

The Neurosteroids Allopregnanolone and DHEA Modulate Neurocircuits  
implicated in Emotion Regulation and Posttraumatic Stress Disorder

by

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## **Abstract**

The neurosteroids dehydroepiandrosterone (DHEA) and allopregnanolone are integral components of the stress response and exert positive modulatory effects on emotion in human and animal studies. Though these antidepressant and anxiolytic effects have been well established, little research to date has examined their neural correlates. In particular, brain imaging techniques have not yet been used to assess the impact of neurosteroid administration on emotion regulation neurocircuitry. In a parallel line of research, growing evidence supports that intrinsic connectivity networks involved in emotion regulation are disrupted in anxiety disorders. However, the impact of neurosteroids on these intrinsic connectivity networks is unknown. Thus, the current studies aim to describe the impact of neurosteroids on emotion regulation neurocircuits and amygdala intrinsic connectivity by measuring the effects of neurosteroid administration on the Shifted-Attention Emotional Appraisal Task and on resting-state fMRI. We demonstrate that during emotion regulation, DHEA and allopregnanolone reduce activity in regions associated with generation of negative emotion and enhance activity in regions linked to regulatory processes. Further, we demonstrate that these neurosteroids modulate amygdala intrinsic connectivity in ways that run counter to aberrations observed in posttraumatic stress disorder. Thus, our results provide initial neuroimaging evidence that DHEA and allopregnanolone may be useful as pharmacological interventions for anxiety disorders and invite further investigation into the brain basis of neurosteroid emotion regulatory effects.

## **Chapter 1 : Introduction**

Anxiety disorders are one of the greatest mental health problems in the U.S., with approximately 18% of individuals experiencing at least one anxiety disorder in the past year (1). Anxiety disorders have been linked to alterations in serotonergic, adrenergic, and HPA systems (2, 3) but little research has investigated the relationship between anxiety disorders and neurosteroids such as dehydroepiandrosterone (DHEA) and allopregnanolone. DHEA and allopregnanolone are endogenously produced steroids with anxiolytic and antidepressant effects, and aberrations in these neurosteroids have been linked to psychopathology. However, the neural underpinnings of these effects have not been investigated. Recently, novel techniques have been developed to probe the etiology of anxiety disorders, including resting-state fMRI and pharmacofMRI. Few studies to date have deployed these new methods to investigate neurosteroid modulation of emotion regulation, a function that is postulated to be disrupted in anxiety disorders (for reviews, see 4, 5). Since deficits in emotion regulation skills may contribute to or maintain anxiety (5), understanding the impact of neurosteroids on emotion regulation neurocircuits may be central to understanding why neurosteroid dysregulation is associated with distress and psychopathology. In this dissertation, we investigate the impact of DHEA and allopregnanolone on the neurocircuitry of emotion by (1) examining emotion regulatory pathways via a novel paradigm that probes emotional response and regulation, and (2) examining anxiety-relevant large-scale networks via resting-state functional connectivity, in order to identify the influence of

neurosteroids on emotion regulation neurocircuits and intrinsic connectivity networks that are disrