulation of synaptic plasticity in the hippocampus is affected by several factors. An inverted U-shape effect of corticosterone partially explains the observed modulation of LTP. Indeed, this hypothesis mainly refers to the activation of intracellular corticosteroid receptors and does not take into account the contribution of membrane-bound steroid receptors. In fact, mMR, which bears a similar corticosterone affinity to that of iGR, will be activated at similar steroid concentrations. This implies that the effect of mMR appears earlier than that of iGR, thus inducing an enhancement of LTP instead of LTD. This might be the case in the VH. An additional factor to be considered is the distribution of MR and GR in specific brain areas, and the ratio of membrane-bound to intracellular receptors expressed therein. This is because at the same affinity value for corticosterone concentration, the receptor that is highly expressed will lead the effects on synaptic plasticity. The molecular structure of corticosterone receptors seems to be important. MRs, for example, exist in different molecular configurations (Joels, 2008), thus these receptors can be very diverse. This diversity in molecular structure could be linked to diverse intracellular pathways that differently influence neuronal functions. Another issue that has to be considered is the clusters of brain areas that are involved in a particular stress situation. Various brain regions have specific properties and are incorporated into unique networks, so that even if corticosterone evokes the same effect at the single cell level, this would not always result in the same effect on network functions such as LTP. For instance, both CA1 pyramidal neurons and granule cells in the DG highly express MR as well as GR (Joels, 2008). In the DH, corticosterone and stress consistently suppress the induction of CA1 LTP *in vivo* and *in vitro*, unlike the case for the DG. High concentration of corticosteroid (Pavlides et al., 1993) or tail shocks (Shors and Dryver, 1994) can indeed suppress LTP; however, in other situations, either no effect (Bramham et al., 1998; Gerges et al., 2001; Alfarez et al., 2003) or enhancement of LTP has been reported (Kavushansky et al., 2006). This is because LTP in the DG seems to be more dependent on indirect inputs from the amygdale (Akirav and Richter-Levin, 2002; Kavushansky and Richter-Levin, 2006). Finally, the response to a stressor is also determined by the history of the organism. For instance, the induction of LTP is impaired in animals that have been exposed to repetitive stress in the weeks prior to the experiment, even if corticosterone levels, at the time of LTP induction, are compatible with the expression of a normal LTP (Alfarez et al., 2003). Studies on the effect of maternal care on synaptic plasticity report that animals that received very little maternal care have poor LTP when they are adult, as opposed to animals that received high maternal care (Champagne et al., 2008). Interestingly, while LTP is suppressed by corticosterone in the latter group, it is enhanced in the former (Champagne et al., 2008). All in all, corticosteroid modulation of synaptic plasticity in the hippocampus seems to be more complex than previously thought and additional experiments are needed to address the role of membrane-bound as well as intracellular receptors on LTP/LTD regulation.

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# Dynamic regulation of NMDAR function in the adult brain by the stress hormone corticosterone

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Stress and corticosteroids dynamically modulate the expression of synaptic plasticity at glutamatergic synapses in the developed brain. Together with alpha-amino-3-hydroxy-methyl-4-isoxazole propionic acid receptors (AMPAR), N-methyl-D-aspartate receptors (NMDAR) are critical mediators of synaptic function and are essential for the induction of many forms of synaptic plasticity. Regulation of NMDAR function by cortisol/corticosterone (CORT) may be fundamental to the effects of stress on synaptic plasticity. Recent reports of the efficacy of NMDAR antagonists in treating certain stress-associated psychopathologies further highlight the importance of understanding the regulation of NMDAR function by CORT. Knowledge of how corticosteroids regulate NMDAR function within the adult brain is relatively sparse, perhaps due to a common belief that NMDAR function is stable in the adult brain. We review recent results from our laboratory and others demonstrating dynamic regulation of NMDAR function by CORT in the adult brain. In addition, we consider the issue of how differences in the early life environment may program differential sensitivity to modulation of NMDAR function by CORT and how this may influence synaptic function during stress. Findings from these studies demonstrate that NMDAR function in the adult hippocampus remains sensitive to even brief exposures to CORT and that the capacity for modulation of NMDAR may be programmed, in part, by the early life environment. Modulation of NMDAR function may contribute to dynamic regulation of synaptic plasticity and adaptation in the face of stress, however, enhanced NMDAR function may be implicated in mechanisms of stress-related psychopathologies including depression.

**Keywords: electrophysiology, synaptic plasticity, stress, receptor trafficking, corticosteroid receptor, learning and memory**

## INTRODUCTION

In developed countries such as Canada, around three quarters of the adult population experience moderate levels of stress (Statistics Canada, 2002). As a potent modulator of memory (McEwen and Sapolsky, 1995; Sandi and Pinelo-Nava, 2007), stress is implicated in the associated cognitive impairment in depressive disorders (Muscatell et al., 2009). However, stress does not always impair memory. Indeed, stress is believed to be crucial to the immutable storage of traumatic memories in post-traumatic stress disorder (PTSD) (Vanitallie, 2002). Investigating how stress exerts both facilitatory and suppressive effects on memory could improve our understanding of abnormal memory function in stress-related psychiatric disorders. At the cellular level, memory is established via persistent alterations in the strength of synaptic transmission through a collection of cellular processes known as synaptic plasticity. In parallel with its impact on memory, stress can both facilitate and suppress synaptic plasticity via the actions of the stress hormone cortisol, or corticosterone (CORT) in rodents. Thus, investigating the mechanisms underlying the impact of CORT on synaptic plasticity could help reveal the physiological basis of cognitive effects of stress.

Activation of glutamate receptors, including AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA subtypes (N-methyl-D-aspartate), is instrumental to the formation and maintenance of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Bliss and Collingridge, 1993; Bear and Abraham, 1996; Malinow and Malenka, 2002). Glutamate receptors could be important cellular targets for stress and CORT to regulate synaptic plasticity in the adult brain. Indeed, at least in developing brain tissue, CORT regulates the trafficking properties of AMPA receptor [AMPAR (Groc et al., 2008; Martin et al., 2009)]. Until recently, NMDA receptor (NMDAR) was widely believed to be highly stable in the adult brain. Recent findings from our laboratory revealed CORT-induced plastic changes in both the function and subunit composition of NMDAR. Given that NMDAR plays critical roles in synaptic plasticity, these findings illustrate a novel mechanism for stress to regulate synaptic plasticity. We also found that CORT-induced changes in NMDAR in adulthood can be programmed by early life adversity such as low maternal care. Since early life stress strongly associates with an increased vulnerability to psychiatric disorders like depression (Kessler et al.,

1997; McLaughlin et al., 2010), our findings support an emerging view that alteration of the plastic properties of NMDAR is a key biological substrate of stress-related brain disorders.

The purpose of this review is, therefore, to summarize findings from our laboratory concerning the influence of CORT on NMDAR function in the adult brain. We first discuss the current understanding of the impact of CORT on hippocampal synaptic plasticity. Next, we describe recent findings demonstrating that plastic changes of NMDAR function after CORT exposure regulate synaptic plasticity in the adult brain. Finally, we summarize findings showing that the impact of CORT on NMDAR function in adulthood can be programmed by early life experience in the form of maternal care.

## CORT AND SYNAPTIC PLASTICITY

CORT is a pleiotropic hormone that regulates cardiovascular, immunologic, metabolic, and neurologic functions (Sapolsky et al., 2000). The cellular actions of CORT are mediated by two types of corticosteroid receptors: low affinity glucocorticoid receptors (GRs) and high-affinity mineralocorticoid receptors (MRs) (Reul and de Kloet, 1985; Joels, 2001). Both GR and MR can be found in the cytosol and function as transcription factors that alter gene expression. Recent findings also suggest the presence of membrane-associated GRs and MRs to mediate fast-acting (<30 min) non-genomic actions of CORT (Prager and Johnson, 2009). Under basal conditions, plasma (Atkinson et al., 2006) and hippocampal (Droste et al., 2008) CORT levels follow a circadian rhythm with a nadir around the start of the light cycle. During the light cycle and part of the dark cycle, CORT levels also show an ultradian pattern (1 cycle/h). During ultradian peaks hippocampal CORT levels reach as high as 15 nM (Droste et al., 2008). Stress also significantly raises the levels of hippocampal CORT. For example, a 15 min period of forced swimming increases hippocampal CORT to approximately 100 nM for around 30 min (Droste et al., 2009). CORT exhibits both facilitating and suppressing effects on memory function and hippocampal synaptic plasticity. LTP (Bliss and Lomo, 1973; Bliss and Collingridge, 1993) and LTD (Dudek and Bear, 1992; Bear and Abraham, 1996) are two forms of synaptic plasticity which are regarded as cellular models of learning and memory (Bliss and Collingridge, 1993; Martin et al., 2000). The impact of CORT on synaptic plasticity depends on various factors, which will be discussed below.

### LEVEL OF CORT

Basal levels of CORT are important for memory function such that insufficient CORT (e.g., in adrenalectomized animals) results in impaired LTP (Diamond et al., 1992) and memory (Vaher et al., 1994). These promnesic influences of CORT are likely mediated by high affinity MRs, since LTP is enhanced by MR agonists (Pavlides et al., 1994, 1996; Rey et al., 1994), and stress-induced facilitation of LTP is blocked by MR antagonists (Korz and Frey, 2003; Avital et al., 2006). Exposure to CORT at stress levels, which activates both MRs and GRs, usually results in impairment of memory. Most of these negative impacts were observed hours after CORT application (Pavlides et al., 1995, 1996; Krugers et al., 2005; Wiegert et al., 2005), suggesting the requirement of GR-induced genomic mechanisms (Tsai and O'Malley, 1994). The detrimental impacts of CORT on memory functions could be partly attributed to GR-mediated LTP suppression. LTP is suppressed by GR agonists (Pavlides et al., 1995) and stress-induced inhibition of LTP is blocked by GR antagonists (Rey et al., 1994; Avital et al., 2006). These findings highlight the inverted-U shape relationship between LTP formation and CORT concentration (Diamond et al., 1992; Rey et al., 1994). Unlike LTP, CORT facilitates LTD via GR activation (Xu et al., 1997, 1998; Yang et al., 2005; Chaouloff et al., 2008).

### TIMING AND DURATION OF CORT APPLICATION

Although CORT is better known for its suppressing effect on LTP, recent findings suggest that depending on the timing of LTP induction, CORT may also facilitate LTP. For instance, a brief application of stress level CORT (100 nM) facilitates LTP if it is applied immediately before tetanus stimulation (Wiegert et al., 2006). This facilitating effect of CORT contrasts with its suppressing action on LTP when plasticity is induced hours later (Krugers et al., 2005; Wiegert et al., 2005). Note that membrane bound corticosteroid receptors (Wiegert et al., 2006) have been implicated in these facilitatory effects of CORT on memory. The rapid, acute facilitatory effect of CORT on LTP may relate to the positive impact of intrinsic stress (stress during learning) on the acquisition and consolidation of memory [for review, see (Sandi and Pinelo-Nava, 2007)].

While LTP is facilitated by acute CORT, prolonged CORT exposure suppresses LTP (Kerr et al., 1994). LTP is also suppressed in chronically stressed rats (Gerges et al., 2001; Pavlides et al., 2002; Alfarez et al., 2003) [but also see (Holderbach et al., 2007)]. In addition, LTD can be facilitated in animals exposed to chronic stress (Yang et al., 2006, 2007; Ma et al., 2007) or chronic CORT infusion (Dumas et al., 2010).

### SUBFIELD OF THE HIPPOCAMPUS

Our understanding of the impact of CORT on synaptic plasticity is primarily informed by studies performed in the hippocampal CA1 region. CORT also affects synaptic plasticity in other hippocampal subfields. For instance, one hour after GR agonist application, LTP is suppressed in the dentate gyrus (DG) (Pavlides et al., 1995). Similar to the CA1 region, CORT induces rapid facilitation of LTP in the DG. Stressing rats with a brief forced swimming 15 min after LTP induction converts a short-lasting DG LTP into a long-lasting form (Korz and Frey, 2003) and this effect is mediated by MR activation. The impact of CORT on DG LTD is less clear. A typical LTP protocol induces LTD in GR agonist-treated DG slices (Pavlides et al., 1995), suggesting that LTD in the DG is also facilitated by CORT. Acute stress also suppresses mossy-fiber LTP in the CA3 region through a GR-mediated pathway (Chen et al., 2010). Whether CORT exerts a rapid-onset facilitatory effect on LTP in the CA3 region remains unclear.

### SUBREGIONS OF THE HIPPOCAMPUS

The hippocampus can also be separated into dorsal (septal) and ventral (temporal) subregions. Not only do these hippocampal subregions receive distinct synaptic inputs from the entorhinal cortex (Dolorfo and Amaral, 1998), they also subserve different

cognitive roles. Lesion of the dorsal hippocampus impairs spatial learning and memory (Moser et al., 1993). However, damage to the ventral hippocampus, which connects with the bed nucleus of the stria terminalis and the amygdala (Swanson and Cowan, 1977; Van and Wyss, 1990; Pitkanen et al., 2000), alters performance in fear- and anxiety-related behavioral tasks (Richmond et al., 1999; McHugh et al., 2004). While spatial learning can be suppressed by stress (Conrad et al., 1996; Diamond et al., 1996), stress typically enhances fear- and anxiety-related behaviors. One would, therefore, expect that stress differentially regulates encoding in these two hippocampal regions through opposing effects on synaptic plasticity. In agreement with this hypothesis, it has been shown that while CORT suppresses LTP in the dorsal hippocampus, this stress hormone facilitates LTP in the ventral hippocampus (Maggio and Segal, 2007). The effects of CORT on different hippocampal subregions are mediated by different corticosteroid receptors. MR activation facilitates LTP in the ventral hippocampus, whereas GR activation is responsible for suppressing LTP in the dorsal hippocampus. Notably, the form of LTP that is facilitated by CORT in the ventral hippocampus is not NMDAR dependent but requires activation of voltage-gated calcium channels. CORT also exerts opposing regulation of LTD in the dorsal and ventral hippocampi (Maggio and Segal, 2009). In the dorsal hippocampus, CORT activates GR to enhance LTD formation. However, LTD is suppressed by CORT in the ventral hippocampus through a MR-mediated mechanism.

### GENDER

Our current understanding of the impact of CORT on synaptic plasticity is dominated by findings obtained from male rodents. Available evidence suggests that gender could affect the impact of CORT on synaptic plasticity. For instance, while chronic restraint stress impairs spatial memory in a radial arm maze in male rats, similar stress enhances performance in female rats in this task (Luine et al., 2007). Gender differences in Morris water maze performance are abolished by adrenalectomy (Beiko et al., 2004), suggesting that these differences are glucocorticoid dependent. Gender differences in stress responsiveness are also observed at the level of synaptic plasticity. For instance, the maintenance of DG LTP induced by stimulation of the lateral perforant path in male and female rats is sensitive to MR (Velisek et al., 2003) and GR blockade (Velisek and Vathy, 2005), respectively. In addition, while hippocampal LTD is facilitated in slices obtained from acutely stressed male rats, similar stress-induced facilitation of LTD cannot be observed in slices from stressed female rats (Huang et al.). Gender-dependent CORT effects on hippocampal function may also be regionally specific: while CORT inhibits neurogenesis in both the dorsal and ventral region of hippocampus in male rats, an inhibitory effect on neurogenesis is only observed in the ventral hippocampus of female rats (Brummelte and Galea, 2010).

## CORT REGULATION OF PRE- AND POST-SYNAPTIC FUNCTION

CORT exerts biphasic effects on synaptic plasticity. These actions may relate to changes in glutamatergic transmission. Existing evidence suggests that the rapid effect of CORT is to enhance neuronal excitability and glutamate release, while the delayed effect is to normalize activity to pre-stimulation levels (Joels et al., 2007). CORT induces rapid alterations in both pre- and post-synaptic function. *In vivo*, CORT enhances extracellular glutamate levels within the hippocampus rapidly (within 15 min) and transiently (return to baseline within 30–45 min) and these effects are insensitive to both GR and MR antagonists (Venero and Borrell, 1999). *In vitro*, CORT-induced increases in the frequency but not the amplitude of mEPSCs in CA1 pyramidal neurons and DG granule neurons after brief CORT treatment point to an effect on presynaptic glutamate transmission (Katz, 1971). This effect is reproduced by membrane impermeable BSA-CORT and the endogenous mineralocorticoid, aldosterone, and blocked by the MR-antagonist spironolactone (Karst et al., 2005; Pasricha et al., 2011) implicating a membrane-bound MR. Similarly, in CA1 pyramidal neurons in acute slices CORT rapidly reduces paired-pulse facilitation (Karst et al., 2005), a measure sensitive to alterations in presynaptic function (Debanne et al., 1996), providing a further demonstration that CORT increases presynaptic glutamate release. In parallel to effects on presynaptic function, CORT rapidly alters postsynaptic function, increasing neuronal excitability via inhibition of $I_A$ conductance of voltage-gated potassium channels. This inhibition is blocked by the MR-antagonist spironolactone or intracellular application of a G-protein inhibitor to the postsynaptic neuron (Karst et al., 2005; Olijslagers et al., 2008).

Following the rapid effects of CORT, the delayed, genomic effects of CORT may compensate for the increased glutamatergic transmission induced by rapid membrane-receptor mediated actions by suppressing neuronal excitability to restore $Ca^{2+}$ homeostasis. Although increased $Ca^{2+}$ influx is maintained by the upregulation of voltage-gated calcium currents (Karst et al., 2000), this enhances the slow after hyperpolarization, reducing neuronal excitability (Joels and de Kloet, 1989). However, recent evidence suggests that delayed upregulation of voltage-gated calcium currents does not occur in the DG highlighting the subfield specific nature of genomic CORT effects in the hippocampus (Van Gemert et al., 2009). Thus, the delayed effects of CORT may act to curtail a period of enhanced plasticity induced by acute stress and limit further changes in synaptic strength.

## CORT AND GLUTAMATE RECEPTORS

Apart from regulating presynaptic release of glutamate and postsynaptic depolarization of neurons, increasing findings suggest that CORT directly alters the functional properties and plasticity of glutamate receptors. Notably, glutamate receptors, including the NMDAR and AMPAR subtypes, are critical mediators of the induction and maintenance of synaptic plasticity. Changes in NMDAR and AMPAR properties after CORT treatment would therefore significantly impact synaptic plasticity. Below we discuss the impact of CORT on these two ionotropic glutamate receptor species.

### GLUTAMATE RECEPTORS AND SYNAPTIC PLASTICITY

NMDAR, AMPAR, and kainate receptors belong to the family of ionotropic glutamate receptors (Dingledine et al., 1999). They are multimeric assemblies of distinct subunits. NMDAR

subunits include GluN1 (Moriyoshi et al., 1991), GluN2 [A–D (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993)], and GluN3 [A–B (Ciabarra et al., 1995; Sucher et al., 1995; Chatterton et al., 2002)]. Functional NMDARs contain GluN1 plus at least one type of GluN2 subunit (Seeburg, 1993; Dingledine et al., 1999). The most common GluN2 subunits in the adult hippocampus are GluN2A and GluN2B (Kirson and Yaari, 1996; Laurie et al., 1997; Wenzel et al., 1997). Four AMPAR subunits (GluA1–4) have been identified (Nakanishi, 1992; Hollmann and Heinemann, 1994; Dingledine et al., 1999). NMDAR plays pivotal roles in LTP (Collingridge et al., 1983) and LTD formation (Dudek and Bear, 1992) because: (1) it is highly conductive to $Ca^{2+}$ (MacDermott et al., 1986), a crucial chemical signal for synaptic plasticity (Bliss and Collingridge, 1993; Bear and Abraham, 1996); (2) its opening is gated by a voltage-sensitive $Mg^{2+}$ blockade that is removed by depolarization (Nowak et al., 1984). The latter property allows NMDAR to serve as a *coincidence detector* of simultaneous presynaptic glutamate release and postsynaptic depolarization, which is fundamental to induction of synaptic plasticity. The presence of $Mg^{2+}$ blockade also limits the contribution of NMDAR to basal synaptic transmission. Thus, long-term alteration of the strength of glutamate synapses after LTP and LTD induction is expressed by changes in the gating (Benke et al., 1998) and/or trafficking (Malenka, 2003; Collingridge et al., 2004) properties of AMPAR in glutamate synapses.

## CORT AND THE PLASTICITY OF AMPAR

CORT facilitates AMPAR-mediated synaptic transmission by increasing the frequency of AMPAR-mediated miniature excitatory postsynaptic currents [EPSC, (Karst and Joels, 2005)]. In addition, CORT enhances the mobility of AMPAR by facilitating exo/endocytotic exchange between cytosolic and surface receptors (Martin et al., 2009) and lateral trafficking between synaptic and extra-synaptic receptors (Groc et al., 2008). The effect of this increased mobility is likely an enrichment of GluA2 subunits in glutamate synapses (Martin et al., 2009). While these changes in AMPAR function could explain the facilitating effect of CORT on synaptic plasticity, they take hours to develop (Karst et al., 2005; Martin et al., 2009). Thus, these slow-onset changes likely contribute little to rapid CORT-induced alterations of LTP (Wiegert et al., 2006) and LTD (within minutes) (Xu et al., 1997). Moreover, how facilitation of AMPAR function contributes to the delayed suppressive effect of CORT on synaptic plasticity remains unclear.

## PLASTICITY OF NMDARs IN THE ADULT HIPPOCAMPUS

Until recently, NMDAR in the adult brain was believed to be highly stable. Electron microscopy studies reveal that the number of immunogold labeled NMDARs per hippocampal synapse from P10 rats is almost identical to that from 5-week-old rats (Petralia et al., 1999). In marked contrast, the number of labeled AMPARs increases 2–3-fold during the same developmental period. The potential for plasticity of NMDAR (i.e., alteration of the expression and/or electrophysiological properties of NMDAR channels) is also reduced across the course of brain development. For instance, plastic changes in NMDAR subunit composition are triggered by high frequency stimulation in developing hippocampal tissue (<P10) but cannot be induced in tissue from 3-week-old rats (Bellone and Nicoll, 2007). In addition, stimulation protocols that induce LTP of AMPAR-mediated synaptic currents do not robustly alter NMDAR-mediated synaptic currents (Muller et al., 1988; Perkel and Nicoll, 1993) [but also see (Bashir et al., 1991; Grosshans et al., 2002)]. The increasing resistance of NMDAR-mediated synaptic currents to plastic alteration across development is likely related to the dramatic alterations of NMDAR subunit composition. In particular, in the first postnatal month there is a switch from GluN2B-enriched to GluN2A-enriched NMDAR in glutamate synapses (Sheng et al., 1994; Ritter et al., 2002). GluN2A-containing NMDARs display less horizontal [between synaptic and extra-synaptic locations (Groc et al., 2006)] and vertical motility [between surface membrane and cytosol (Barria and Malinow, 2002)] than GluN2B-containing receptors. Although GluN2B-containing NMDARs can still be found in adult glutamate synapses (Erisir and Harris, 2003), the developmental increase in GluN2A subunits could greatly enhance NMDAR stability. Taken together, these findings suggest that a high stability of NMDAR function is actively maintained in adult glutamate synapses. Despite this, recent findings have challenged the long-held assumption that plasticity of NMDAR function is difficult to induce in the adult brain.

Several lines of evidence suggest that plastic changes of NMDAR are induced in an experience dependent manner in the adult brain. Dopamine alters NMDAR-mediated synaptic currents in the adult brain (Varela et al., 2009). Apart from changing the size of NMDAR-mediated currents, NMDAR subunit composition is also subject to plasticity in the adult brain. For instance, the ratio of GluN2A/GluN2B mRNA expression varies with the reproductive cycle of female rats (Gore et al., 2000), seasonal testosterone levels of male song birds (Singh et al., 2003), and chronic stress exposure (Qin et al., 2004). Although the functional consequences of these subunit modifications remain unknown, these findings raise two important points. Firstly, steroidal hormones, including CORT, could be potent biological modulators of NMDAR function in the adult brain. Secondly, even after the developmental switch of NMDAR from GluN2B-enriched to a GluN2A-enriched, the potential remains for further GluN2 subunit change in response to stressful experiences. The effect of CORT on NMDAR function is supported by findings obtained in young (acute slices prepared from early postnatal brains) and developing brain tissue (cultured neurons prepared from embryonic brains). CORT can both facilitate (Takahashi et al., 2002) and attenuate (Sato et al., 2004; Liu et al., 2007) NMDAR-mediated $Ca^{2+}$ influx and current in both cultured neurons and young hippocampal slices. How CORT mediates bidirectional changes in the electrophysiological properties of NMDAR remains unclear. Notably, whether NMDAR function in the adult brain remains sensitive to modulation by CORT is yet to be widely investigated.

## CORT-INDUCED ENHANCEMENT OF NMDAR FUNCTION IN THE ADULT HIPPOCAMPUS

Recently, we addressed the issue of the capacity of CORT to induce changes in synaptic NMDAR function in the adult brain

using an adult (3-month old) rat hippocampal slice preparation (Tse et al., 2011). The findings of these studies identified both a fast-onset and long-lasting increase in synaptic NMDAR function following a 30 min exposure to stress level (100 nM) CORT. Note that 100 nM CORT approximates CORT levels measured by microdialysis *in vivo* in rat hippocampus shortly after exposure to an intense stressor such as forced swimming (Droste et al., 2009). A single 30 min CORT application increased NMDAR function at glutamate synapses in the dorsal hippocampal CA1 region as reflected by an increase in normalized NMDAR-mediated field excitatory postsynaptic potentials (NMDAR-fEPSPs). Surprisingly, using a similar methodology we did not observe an effect of CORT on AMPAR function measured by normalized AMPAR-fEPSPs. Importantly, there was a parallel increase in the ratio of evoked NMDAR-mediated EPSCs (NMDAR-EPSCs) vs. AMPAR-EPSC after CORT treatment using whole-cell patch clamp recording. This further confirms that CORT enhances NMDAR function. Moreover, although CORT treatment was limited to 30 min, we found that the rapid CORT-induced increase in NMDAR/AMPAR ratio lasted for at least two hours after wash-out of CORT. It is important to consider the specific temporal parameters used when interpreting the lack of AMPAR changes in this study. Since CORT-induced alterations in AMPAR expression and function were previously observed 2–3 h post-treatment (Karst and Joels, 2005; Groc et al., 2008; Martin et al., 2009), the time window in which we observed alterations in NMDAR function may precede these slow-onset changes.

As reviewed above, NMDAR function is critically implicated in synaptic plasticity and CORT exerts a complex regulation of bidirectional synaptic plasticity. Thus, we asked how the acute modulation of NMDAR function by CORT might manifest in regulation of bidirectional synaptic plasticity. We found that during CORT treatment, both LTP and LTD of AMPAR fEPSPs were facilitated relative to vehicle treated slices. This finding is consistent with the fast-onset facilitation of bidirectional synaptic plasticity by stress (Xu et al., 1997) and CORT (Xu et al., 1998; Wiegert et al., 2006). This phenomenon might be attributable to enhanced NMDAR function in the presence of CORT. This would increase calcium influx during LTP and LTD induction and increase the magnitude of plastic change. However, the completeness of this explanation is challenged by our finding that synaptic plasticity was not facilitated 1–2 h after a brief CORT treatment despite sustained enhancement of NMDAR function. To resolve the question of why CORT no longer facilitated synaptic plasticity although synaptic NMDAR function remained enhanced, a more thorough characterization of CORT effects on NMDAR was required.

### CORT-INDUCED ALTERATION OF NMDAR SUBUNIT COMPOSITION IN THE ADULT HIPPOCAMPUS

Apart from modulating the synaptic currents mediated by NMDAR, CORT might also regulate the GluN2 subunit composition of NMDAR to modulate induction of bidirectional synaptic plasticity. In the hippocampus, GluN2A and GluN2B are the two most common GluN2 subunits. Expression of GluN2 subunits is developmentally regulated. In early postnatal stages (e.g., $<1$ month), hippocampal NMDARs are mostly GluN2B-containing (Monyer et al., 1994). GluN2A expression increases with development and predominates in the adult hippocampus (Wenzel et al., 1997). GluN2 subunits play important roles in determining NMDAR function (Monyer et al., 1994). For example, blocking GluN2B-containing NMDAR using Ro25–6981 inhibits LTD formation *in vitro* and *in vivo*, whereas blocking GluN2A-containing NMDAR selectively abolishes LTP (Liu et al., 2004; Ge et al., 2010). Nonetheless, how different GluN2 subunits contribute to bidirectional synaptic plasticity is still extensively debated [for review, see (Yashiro and Philpot, 2008; Fetterolf and Foster, 2011)]. Differences in biophysical properties and signaling between GluN2A and GluN2B may be responsible for their differential roles in synaptic plasticity.

GluN2A-containing NMDAR displays larger conductance and faster decay kinetics than GluN2B-containing NMDAR (Monyer et al., 1994). Findings from single channel studies also reveal higher opening probability and faster conformational changes in GluN2A-containing NMDAR compared with GluN2B-containing NMDAR (Erreger et al., 2005). Due to their more rapid conformational change, GluN2A-containing NMDARs may contribute more to calcium transfer than GluN2B-containing NMDARs during LTP-inducing high frequency stimulation. In contrast, LTD-inducing low frequency stimulation protocols would favor charge transfer through GluN2B-containing NMDAR. Alternatively, the carboxyl terminal of GluN2 subunit, which interacts with different scaffolding or signaling proteins, could determine the polarity of synaptic plasticity. For instance, mice expressing GluN2A subunit without the carboxyl terminal are deficient in hippocampal LTP formation (Kohr et al., 2003). This finding suggests that the carboxyl terminal of GluN2A may recruit signaling proteins that are responsible for LTP formation. However, the contribution of GluN2A to LTP may follow an inverted U-shape relationship. Overexpression of GluN2A subunit in cultured hippocampal slices impairs LTP (Foster et al., 2010). Overexpressing carboxyl-terminal truncated GluN2A subunit does not affect LTP formation, suggesting that excessive GluN2A impairs LTP through recruiting LTP-blocking signaling proteins that bind the carboxyl terminal of GluN2A subunit. The identity of proteins that bind to the carboxyl terminal of GluN2A subunit to facilitate or suppress LTP formation remain unknown. Although knocking down GluN2B abolishes LTD formation (Brigman et al., 2010), little is known about the contribution of GluN2B carboxyl terminal to LTD formation.

When we assessed glutamate receptor surface membrane expression the data strongly suggested that CORT increased the ratio of GluN2A/GluN2B. CORT increased the surface GluN2A and GluN1 expression measured in hippocampal synaptosomes yet did not affect the expression of GluN2B or GluA1, an AMPAR subunit. GluN2A and GluN1 expression was not increased during CORT treatment but only 1–2 h after the cessation of CORT treatment. Interestingly, the time-course of increased GluN2A corresponds to the time-course of the attenuation of CORT-induced facilitation of bidirectional synaptic plasticity. An increase in GluN2A could inhibit both LTP and LTD formation. Increased GluN2A/GluN2B ratio lowers the synaptic contribution of GluN2B-containing NMDAR. This could reduce LTD which requires GluN2B-NMDAR activation (Liu et al., 2004;

Ge et al., 2010). As mentioned earlier, excess GluN2A expression inhibits LTP formation (Foster et al., 2010). Increased synaptic expression of GluN2A subunit could be one mechanism through which rapid facilitation of synaptic plasticity is attenuated in the period hours after CORT or stress exposure.

Since GluN2 subunits undergo substantial developmental changes, CORT-induced changes in NMDAR subunit composition may differ with developmental stage. In one-month-old rats that exhibit high GluN2B expression, acute stress increases synaptic NMDAR function in the prefrontal cortex (PFC) (Yuen et al., 2009). In contrast to the selective increase of GluN2A expression in CORT-treated adult hippocampus, both GluN2A and GluN2B expression are enhanced by stress in juvenile PFC synapses. However, these findings could also suggest a regional difference in the regulation of NMDAR expression by CORT between the PFC and the hippocampus. Future studies, are necessary to determine the impact of CORT on NMDAR subunit composition in young hippocampal tissue.

Our findings suggest that NMDAR in the adult hippocampus is altered by brief exposure to stress level CORT (**Figure 1**). The enhancement of NMDAR occurs rapidly during CORT treatment. This rapid enhancement associates with facilitation of bidirectional synaptic plasticity. However, increased NMDAR function is followed by increased synaptic expression of GluN1 and GluN2A subunits. This secondary effect associates with the loss of synaptic plasticity facilitation. We suggest that plastic alteration of synaptic NMDAR in the adult hippocampus is instrumental to CORT regulation of synaptic plasticity. Regulation of synaptic plasticity by CORT in adulthood is programmed by early life experience.

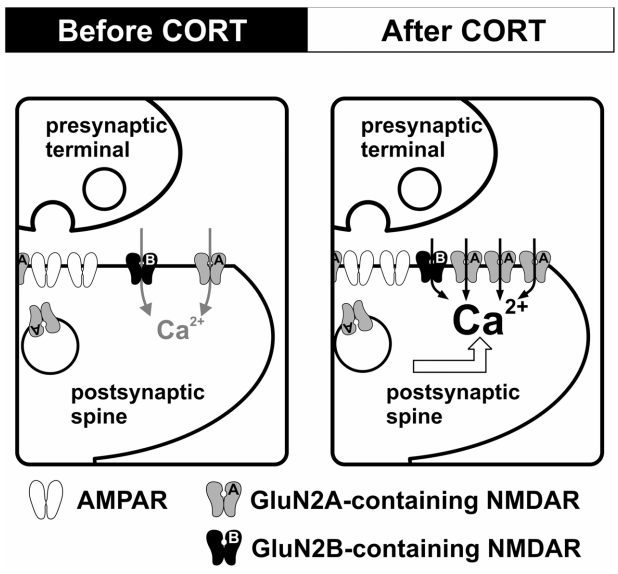


**FIGURE 1 | CORT-induced dynamic regulation of synaptic NMDARs in the adult hippocampus.** Schematic diagrams summarize the impact of CORT on NMDAR function. Compared with controls (*left*), stress level CORT treatment (100 nM, 30 min) induces a fast-onset increase in synaptic NMDAR function and a slow-onset (1–2 h after CORT treatment) enhancement of the surface expression of GluN2A-containing NMDAR (*right*).

As we will discuss below, maternal care exerts a lasting impact on stress effects on hippocampal synaptic plasticity.

## HIPPOCAMPAL COGNITIVE DEVELOPMENT AND MATERNAL CARE

The early environment exerts profound and enduring effects on hippocampal development and function (Bornstein and Tamis-LeMonda, 1989; Liu et al., 2000; Champagne et al., 2008). In rodents, the tactile stimulation provided by maternal pup-directed licking/grooming (LG) is an important component of the early environment (Schanberg et al., 1984). Intensive characterization of naturally occurring variations in maternal behavior in outbred Long–Evans rats reveals that the frequency of LG is normally distributed within the population and the relative frequency with which a rat dam licks and grooms her pups is stably maintained across subsequent litters (Champagne et al., 2003). The frequency of LG behavior can be used to identify two populations of rats in which to examine the consequences for offspring development of comparatively low (Low LG) and high (High LG) levels of maternal stimulation.

Maternal LG frequency is positively correlated with hippocampus-dependent learning in adult male offspring. Compared to Low LG offspring, offspring of High LG mothers learn the location of a hidden platform in the Morris water maze in fewer trials and exhibit enhanced recall of the platform location in probe tests (Liu et al., 2000). The offspring of High LG mothers also show enhanced memory in an object recognition task (Bredy et al., 2003). Consistent with enhanced hippocampal-dependent learning and memory, the magnitude of LTP in the hippocampal DG of High LG offspring is greater than in Low LG offspring (Bredy et al., 2003; Champagne et al., 2008; Bagot et al., 2009). Maternal effects on hippocampal synaptic plasticity and memory associate with increases in hippocampal NMDAR and AMPAR mRNA subunit expression and receptor binding as well as enhanced cholinergic innervation of the hippocampus (Liu et al., 2000; Bredy et al., 2003, 2004). Furthermore, hippocampal morphology is influenced by maternal care and dendritic arborization and spine density is also increased in the hippocampal CA1 of High LG offspring (Champagne et al., 2008; Bagot et al., 2009).

### MATERNAL CARE AND STRESS RESPONSIVITY

In addition to effects on cognitive development, maternal care influences stress reactivity and the hypothalamic-pituitary-adrenal (HPA) stress axis. High levels of pup LG in early life are associated with reduced stress responsivity in adulthood. Compared to the adult offspring of Low LG mothers, those of High LG dams show lower plasma levels of adrenocorticotropic hormone (ACTH) and CORT both during and following the termination of acute restraint stress (Liu et al., 1997). Up-regulation of GR expression in all hippocampal subfields is an important mediator of the enhanced negative feedback control in adult animals exposed to high levels of maternal LG (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004). During stress-induced elevations in CORT, GRs become progressively occupied and thus hippocampal control of stress-induced HPA-axis activity is mediated by stimulation of GR activity by CORT

(de Kloet et al., 1998; Furay et al., 2008). Manipulations that increase hippocampal GR expression, such as early-life handling are associated with attenuated post-stress plasma ACTH and CORT levels (Meaney et al., 1985; Viau et al., 1993). Reductions in GR expression, such as occur in aged animals, are associated with prolonged increases in stress-induced plasma CORT (Morano et al., 1994). The central role of the hippocampus as target and regulator of the HPA-axis suggests that alterations of HPA-axis activity should have wide ranging consequences for hippocampal learning and plasticity. Indeed, brief CORT treatment suppresses LTP formation in the dorsal hippocampal CA1 (Champagne et al., 2008) and DG (Bagot et al., 2009) of High LG offspring. However, LTP is facilitated by CORT in Low LG offspring. Stress also enhances hippocampus-dependent learning in Low LG offspring in contextual fear-conditioning (Bagot et al., 2009). Thus the maternal effect on stress responsivity influences hippocampus-dependent learning and synaptic plasticity. Given the fundamental roles of NMDAR in synaptic plasticity, maternal care might regulate hippocampal function through actions on this glutamate receptor. Findings from expression and binding studies suggest LG experience enhances the expression of NMDAR subunits GluN1, GluN2A, and GluN2B in the hippocampus (Liu et al., 2000). Nonetheless, changes in NMDAR expression and binding do not directly reflect the functional properties of NMDAR activation in synapses, which is crucial to synaptic plasticity.

#### NMDAR SYNAPTIC FUNCTION IS INCREASED IN LOW LG OFFSPRING

In contrast to earlier studies of receptor expression, recent work in our laboratory employing functional measures of glutamate receptor activity suggest that NMDAR function is enhanced in Low LG offspring (Bagot et al.). In the dorsal DG, normalized NMDAR-fEPSPs are significantly larger in Low LG than High LG offspring. However, AMPAR-fEPSPs do not differ between High and Low LG offspring indicating the maternal effect is specific to NMDAR function. Whole-cell recording experiments further support this conclusion. The ratio of the amplitude of NMDAR-EPSCs vs. the amplitude of AMPAR-EPSCs is significantly increased in Low LG offspring. Given that Low LG offspring also exhibit deficits in LTP (Bredy et al., 2003; Champagne et al., 2008; Bagot et al., 2009) this increase in NMDAR function is surprising. Enhanced NMDAR function could be expected to reduce the threshold and enhance the magnitude of LTP. However, over-activation of NMDAR induced by low extracellular $Mg^{2+}$ conditions during LTP induction (Coan et al., 1989; Frankiewicz and Parsons, 1999) or excessive cleft glutamate (Katagiri et al., 2001) impairs LTP. Thus, excessive NMDAR activation during LTP induction might underlie the loss of LTP in offspring of Low LG mothers.

#### MATERNAL CARE ALTERS CORT-REGULATION OF NMDAR FUNCTION

Although maternal care might be expected to differentially affect CORT-regulation of NMDAR function, the direction of such an effect is difficult to predict based on previous findings. Since High LG offspring are less stress responsive than Low LG offspring, one might expect CORT to exert a stronger impact on NMDAR in Low LG offspring. Alternatively, since High LG offspring express higher levels of GR in the hippocampus, and GR activation is necessary for CORT-induced enhancement of NMDAR function (Tse et al., 2011), CORT may more potently regulate NMDAR function in High LG offspring. In fact, we found that stress-level CORT (100 nM) significantly enhanced NMDAR function in High LG offspring and increased the normalized NMDAR-fEPSP. In contrast, CORT treatment had no detectable effect on NMDAR-fEPSPs in Low LG offspring. The mechanism underlying the loss of CORT-regulation of NMDAR in Low LG offspring is unclear. Since NMDAR function is maintained at a high and possibly saturated level in Low LG offspring in basal conditions, the capacity for further enhancement of NMDAR function after CORT treatment could be limited. Interestingly, the time-course of CORT-induced enhancement of NMDAR function (within 20 min) suggested that a classical genomic action requiring cytoplasmic corticosteroid receptors is not involved. Indeed the CORT effect was reproduced by a BSA-CORT conjugate, implicating the involvement of a membrane-bound corticosteroid receptor. Thus, similar to the non-genomic effects of CORT in facilitating AMPAR (Karst et al., 2005) and LTP formation (Wiegert et al., 2006), CORT-induced facilitation of synaptic NMDAR in the adult hippocampus of High LG offspring is likely mediated by non-genomic mechanisms.

Almost all NMDARs in the adult hippocampus are GluN2A- and GluN2B-containing, and these two subunits exhibit fast and slow decay properties (Monyer et al., 1994). Our findings suggest that GluN2A expression in the hippocampal synapses of Low LG offspring may be higher than High LG offspring although this requires further investigation. After CORT treatment the decay time constant of NMDAR current is significantly reduced only in High LG offspring. Thus, the decay properties of NMDAR current in Low LG offspring are unresponsive to CORT treatment, similar to the lack of effect of CORT on synaptic NMDAR currents. Insertion of fast-decaying GluN2A subunit may occur in the hippocampal synapses of High LG offspring after CORT treatment although this has not been examined.

### POSSIBLE MECHANISMS OF CORT-INDUCED CHANGES IN NMDAR IN THE ADULT BRAIN

Stress level CORT induces a rapid (within 30 min) long-lasting enhancement and faster decay kinetics of synaptic NMDAR function in hippocampal synapses of High LG offspring. This rapid effect of CORT is mediated by membrane-bound corticosteroid receptors (**Figure 2**). Although rapid enhancement of NMDAR-mediated $Ca^{2+}$ influx by CORT has been reported (Takahashi et al., 2002; Xiao et al., 2010), the mechanism is unclear. Evidence of very rapid effects of CORT [seconds to minutes (Dallman and Yates, 1969)] inconsistent with the temporal requirements for transcription and translation has long suggested the existence of non-genomic actions of CORT. The existence of a putative membrane-receptor is supported by membrane-localized GR-antibody staining in rat hippocampal, hypothalamic, and amygdala neurons (Liposits and Bohn, 1993; Johnson et al., 2005). Additionally, membrane-impermeable BSA-CORT efficiently reproduces certain CORT effects on neuronal excitability, memory consolidation, and neurotoxicity (Takahashi et al., 2002; Karst et al., 2005; Roozendaal et al.,

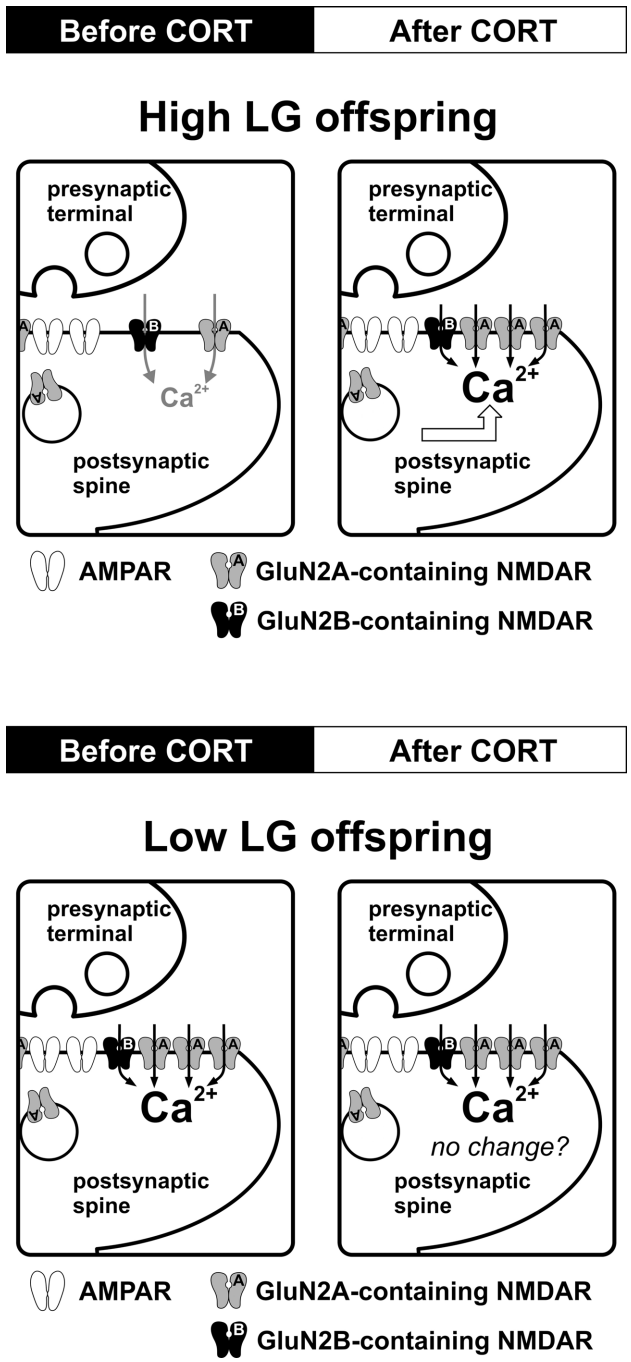


**FIGURE 2 | Effects of maternal care on CORT-induced regulation of synaptic NMDARs in the adult hippocampus.** Schematic diagrams summarize the impact of CORT on NMDAR function and synaptic plasticity. In High LG offspring, stress level CORT (100 nM, 30 min) induces a fast-onset increase in synaptic NMDAR current and a reduction of NMDAR decay kinetics, which may result from an increase in synaptic GluN2A expression. The same CORT treatment produces no observable alteration of NMDAR function or decay kinetics in Low LG offspring. Potential alteration of other ionotropic receptors species (e.g., lateral trafficking of AMPAR) after CORT treatment in Low LG offspring has not been investigated.

2010; Xiao et al., 2010). However, the identity of a putative membrane-corticosteroid receptor is debated (Riedemann et al., 2010; Groeneweg et al., 2011) and as such, discussion of the mechanism by which CORT rapidly enhances NMDAR function is speculative. A rapid, specific potentiation of NMDAR current could be mediated by alterations in the properties of existing synaptic NMDARs or by addition of receptors to the postsynaptic density. Although less mobile than AMPARs, the population of synaptic NMDARs is dynamically regulated by processes of lateral diffusion and receptor insertion (Tovar and Westbrook, 2002). Whether such a process is rapidly modulated by CORT is unknown. However, it is interesting to note that PKC enhances lateral diffusion of NMDARs (Groc et al., 2004) and PKC activation by CORT is implicated in the signal transduction mechanisms of putative membrane-corticosteroid receptors in hippocampal neuronal cultures (Qi et al., 2005).

Findings obtained from non-hippocampal regions may also shed light on mechanisms underlying CORT-induced regulation of NMDAR (Yuen et al., 2009, 2011). Acute stress increases synaptic NMDAR and AMPAR function in the PFC of young rats (one-month old) by enhancing synaptic expression of these receptors. This stress effect is blocked by a GR antagonist, suggesting involvement of CORT. In addition, the impact of CORT on NMDAR trafficking requires activation of serum- and glucocorticoid-inducible kinase and Rab4, which regulates receptor trafficking. Further studies are needed to reveal the involvement of these signaling pathways in CORT-induced regulation of NMDAR in the adult hippocampus.

## FUNCTIONAL IMPLICATIONS OF CORT-INDUCED INCREASE IN NMDAR IN THE ADULT BRAIN

CORT-induced enhancement of NMDAR facilitates both LTP and LTD formation. These facilitating effects of CORT on synaptic plasticity could aid survival in threatening environments by fulfilling increased cognitive demands and supporting encoding of threat-relevant information that may enhance recognition of future threats. Critically, the facilitating effect of CORT on synaptic plasticity is short lasting, returning to basal conditions within one hour of the end of CORT exposure. Prolonged facilitation of hippocampal plasticity could enhance encoding of non-pertinent information, interfering with new memory traces formed during stress. Curtailing synaptic plasticity facilitation after CORT may be essential for appropriate encoding and storage of information relevant to the context in which stress is experienced. The delayed curtailment of the facilitation of synaptic plasticity after CORT could have a homeostatic role, resetting the threshold for synaptic plasticity to ensure the continued capacity for information storage in the hippocampus. The slow-onset increase in synaptic GluN2A expression may be one mechanism of such homeostatic regulation.

### INFLUENCE OF HIPPOCAMPAL SUBFIELD, SUBREGION, AND GENDER ON CORT-INDUCED ALTERATION OF NMDAR

Our findings obtained from the dorsal CA1 and DG of adult rats reveal comparable enhancement of NMDAR function by

CORT in both hippocampal subfields. These findings parallel the similar impact of acute stress and CORT on LTP in CA1 and DG (see section "Subfield of the Hippocampus"), suggesting that plastic changes of NMDAR are relevant to the regulation of synaptic plasticity in CA1 and DG. Whether CORT exerts similar enhancement of NMDAR function in CA3 is not known. Although the expression of GR, which is responsible for CORT-induced changes in NMDAR function (Yuen et al., 2009; Tse et al., 2011), in CA3 is reduced relative to CA1 and DG (Van Eekelen et al., 1988), CA3 neurons show profound reductions in dendritic arborization after chronic CORT or stress exposure (Woolley et al., 1990; Watanabe et al., 1992). CORT may also enhance NMDAR function in CA3. Recent findings suggest that metaplastic increases in NMDAR function caused by high frequency stimulation in the CA3 region support formation of NMDAR-dependent LTP in this hippocampal subfield (Rebola et al., 2011). Future experiments should investigate if CORT or acute stress also enhances NMDAR function in the CA3 region to regulate metaplasticity.

How factors such as hippocampal subregion and gender (see sections "Subregions of the Hippocampus" and "Gender") influence the CORT effects on NMDAR function has not been investigated. Dorsal and ventral hippocampus exhibit differential NMDAR expression. Both mRNA and protein expression of GluN2A and GluN2B in the dorsal hippocampus is increased relative to the ventral hippocampus (Pandis et al., 2006; Liu et al., 2008). Moreover, NMDAR function is likely not uniform along the dorsal-ventral axis of the hippocampus. For instance, NMDAR-dependent high frequency oscillations are more frequent in ventral hippocampus than in the dorsal hippocampus (Papatheodoropoulos, 2007). Hippocampal NMDAR subunit expression displays gender-specific differences (Palomero-Gallagher et al., 2003) and mRNA expression of GluN1 and GluN2A is also regulated by estrogen in female rats (Adams et al., 2001). Corticosteroid receptor expression also displays regional- and gender-specific differences. For instance, MR but not GR mRNA expression in the ventral hippocampus is higher than that in the dorsal hippocampus (Robertson et al., 2005). Although similar mRNA expression of MR and GR was found between the hippocampus of male and female rats, stress-induced changes in the expression of these receptors are greatly influenced by gender (Kitraki et al., 2004). Taken together these findings suggest that regional and gender differences could influence CORT-induced regulation of NMDAR function.

## CHRONIC STRESS AND CORT-INDUCED ALTERATION OF NMDAR IN THE ADULT BRAIN

CORT-induced changes in NMDAR could have pathological consequences. Sustained, excessive activation of NMDAR leads to excitotoxicity (Choi, 1988), especially in the CA1 region (Ikegaya and Matsuki, 2002). Chronic stress is associated with atrophy of dendritic arbors of CA3 neurons (McEwen, 1999; Sapolsky, 2000). Along the longitudinal axis of CA3, chronic stress produces more extensive atrophy in the ventral (reduction in dendritic length and branches) than in the dorsal hippocampus (reduction in dendritic length only) (Christian et al., 2011). Stress-related hippocampal atrophy is ameliorated by pharmacological blockade of NMDAR function (Magarinos and McEwen, 1995) and genetic ablation of GluN1 in the CA3 region (Christian et al., 2011). However, AMPAR blockade is ineffective. We suggest that exposure to high levels of glucocorticoids during stress may render the hippocampus vulnerable to NMDAR-induced excitotoxicity. This increased vulnerability to excitotoxicity may arise from NMDAR hyperfunction in the chronically stressed hippocampus. For instance, three weeks of daily restraint stress increased synaptic NMDAR, but not AMPAR, currents in CA3 pyramidal neurons (Kole et al., 2002). Chronic stress also affects GluN2 subunit expression by decreasing GluN2B expression (Cui et al., 2009). In parallel with this finding, we have observed significant increases in synaptic expression of GluN2A subunit after brief CORT exposure (**Figure 1**). Taken together, these findings suggest that an increase in GluN2A/GluN2B ratio could be a neurobiological signature of chronic stress. It is interesting to note that increased GluN2A is implicated in the formation of depression-related behaviors in rodents (Taniguchi et al., 2009). Conversely, depression-related behavior is reduced in transgenic mice lacking the GluN2A subunit (Boyce-Rustay and Holmes, 2006).

## NMDAR HYPERFUNCTION AND DEPRESSION

The World Health Organization estimates that by 2015 mood disorders, such as depression, will be the leading cause of health burden in the world. However, the clinical efficacy of pharmacological interventions has improved only modestly since the introduction of tricyclics in the late 1970's. Thus, recent findings of the fast acting antidepressant effect of the NMDAR antagonist ketamine have drawn a lot of attention (Pittenger et al., 2007; Skolnick et al., 2009). The antidepressant effects of ketamine are linked to the activation of BDNF (Machado-Vieira et al., 2009) [but also see (Lindholm et al., 2012)] and mTOR pathways (Li et al., 2010). These antidepressant effects also suggest a state of NMDAR hyperfunction in the brain of depression patients. Findings obtained from Low LG offspring also point to a link between hippocampal NMDAR hyperfunction and depression. Low LG offspring have high levels of basal NMDAR function and exhibit depression-like behaviors in forced swimming and novelty suppression of feeding tests (Caldji et al., 1998; Weaver et al., 2005). Potentially, risk factors for depressive disorders, including early life adversity and chronic stress, could induce depression-related behavior by enhancing NMDAR function in the hippocampus. Future studies should validate this hypothesis by examining the antidepressant effect of NMDAR antagonists in Low LG offspring. Further understanding of the mechanisms underlying CORT-induced increases in NMDAR function could identify molecular targets to ameliorate NMDAR changes caused by chronic stress. Associated pharmacological advances may lead to novel therapeutic tools to treat depression and other stress-related mood disorders that are highly resistant to current therapies (Meltzer and McGurk, 1999; Butters et al., 2000).

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# Gestational or acute restraint in adulthood reduces levels of 5α-reduced testosterone metabolites in the hippocampus and produces behavioral inhibition of adult male rats

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Stressors, during early life or adulthood, can alter steroid-sensitive behaviors, such as exploration, anxiety, and/or cognitive processes. We investigated if exposure to acute stressors in adulthood may alter behavioral and neuroendocrine responses of male rats that were exposed to gestational stress or not. We hypothesized that rats exposed to gestational and acute stress may show behavioral inhibition, increased corticosterone, and altered androgen levels in the hippocampus. Subjects were adult, male offspring of rat dams that were restrained daily on gestational days 14–20, or did not experience this manipulation. Immediately before testing, rats were restraint stressed for 20 min or not. During week 1, rats were tested in a battery of tasks, including the open field, elevated plus maze, social interaction, tailflick, pawlick, and defensive burying tasks. During week 2, rats were trained and tested 24 h later in the inhibitory avoidance task. Plasma corticosterone and androgen levels, and hippocampal androgen levels, were measured in all subjects. Gestational and acute restraint stress increased plasma levels of corticosterone, and reduced levels of testosterone's 5α-reduced metabolites, dihydrotestosterone (DHT) and 3α-androstanediol (3α-diol), but not the aromatized metabolite, estradiol ($E_2$), in plasma or the hippocampus. Gestational and acute restraint stress reduced central entries made in the open field, and latencies to enter the shock-associated side of the inhibitory avoidance chamber during testing. Gestational stress reduced time spent interacting with a conspecific. These data suggest that gestational and acute restraint stress can have actions to produce behavioral inhibition coincident with increased corticosterone and decreased 5α-reduced androgens of adult male rats. Thus, gestational stress altered neural circuits involved in the neuroendocrine response to acute stress in early adulthood.

**Keywords: testosterone, androgens, anxiety, affect, inhibitory avoidance, prenatal stress**

## INTRODUCTION

The profound effects of stress on the nervous, immune, metabolic, and cardiovascular systems for health-related outcomes throughout development may depend in part upon the timing of exposure to stressors. On a basic level, acute stress has adaptive short-acting effects on systems that can mobilize individuals from stimuli that challenge homeostasis. Early life, chronic stress has pervasive physiological, neuroendocrine, and behavioral consequences, involving hypothalamic-pituitary-adrenal axis (HPA) dysfunction, that may contribute to pathological conditions [e.g., depression, posttraumatic stress disorder (PTSD), premature aging, hypertension, insulin resistance, etc.; Barker et al., 1993; Bremner et al., 1997; Seckl, 2004; Entringer et al., 2008]. Thus, the nature of stressors' effects may depend upon the timing of exposure.

Animal models of early life stress are useful to elucidate some of the perinatal determinants of adult psychopathology. One valid model of early life stress involves exposing rat dams to restraint stress during late gestation and then assessing the developmental and behavioral outcomes of their offspring. The construct validity of this model of HPA dysfunction is supported by rats exposed to gestational stress having higher baseline and stress-induced corticosterone levels (reviewed in Weinstock, 2007). There is also face validity in this model. There are reports in the clinical literature that gestational stress can produce cognitive impairments and deficits in affective responses, as well as increased risk for diagnoses of psychopathologies, in addition to these alterations in the HPA (reviewed in Weinstock, 2007). Despite the clear validity of this model, the nature of these effects may depend upon sex/gender, developmental stage, and other factors.

Sex differences, or the early organizing role of gonadal steroids, need to be considered in the context of gestational stress. In support, our laboratory, and others, has shown that young adult female rats may experience more deleterious physiological, neuroendocrine, and behavioral effects of gestational stress than do their male littermates (Koehl et al., 1991; Weinstock et al., 1992; McCormick et al., 1995; Alonso et al., 1997; Sternberg, 1999; Szuran et al., 2000; Frye and Wawrzycki, 2003). Furthermore, restraint stress on gestational day 18 decreases the volume of the hippocampus of adult female rats, compared to male littermates as adults, or age-matched, non-prenatally stressed controls (Schmitz et al., 2002). However, not all studies show this pattern of effects. For instance, there are clear effects of gestational stressors among rats to increase anxiety behavior in the elevated plus maze of male, but not female, rats compared to non-stressed controls (Zuena et al., 2008; Brunton and Russell, 2010). An important consideration is the timing of the stressor effects (Andersen and Teicher, 2004). Early gestational stress (days 1–7) produces performance deficits in the Barnes maze of adult male mice compared to that of non-stressed controls; whereas early gestational stress enhances females' performance (Mueller and Bale, 2007). Additionally, male, but not female, mice exposed to stress during gestational days 1–7 have increased depression-like behavior in the tail suspension and forced swim tasks and reduced sucrose preference and greater HPA activity, compared to non-stressed mice (Mueller and Bale, 2008). These effects were not observed in mice that were gestationally stressed during mid to late pregnancy. Another notion to consider is that vulnerability to pervasive effects of early stress may occur at a later stage of development in males than in females. For example, there are sex differences in response to early adversity among rhesus macaques (Cirulli et al., 2009). On postnatal day (PND) 60, but not before as observed in females, male rhesus macaques showed effects of peer-rearing stress (e.g., had increased cortisol and reduced play behavior) compared to mother-reared controls (Cirulli et al., 2009). Overall, these and other data, suggest that there are sex differences and timing effects of stressor exposure for adult stress responding and behavior (Bowman et al., 2004; Bowman, 2005). Androgens have well-known organizing and activating effects on neural and behavioral outcomes. Of interest is the extent to which gestational stress may alter later androgen secretion and androgen-mediated behavioral effects.

Recent studies have suggested that early challenges may alter later secretion and effects of pregnane steroids produced *de novo* in the brain ("neurosteroids"). In support, male and female rats that were exposed to an immune challenge stressor during late gestation had lower levels of a pregnane neurosteroid, 5α-pregnan-3α-ol-20-one (3α,5α-THP), than did control rats when they were assessed at PND 28–30 (Paris et al., 2011a). Moreover, male rats exposed to such a stressor during gestation show a more female-like pattern of anxiety-like behavior in the open field, compared to control male rats, tested at PND 28–30. A similar pattern of effects was observed for restraint stress, variable stressors, or administration of finasteride, a 5α-reductase inhibitor, during late gestation, to produce deficits in object recognition memory and lower pregnane neurosteroids among juvenile male and female rats (Paris and Frye, 2011a,b; Paris et al., 2011a,b). In these studies, androstane neurosteroids were not measured, but finasteride would be expected to similarly reduce levels of androstane neurosteroids. The effects of gestational stress on neurosteroidogenesis persist into adulthood. For example, adult female rats that were exposed to gestational stress have lower hippocampus levels of 3α,5α-THP, as well as increased depressive-like responding in the forced swim test, compared to controls (Frye and Walf, 2004). These studies show clear behavioral deficits coincident with decrements in neuroendocrine responding (i.e., lower pregnane neurosteroids) among gestationally stressed offspring. Although both males and females secrete pregnane neurosteroids, it may be that males are less sensitive to pregnane neurosteroids than to androstane neurosteroids, which are produced at greater levels among males than females.

It is of interest to determine the extent to which some of the effects of gestational stress on male offspring are related to androstane neurosteroids. Some sex differences noted in adult rodents for HPA axis activity may be related to actions of androgens. For example, there is greater activity in brain regions known to inhibit the HPA, such as the hippocampus, among gonadally intact males compared to females or gonadectomized male mice (Goel et al., 2011). Moreover, studies conducted in our laboratory and others have demonstrated that androgens can have activational effects to reduce anxiety- and depression-like behaviors and enhance cognitive performance of adult male rats and mice (Frye and Seliga, 2001; Aikey et al., 2002; Edinger and Frye, 2004; Fernández-Guasti and Martínez-Mota, 2005; Buddenberg et al., 2009). These effects may be due to actions of testosterone (T) and/or its metabolites in the hippocampus. T is aromatized to produce estradiol ($E_2$), and metabolized by 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) to form dihydrotestosterone (DHT) and 3α-androstanediol (3α-diol), respectively. DHT and 3α-diol are androstane neurosteroids produced locally in the brain, in areas, such as the hippocampus, which has high levels of expression of the requisite enzymes (Tsuruo, 2005). The hippocampus is sensitive to the effects of T metabolites to enhance neurogenesis in adult rats (Spritzer and Galea, 2007; Galea, 2008). There are clear effects of gestational stress on hippocampus structure and/or function of rodents (Schmitz et al., 2002; Kim et al., 2006; Setiawan et al., 2007; Weinstock, 2007). The importance of 3α-diol in the hippocampus for the behavioral effects of androgens has been reported in non-stressed adult male rats (Frye et al., 2010). A question is the role of gestational stress, coinciding with the development of the hippocampus (i.e., late pregnancy), for later androgen responses. We hypothesized that: (1) exposure to gestational stress of male rats during late pregnancy would alter neuroendocrine function (increase corticosterone, decrease androstane neurosteroids in the hippocampus) and behavior (decrease exploration, social interaction, and inhibitory avoidance), (2) there would be similar effect of acute restraint stress in adulthood to increase corticosterone, decrease neurosteroidogesis, and produce behavioral inhibition, and (3) gestational stress may alter later responses to acute restraint stress of adults for these neuroendocrine and behavioral measures.

## MATERIALS AND METHODS

All methods utilized in this study using animal subjects were pre-approved by the Institutional Animal Care and Use Committee at the University at Albany-SUNY.

### SUBJECTS AND HOUSING

Gonadally-intact, adult male Long-Evans rats (∼55 days of age; 250–300 g) were experimental subjects in this study ($N = 48$). Rats were obtained from our breeder colony (original breeders from Taconic, Germantown, NY) in the Social Sciences Laboratory Animal Care Facility at the University at Albany-SUNY following gestational stress or not (described below). They were group-housed 3–4 per cage with continuous access to Purina Rat Chow and tap water on a 12/12 h reversed light-dark cycle (lights off at 0800 h). Experimental rats were picked up by animal care staff and placed into clean cages once a week.

### GESTATIONAL STRESS

Female breeder rats ($n = 60$) were cycled through two normal, 4–5 day estrous cycles and mated on behavioral estrus. Pregnant rats were then randomly assigned to the control ($n = 26$) or restraint stress ($n = 34$) condition. Rats in the control condition remained undisturbed in their home cages throughout pregnancy, except for weekly cage cleaning by animal care staff. The pregnant rats that were restraint stressed experienced weekly cage cleaning in the same manner as did the control breeders, but were restraint stressed by being placed in a Plexiglas restrainer (7.5 cm diameter × 19.5 cm length) under a 60-watt light for 45 min daily from gestational days 14–20 (Frye and Walf, 2004). Although it was not systematically examined, overt differences in body weight of the dams, or weight or length of the pups, were not apparent. However, this type of chronic stress during gestation can produce profound effects to interfere with reproductive outcomes and reduce fertility, length of gestation, and litter size (Paris and Frye, 2011a,b). Additionally, gestational stress can have long-lasting effects on HPA responding, such that restraint stress from gestational day 17–21 increases corticosterone in dams at the time of birth compared to control dams (Paris and Frye, 2011b). To control for potential litter effects, which may be due to differences in maternal care, one pup from each litter in the control or gestational stress conditions were utilized so that there was not over-representation of any one litter in the experimental groups. Cross-fostering was not utilized as this produced confounds and/or detrimental effects in some studies (Macrì et al., 2010).

### ACUTE RESTRAINT STRESS

As adults, rats were randomly assigned to be in the control, non-restraint stressed condition ($n = 24$), or they experienced acute restraint stress ($n = 24$). Restraint stress consisted of placing rats in Plexiglas restrainers (7.5 cm diameter × 19.5 cm length) for 20 min, under a 60-watt light (Walf and Frye, 2005). Temperatures of the restraint tube, when placed 30.5 cm under such a lamp, rise from 20 to 21°C within 1 min and remain at this temperature 20 and 45 min later. As such, this is not considered a heat stressor when utilized for 45 min as a gestational stressor, or when utilized for 20 min as an acute stressor in adults. We have verified that this acute restraint stress protocol increases corticosterone levels following restraint stress and 20 min of behavioral testing (open field, elevated plus maze, forced swim test) compared to behavioral testing in these tasks alone among female rats (Walf and Frye, 2005). Thus, there were four experimental conditions ($n = 12$/condition): (1) Non-gestationally stressed, non-acute stressed control, (2) Non-gestationally stressed, acute restraint stressed, (3) Gestationally stressed, non-acute stressed, (4) Gestationally stressed, acute restraint stressed.

### BEHAVIORAL TESTING

Traditional measures of stress/anxiety of rodents were utilized as behavioral indices of hippocampal function (open field, elevated plus maze, defensive freezing) and hippocampal/amygdala function (inhibitory avoidance task). Because footshock was utilized as stimuli, pain thresholds (tailflick and pawlick latencies) were assessed. Handling can alter behavioral responses, so rats received the same amount of handling before testing. Each rat was picked up once each week by the animal care staff for cage changing, and then consistently picked up by the experimenter immediately before behavioral testing. Rats had behavioral assessments in a battery of tasks (open field, elevated plus maze, social interaction, tailflick, pawlick, and defensive freezing) during the first week of testing. The next week, rats were habituated and trained in the inhibitory avoidance task and then tested the following day. All behavioral tasks were run by observers blind to the hypothesized outcome of the study and/or gestational stress condition of rats. Testing chambers were thoroughly cleaned with dilute Quatricide and dried with paper towels between each test. The bars on the grid floor of the inhibitory avoidance chamber were also cleaned with 70% isopropyl alcohol.

#### *Open field*

Rats were placed in the southeast corner of the open field. Entries into central and peripheral squares of the open field (76 × 57 × 35 cm) were recorded during the 5-min task. Entries were defined by placement of all four paws in the square. The total and central square entries made in the open field are utilized as indices of general motor/exploratory and reduced anxiety-like behavior, respectively (Walf and Frye, 2005).

#### *Elevated plus maze*

In the elevated plus maze, rats were placed in the junction of the four arms (two alleyways with walls, and two alleyways without walls) of the elevated plus maze (Walf and Frye, 2005). The time spent by rats on the open and closed arms was recorded during this 5-min task. Open arm time is considered an index of reduced anxiety-like behavior.

#### *Social interaction*

A stimulus male from the breeder colony that was gonadally intact was placed in the open field for this task. This male rat had been habituated to this task and similar tasks so that the behavior of the experimental animal did not depend upon that of the stimulus male conspecific. The time spent by the experimental rat engaging in social interaction with the conspecific (with the experimental male making the contact) was recorded during this 5 min task (Frye and Seliga, 2001). Social interaction was defined

by grooming, sniffing, touching, and following with contact when it was initiated by the experimental rat. The time spent in social interaction is utilized as a measure of social behavior.

#### *Pawlick task*

The latency for rats to lick their front or back paws following placement on a heated surface (Fisherbrand test tube warmer; 50°C) was recorded (Frye and Seliga, 2001). The maximum latency in this task was 180 s. This latency is utilized as an index of anti-nociceptive behavior in this task.

#### *Tailflick task*

The latency for rats to reflexively move their tails from a heat source (San Diego Instruments; 50°C) was recorded for three consecutive trials and averaged (Frye and Seliga, 2001). The maximum latency for each trial was 10 s. The average tailflick latency is utilized as an index of anti-nociceptive behavior in this task.

#### *Defensive burying task*

Rats were tested in the defensive burying task as per published methods (Frye and Seliga, 2001). Rats were placed in the southeast corner of a testing chamber (clear Plexiglas, 26.0 × 21.2 × 24.7 cm, with woodchip bedding). In the northwest corner of the chamber, there was a cylindrical pedestal (2.5 cm diameter, 10.0 cm height) wrapped by wires connected to a shock source (Lafayette Model A615B, Lafayette, IN) set to deliver 0.66 mA of unscrambled shock. When rats touched the pedestal, a brief footshock was delivered, which was terminated immediately following the rat's withdrawal of its paw from the pedestal. The duration spent burying the pedestal with the woodchip bedding in response to the footshock received by the rat was recorded for 15 min following shock. The time spent burying was utilized as an index of anxiety-like responding.

#### *Inhibitory avoidance task*

The inhibitory avoidance task was conducted in accordance with previously published methods (Edinger and Frye, 2004). The chamber consisted of two compartments (a white illuminated compartment and a black dark compartment) divided by a guillotine door. All rats were habituated for 2 min on the white side of the box. During training rats were placed into the white side of the box for 1 min before the guillotine door was lifted by the experimenter. The latency for rats to crossover to the dark side of the chamber (max. latency 20 min) was recorded and the door was closed. Rats were then administered a mild footshock (0.2 mA, 5 s duration) through a grid floor, and left in the dark side of the chamber for 1 min. The next day, rats were placed in the white chamber for 1 min, the door was lifted, and the latency to move to the dark side was recorded (max latency 5 mins). Longer crossover latencies indicate better inhibitory avoidance performance.

### MEASUREMENT OF CORTICOSTERONE AND ANDROGEN LEVELS

#### *Tissue collection, storage, and preparation for radioimmunoassay*

After testing in the inhibitory avoidance task, rats were rapidly decapitated and trunk blood and whole brains were collected. Blood was spun in a refrigerated centrifuge at 3000 g at 4°C. Whole brains were rapidly frozen on dry ice immediately after dissection from the skull. Tissues were placed in long-term storage in a −80°C freezer. Brains were thawed on weigh boats placed on ice and the entire hippocampus was dissected out. Hippocampus samples were homogenized with a glass/Teflon homogenizer in distilled water and trace amounts of [$^3$H] steroid.

#### *Steroid extraction for radioimmunoassay*

Steroids were extracted as follows to measure corticosterone in plasma and androgens (T, $E_2$, DHT, and 3α-diol) in plasma and hippocampus (Edinger and Frye, 2004; Frye et al., 2010). Corticosterone was extracted from 10 µl of plasma by heating plasma samples at 60°C for 30 min. Plasma samples for extraction of $E_2$, T, DHT, and 3α-diol were incubated at room temperature with distilled water and 800 cpms of [$^3$H] steroid. Plasma samples were then snap frozen twice by placing an acetone bath with dry ice, and then test tubes were placed in a savant to evaporate ether. Dried down samples were reconstituted by adding the same volume of 0.1 M phosphate assay buffer (pH 7.4) as the original plasma volume immediately before set-up of radioimmunoassays. Androgens were extracted from the hippocampus homogenate with diethyl ether, which was subsequently evaporated. Samples were reconstituted in 0.1 M phosphate assay buffer (pH 7.4).

#### *Radioimmunoassay of corticosterone and androgens*

Typical radioimmunoassay methods for plasma corticosterone and plasma and brain androgens were employed (Edinger and Frye, 2004; Frye et al., 2010). The range of the standard curves, prepared in duplicate, was 0–4 ng for corticosterone, 12.5–1000 for $E_2$ 50–2000 pg for T and DHT and 0–2000 pg for 3α-diol. Samples were added to assay buffer followed by addition of the appropriate antibody and [$^3$H] steroid (PerkinElmer). For corticosterone measurement, samples were incubated at room temperature for 60 min with [$^3$H] corticosterone (NET 182: specific activity = 48.2 ci/mmol; New England Nuclear) and corticosterone antibody (B#3-163; Esoterix Endocrinology, Calabasas Hills, CA), which binds 40–60% of corticosterone at a 1:20,000 concentration. T, DHT, and 3α-diol assays were incubated overnight at 4°C. $E_2$ was incubated for 60 mins at room temperature. The $E_2$ antibody (#244; Dr. Niswender, Colorado State University, Fort Collins, CO) binds approximately 90% of [$^3$H] $E_2$ (NET-317, 51.3 Ci/mmol) in a 1:40,000 dilution. The T antibody (T3–125; Esoterix Endocrinology) only has modest cross reactivity with DHT, and binds between 60 and 65% of [$^3$H] T (NET-387: specific activity = 51.0 ci/mmol) in a 1:20,000 dilution. The DHT antibody (DT3-351; Esoterix Endocrinology; 1:20,000 dilution) is moderately specific to DHT, but there is some cross-reactivity with T and binds 60–65% of [$^3$H] DHT (NET-302; specific activity = 43.5 Ci/mmol). The 3α-diol antibody (X-144; Dr. P.N. Rao, Southwest Foundation for Biomedical Research, San Antonio, TX) is highly specific to 3α-diol and binds about 96% of [$^3$H] 3α-diol (NET-806: specific activity = 41.00 ci/mmol) when used in a 1:20,000 dilution. Dextran-coated charcoal in assay buffer was rapidly added to assay tubes and samples were incubated with charcoal for 20 min. Samples were then spun in a refrigerated centrifuge at 3000 g at 4°C for 20 min to separate bound and free steroid. Supernatant was decanted

into a glass scintillation vials with 5 ml Scintiverse BD (Fisher Scientific). Total assay volumes were 950 µl for corticosterone and 1200 µl for androgens. The concentration of the samples was determined by using the logit-log method of Rodbard and Hutt (1974), interpolation of the standards, and correction for recovery with Assay Zap. Intra- and inter-assay coefficients of variance for these assays are: corticosterone: 5% and 8%; T: 5% and 5%; $E_2$: 8% and 10%; DHT: 2% and 10%; 3α-diol: 9% and 10%.

### DATA ANALYSES

A MANOVA was utilized to determine the extent to which there was a pattern of the independent variables of stressor exposure for behavior across the several tasks that were utilized. These results suggested that there was a difference between measures utilized, with the most robust effects noted in the tasks that rats were exposed to immediately after restraint stress (open field, inhibitory avoidance) or a highly androgen sensitive task (social interaction). Two-way analyses of variance (ANOVAs) with Fisher's *post-hoc* tests were used to evaluate effects of gestational and acute restraint stress on behavioral indices and steroid levels. Given evidence for the nature and timing of the task to influence outcomes, we will focus the discussion of the results on the open field, inhibitory avoidance, and social interaction tasks. As a proxy of metabolism enzyme activity, ratios of the metabolites, DHT and 3α-diol, to the parent hormone, T, in the hippocampus were calculated and compared. The α level for statistical significance was $p < 0.05$, and a trend was considered when $p < 0.10$.

## RESULTS

### EFFECTS OF GESTATIONAL STRESS

Gestational stress had pervasive effects to alter HPA responding as demonstrated by a main effect of gestational stress on plasma corticosterone levels [$F_{(1, 44)} = 16.87$, $p < 0.01$]. *Post hoc* analyses demonstrated that rats exposed to gestational stress had significantly higher plasma corticosterone levels than did non-stressed, control rats (**Figure 1**). Corticosterone levels in the non-stressed control group were akin perhaps to those reportedly in similarly non-stressed adult male rats (e.g., 2.9 µg/dl ± 1.3 s.e.m.; Frye et al., 2010).

Although there were no differences due to gestational stress for plasma levels of T, DHT, or $E_2$ (**Table 1**), there were differences in plasma levels of 3α-diol [$F_{(1, 44)} = 3.69$, $p < 0.06$]. Gestational stress tended to reduce plasma 3α-diol levels compared to that observed in non-stressed controls (**Figure 2**). Plasma levels of T, DHT, and 3α-diol were in the ranges reported in the literature of gonadally intact adult male rats (T: 12.0–5.0 ng/ml, DHT: 6.0–3.5 ng/ml; 3α-diol: 15.0–1.5 ng/ml; Frye and Edinger, 2004; Edinger and Frye, 2007a; Frye et al., 2010), but plasma $E_2$ levels tended to be higher than a previous study (0.8 pg/ml ± 0.5 s.e.m.; Frye et al., 2010).

There were significant effects of gestational stress for hippocampal levels of DHT [$F_{(1, 44)} = 5.18$, $p < 0.03$], but not T, $E_2$, or 3α-diol. Gestational restraint stress significantly reduced hippocampal DHT levels, compared to non-stressed controls (**Figure 2**). Hippocampus levels of T, $E_2$, DHT, and 3α-diol were similar to ranges of levels reported in the literature of gonadally intact adult male rats (T: 4.0–7.0 ng/mg, $E_2$: 1.3 pg/mg ± 0.3 s.e.m., DHT: 3.0–1.5 ng/mg; 3α-diol: 5.0–2.7 ng/mg; Frye and Edinger, 2004; Edinger and Frye, 2007a; Frye et al., 2010).

There were significant effects of gestational stress for behavioral responses in the open field, inhibitory avoidance, and social interaction tasks. There were significant effects of gestational stress for central [$F_{(1, 44)} = 4.84$, $p < 0.03$], but not total, entries

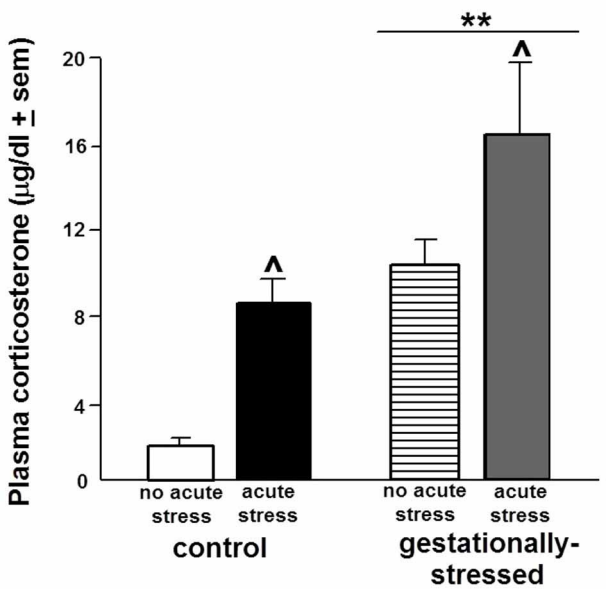


**FIGURE 1 | Plasma Corticosterone Levels.** Figure depicts the plasma levels of corticosterone (mean ± s.e.m.) of adult male rats that were gestationally stressed or not, and then restraint stressed, or not, immediately before testing in the open field. **Above line indicates a significant difference of gestational stress compared to non-gestationally stressed (control) rats ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). ^ Indicates a significant difference of acute restraint stress compared to no acute stress group ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). There was no significant interaction between stress variables for plasma corticosterone levels.

**Table 1 | Plasma and hippocampal levels of testosterone (T) and its aromatized metabolite, estradiol ($E_2$).**

| Endocrine measures | Condition | | | |
|---|---|---|---|---|
| | Control | | Gestationally-stressed | |
| | No acute stress | Acute stress | No acute stress | Acute stress |
| Plasma T (ng/ml) | 9.2 ± 3.4 | 4.2 ± 1.0 | 7.0 ± 1.7 | 5.6 ± 1.1 |
| Hippocampus T (ng/mg) | 7.1 ± 1.9 | 5.2 ± 1.4 | 4.4 ± 0.8 | 4.6 ± 1.2 |
| Plasma $E_2$ (pg/ml) | 4.8 ± 1.0 | 4.0 ± 0.8 | 4.7 ± 1.1 | 6.3 ± 1.4 |
| Hippocampus $E_2$ (pg/mg) | 1.3 ± 0.2 | 2.6 ± 1.0 | 1.9 ± 0.8 | 1.2 ± 0.2 |

*Data are expressed as mean + S.E.M. There were no significant effects of gestational or acute stress, or interactions of these variables, to report for these measures.*

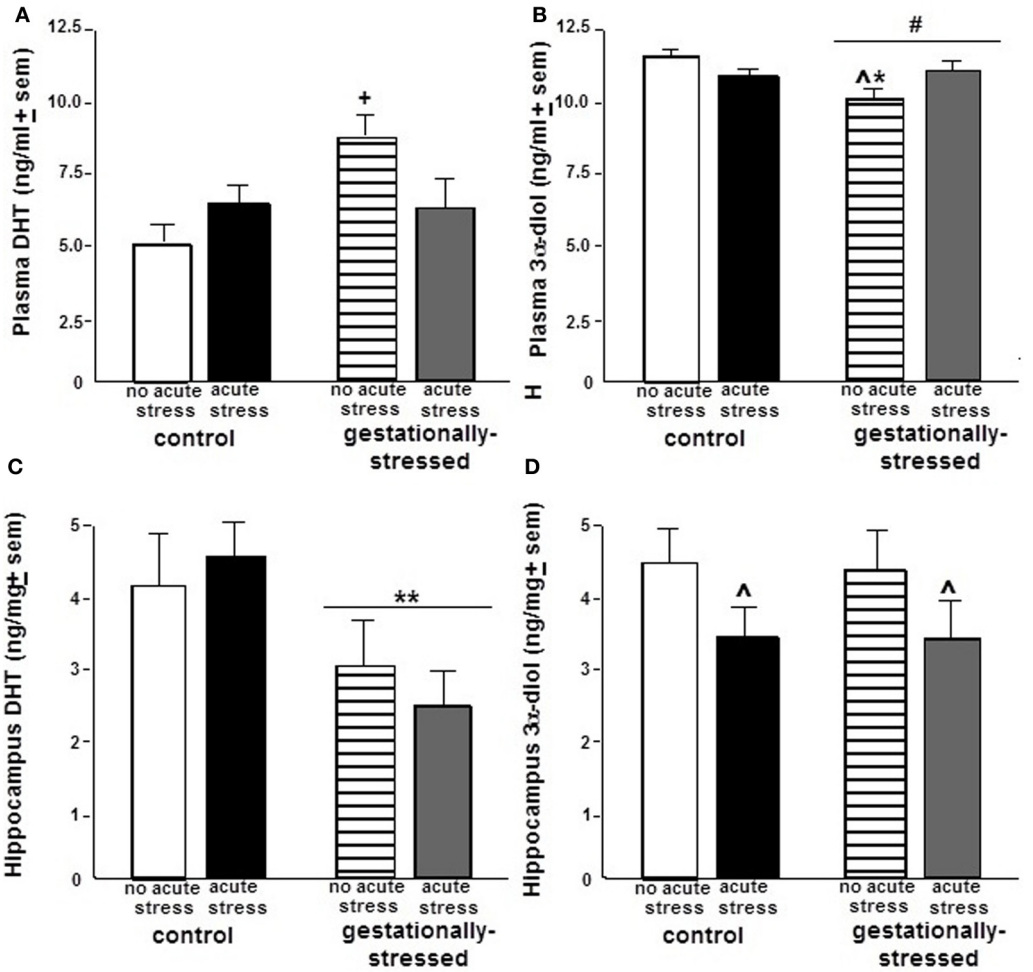


**FIGURE 2 | Plasma and Hippocampal Dihydrotestosterone and 3α-Androstanediol Levels.** Figure depicts the plasma levels (mean ± s.e.m.) of dihydrotestosterone (DHT; panel **A**) and 3α-androstanediol (3α-diol; panel **B**) and hippocampal levels (mean ± s.e.m.) of DHT (panel **C**) and 3α-diol (panel **D**) of adult male rats that were gestationally stressed or not, and then restraint stressed, or not, immediately before testing in the open field. ** Above line indicates a significant effect of gestational stress vs. no gestational stress ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). ^ Indicates a significant effect of restraint stress vs. no acute stress ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). *^ Indicates an interaction between gestational and restraint stress ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). + Indicates a tendency for an interaction between gestational and restraint stress ($p < 0.10$ for main effect and $p < 0.05$ for Fisher's PLSD *post-hoc* tests). # Indicates a tendency for difference of gestational stress compared to non-stress control condition ($p < 0.10$ for main effect and $p < 0.05$ for Fisher's PLSD *post-hoc* tests).

in the open field. Gestational stress decreased central open field entries compared to that observed in the non-stress condition (**Figure 3**). A similar pattern was observed in the inhibitory avoidance task. Rats that were gestationally stressed [$F_{(1,\ 35)} = 6.29$, $p < 0.02$] had lower crossover latencies in the inhibitory avoidance task compared to non-stressed control rats (**Figure 4**). Similarly, in the social interaction task, gestationally stressed rats, compared to control rats, spent significantly less time engaging in social interaction with a conspecific [$F_{(1,\ 44)} = 7.29$, $p < 0.01$; **Figure 5**].

#### EFFECTS OF ACUTE RESTRAINT STRESS

The effects of the acute restraint stress paradigm utilized were validated by a demonstrated increase in plasma corticosterone [$F_{(1,\ 44)} = 10.43$, $p < 0.01$]. Compared to the non-stressed control rats, acute restraint stress significantly increased plasma corticosterone levels (**Figure 1**).

There were no differences due to acute restraint stress for plasma or hippocampus levels of T, $E_2$, or DHT, or plasma 3α-diol levels (**Table 1**, **Figure 2**), there were differences in hippocampal levels of 3α-diol [$F_{(1,\ 44)} = 10.43$, $p < 0.01$]. Rats that were exposed to acute restraint stress had significantly reduced levels of 3α-diol in the hippocampus compared to the non-stressed rats (**Figure 2**).

There were significant effects of acute restraint stress for behavioral responses in the open field and inhibitory avoidance task. Acute restraint stress decreased total [$F_{(1,\ 44)} = 15.36$, $p < 0.01$] and central [$F_{(1,\ 44)} = 3.80$, $p < 0.05$] open field

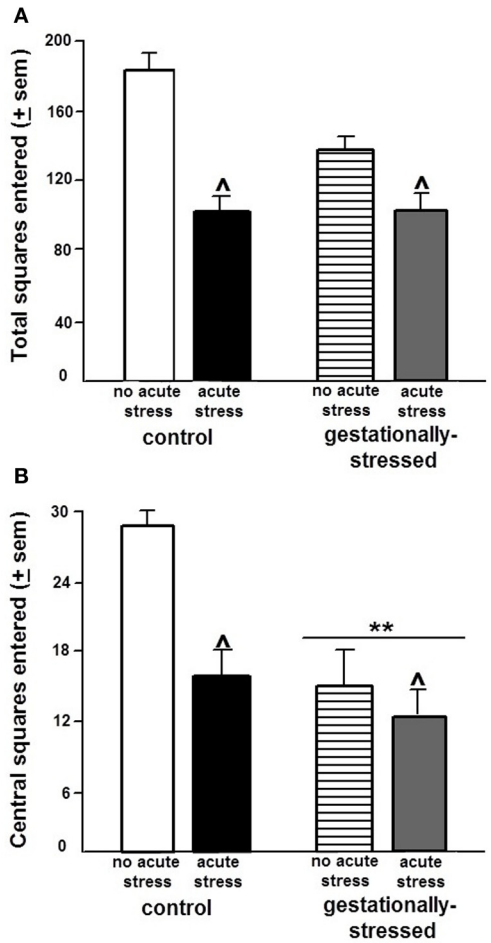


**FIGURE 3 | Open Field Behavior.** Figure depicts total (panel **A**) and central (panel **B**) entries made (mean ± s.e.m.) made in the open field of adult male rats that were gestationally stressed or not, and then restraint stressed, or not, immediately before testing in the open field. ** Above line indicates a significant difference of gestational stress compared to non-gestationally stressed (control) rats ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). ^ Indicates a significant difference of acute restraint stress compared to no acute stress group ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). There were no significant interactions between stress variables for performance in the open field.

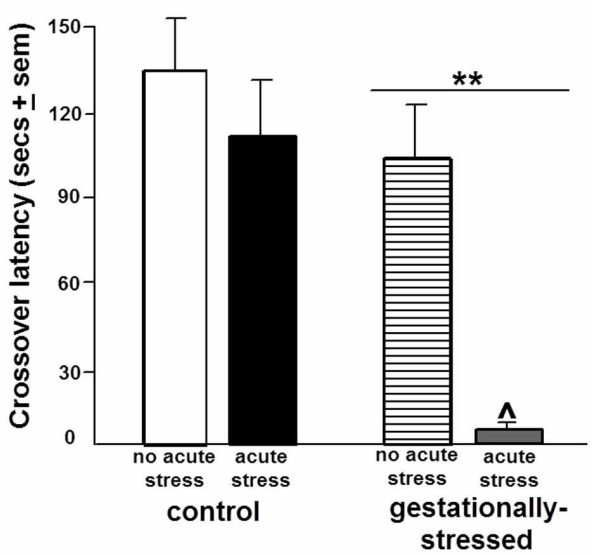


**FIGURE 4 | Inhibitory Avoidance Performance.** Figure depicts the crossover latencies (mean in secs ± s.e.m.) during testing of adult male rats that were gestationally stressed or not, and then restraint stressed, or not, immediately before training in the inhibitory avoidance task. ** Above line indicates a significant difference of gestational stress compared to non-gestationally stressed (control) rats ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). ^ Indicates a significant difference of acute restraint stress compared to no acute stress group ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). There was no significant interaction between stress variables for performance in the inhibitory avoidance task.

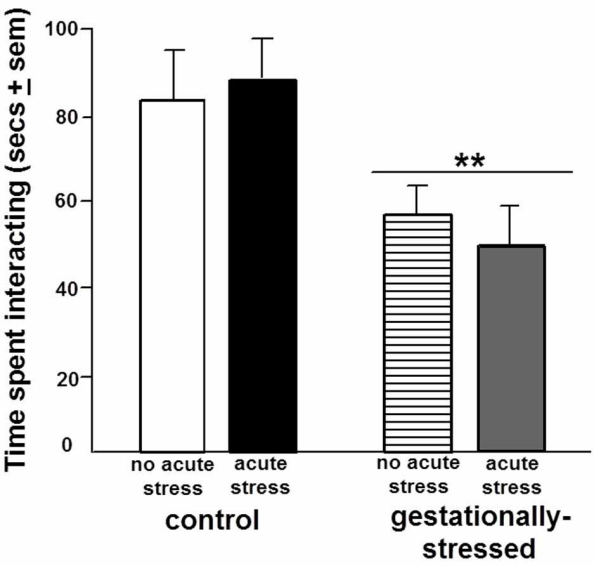


**FIGURE 5 | Social Interaction.** Figure depicts the duration of time spent in social interaction with a conspecific (mean in secs ± s.e.m.) of adult male rats that were gestationally stressed or not, and then restraint stressed, or not, immediately before testing. ** Above line indicates a significant difference of gestational stress compared to non-gestationally stressed (control) rats ($p < 0.05$ for main effect and Fisher's PLSD *post-hoc* tests). There was no significant interaction between stress variables for social interaction.

entries compared to the non-stress control condition (**Figure 3**). Similarly, in the inhibitory avoidance task, acute restraint stress [$F_{(1,\ 35)} = 4.25$, $p < 0.04$] decreased crossover latencies in the inhibitory avoidance task compared to the non-stress condition (**Figure 4**). There were no significant effects of gestational stress for behavioral responses in the social interaction (**Figure 5**) elevated plus maze, paw lick, tailflick, or defensive freezing tasks (**Table 2**).

### INTERACTIONS BETWEEN GESTATIONAL AND ACUTE STRESS

There was a tendency for an interaction between gestational and acute stress to alter plasma DHT levels. Plasma levels of DHT tended to be reduced most greatly among gestationally stressed rats following acute restraint stress [$F_{(1,\ 44)} = 3.17$, $p < 0.08$],

**Table 2 | Behavioral data in the elevated plus maze, pawlick, tailflick, and defensive burying task.**

| Behavioral measures | Condition | | | |
|---|---|---|---|---|
| | Control | | Gestationally-stressed | |
| | No acute stress | Acute stress | No acute stress | Acute stress |
| Elevated plus maze—*open arm time (s)* | 25.4 ± 6.0 | 13.2 ± 4.5 | 10.3 ± 3.9 | 13.8 ± 6.6 |
| Pawlick—*front paw latency (s)* | 137.3 ± 14.3 | 125.7 ± 17.0 | 122.2 ± 10.9 | 106.2 ± 16.6 |
| Pawlick—*back paw latency (s)* | 167.0 ± 7.7 | 149.0 ± 12.6 | 149.0 ± 10.3 | 135.4 ± 15.9 |
| Tailflick—*average latency (s)* | 5.9 ± 0.4 | 5.3 ± 0.3 | 5.4 ± 0.6 | 6.1 ± 0.5 |
| Defensive burying—*time spent burying (s)* | 101.3 ± 40.4 | 87.5 ± 41.9 | 49.0 ± 28.0 | 46.2 ± 31.1 |

*Data are expressed as mean + S.E.M. There were no significant effects of gestational or acute stress, or interactions of these variables, to report for these measures.*

compared to controls. Similarly, there was a significant interaction for gestational and acute restraint stress for plasma 3α-diol levels [$F_{(1,\ 44)} = 6.98$, $p < 0.01$]. Plasma 3α-diol levels were reduced particularly in rats that were gestationally stressed, and not exposed to restraint stress as adults, compared to non-stressed rats.

Albeit not statistically significant, there was evidence for an interaction between gestational and acute restraint stress for activity of 5α-reductase and 3α-HSD in the hippocampus. Gestationally stressed rats that were exposed to acute stress as adults had the lowest 5α-reductase (2.5 ± 1.2), and highest 3α-HSD (2.7 ± 0.8), conversion ratios, compared to all other groups: gestationally stressed and no acute stress (5α-reductase 3.1 ± 1.0; 3α-HSD 2.2 ± 0.7), no gestational stress and acute restraint stress (5α-reductase 2.9 ± 0.8; 3α-HSD 1.2 ± 0.5), and no restraint stress during gestation or adulthood (5α-reductase 2.9 ± 0.8; 3α-HSD 1.7 ± 0.6).

## DISCUSSION

Our hypotheses that rats exposed to gestational and acute stress may increase corticosterone secretion, alter androgen levels, and produce behavioral inhibition, and gestational stress may potentiate the effects of acute stress exposure in adulthood, was supported in the following ways. Gestational stress increased corticosterone levels, decreased plasma 3α-diol levels, decreased hippocampal DHT levels and produced behavioral inhibition in the open field, inhibitory avoidance, and social interaction tasks. Acute restraint stress increased corticosterone levels, decreased hippocampal 3α-diol levels and produced behavioral inhibition in the open field and inhibitory avoidance task. There was evidence that gestational stress exposure altered later neuroendocrine, but not behavioral, responses of acutely restraint stressed rats. Plasma levels of DHT and 3α-diol were lowest, hippocampal 5α-reductase activity was lowest, and hippocampal 3α-HSD activity was highest among gestationally stressed rats that were acutely restraint stressed. No group differences were noted for plasma or hippocampal levels of T, or its aromatized metabolite. Together these data show that gestational and acute restraint stressors have actions to increase HPA responding as measured by plasma corticosterone, alter 5α-reduced T metabolite levels in plasma and hippocampus, and produce behavioral inhibition. Further, gestational stress may impose organizational effects to alter androstane neurosteroid responses to acute stress exposure in adulthood.

The present study confirms and extends the previously reported effects of gestational stress to produce behavioral inhibition and alter functional effects of androgens in the open field and inhibitory avoidance task. In the present study, gestational stress reduced central entries made in the open field. In previous studies, gestationally stressed male rodents have greater depression-like behavior in the forced swim or sucrose anhedonia test (Frye and Wawrzycki, 2003; Mueller and Bale, 2008). As well, rats that were gestationally stressed had poorer performance than did their non-stressed controls in the inhibitory avoidance task. This pattern confirms previous reports on the role of HPA dysregulation for cognitive and/or emotional memory task performance of rats. In support, gestationally stressed female rats have poorer performance in the inhibitory avoidance task compared to their non-stressed controls (Walf and Frye, 2007) and gestationally stressed male rats have poorer spatial performance (Lemaire et al., 2000; Zagron and Weinstock, 2006). Gestational stress reduced time spent in social interaction with a conspecific. Other studies have demonstrated gestational challenges alter social interaction and reproductive behaviors (Ward et al., 1994, 1996, 1999; Lee et al., 2007). In the present study, there were no effects of acute restraint stress for social interaction. These data suggest that there may be differences in the behavioral outcomes of restraint during early development versus later in life for this androgen-sensitive behavior. A question is whether more robust differences for social behavior would have been observed in more challenging and androgen-sensitive situations, such as mating and/or agonistic encounters (DeBold and Miczek, 1981; Lumia et al., 1994; McGinnis, 2004). Individual differences in mating responses and subsequent central production of androstane neurosteroids in the brain mediate anxiety-responding of adult male rats (Edinger and Frye, 2007a). In the present study, there was reduced androstane neurosteroids and social interaction among rats that were gestationally stressed. A question to address in future studies is the extent to which gestational stress may have pervasive effects to alter mating and mating-induced neurosteroidogenesis among males.

The present study confirms and extends the previously reported pattern of acute stress altering steroid-mediated responding among rats. In the present study, we found that male rats that were restraint stressed for 20 min before behavioral assessments had behavioral inhibition, as evidenced by fewer total and central square entries made, compared to their non-stressed counterparts. We have observed a similar pattern of behavioral inhibition in the open field and elevated plus maze among adult, ovariectomized, $E_2$-primed following 20 min of restraint stress, compared to their non-stressed counterparts (Walf and Frye, 2005). Interestingly, in the present study, the effects of acute stress were more robust in the open field than in other anxiety tasks assessed, the elevated plus maze or defensive burying task. Although differences between these anxiety tasks were not anticipated, the timing of when rats were tested in these tasks suggests that the greatest amount of behavioral inhibition occurred immediately following acute restraint stress. Rats were exposed to acute restraint stress immediately before testing in the open field; whereas, stressor exposure was approximately 5 and 20 min before testing in the elevated plus maze and defensive freezing task, respectively. Additionally, rats that were acutely restraint stressed before training in the inhibitory avoidance task demonstrated memory impairments in this task when tested 24 h later. Although we did not measure corticosterone following training, we predict that corticosterone levels were high among restraint stressed rats during training and in the period afterward, thus, interfering with memory consolidation. Additionally, restraint stress reduced levels of 3α-diol in the hippocampus. Previous studies have demonstrated that 3α-diol has actions in the hippocampus to improve cognitive function and decrease anxiety-like responding of male rats (Edinger and Frye, 2004, 2007b; Frye and Edinger, 2004; Frye et al., 2008, 2010). Thus, restraint stress produced behavioral inhibition in the open field task and performance deficits in the inhibitory avoidance task, and reduced hippocampus levels of 3α-diol.

The present data show that gestational stress can have pervasive effects on adult responding to an acute restraint stressor. These effects were apparent for rats' neuroendocrine responses, rather than behavioral effects. Rather than alterations in T or $E_2$ levels among male rats in the present study, salient reductions in plasma levels of DHT and 3α-diol were observed for gestationally stressed rats exposed to acute restraint stress. As well, these results of lower levels of DHT and 3α-diol suggest that stress exposure during gestation and adulthood may have reduced expression or activity of the requisite enzymes, 5α-reductase and 3α-HSD, respectively. Although expression and activity of 5α-reductase and 3α-HSD were not measured directly, calculated conversion ratios suggested a pattern of decreased 5α-reductase and increased 3α-HSD activity among gestationally stressed rats that were acutely restraint stressed as adults. Enzymes, such as the 5α-reductase isozymes, are involved in organizational effects of steroids on the brain during early development, and there are sex differences in adulthood as to how androgens modify these enzymes (Torres and Ortega, 2003, 2006). Neonatal manipulations of T irreversibly program the expression of these enzymes that convert T to DHT and 3α-diol in the liver of rats (Gustafsson and Stenberg, 1974a,b). The role of stressors for regulating 5α-reductase and other steroidogenic enzymes, and their neurosteroid products, throughout development has been described. Prenatal immobilization stress on gestational days 15–18 is associated with initial decreases in 5α-reductase activity in the cerebral cortex and hypothalamus of PND 1 male pups, but elevated 5α-reductase activity in the cortex, hippocampus, and hypothalamus on PND 5 (Ordyan and Pivina, 2005). Another model of early life stress, isolation rearing, for 5–8 weeks reduces expression of 5α-reductase and levels of 3α,5α-THP in the nucleus accumbens and medial prefontal cortex of male rats (Bortolato et al., 2011). Among PND 7 male and female rats, high expression of 3 alpha-HSD mRNA was found, which is coincident with the stress hyporesponsive period in the rat (Mitev et al., 2003). Conversely, acute swim or environmental stress among adult male rats increases prefrontal cortex expression of 5α-reductase (Sánchez et al., 2008, 2009). In our laboratory, social challenges, such as paced mating, reliably increase production of the pregnane neurosteroid, 3α,5α-THP, in the midbrain, hippocampus, and prefrontal cortex of female rats (Frye et al., 2007). As such, the present results may be related to the pervasive effects of acute and chronic stressors on activity and/or expression of metabolism enzymes. Moreover, 3α-diol is a positive allosteric modulator of GABA/benzodiazepine receptor complexes (Gee, 1988), and like the pregnane neurosteroid, 3α,5α-THP, may be released with stressors to dampen the HPA response and restore homeostasis (Erskine and Kornberg, 1992; Patchev et al., 1994, 1996; Frye, 2009). There is recent evidence for 3α-diol to reduce HPA hyper-responsiveness to a physical, stressor, IL-1β administration, of gestationally stressed male rats (Brunton and Russell, 2010). Together, these data further provide evidence supporting a role of neurosteroids as modulators of the HPA (Purdy et al., 1991; Patchev et al., 1994, 1996; Guo et al., 1995). Future studies will further investigate this notion that some of these behavioral deficits with stress could be related to differences in capacity for androgens to be metabolized.

In summary, the present study demonstrated that gestational and acute restraint stress increased corticosterone secretion, reduced levels of androstane neurosteroids, and produced behavioral inhibition of adult male rats. It is important to determine how sex/gender and gonadal hormones may mitigate stress responses following early life adversity because these factors influence the individual's developmental trajectory and pathophysiological states. Neuropsychiatric disorders, such as anxiety, depression, and PTSD, are stress-related disorders that are influenced by sex/gender and gonadal hormones. Indeed, neurodegeneration, as can occur with aging or disease, can be exacerbated by stress and influenced by sex/gender and gonadal hormones. Of clinical significance is that some males may particularly be sensitive to stressors in adulthood when androgen levels are perturbed. Examples of this may be "roids rage" and/or post-finasteride syndrome. The understanding of these pathophysiological states is important to reveal the etiology of disorders, but also for elucidating the possible mechanisms of the normative state, which may be influenced by interactions between adrenal and gonadal

hormones, and their metabolism, during different developmental periods.

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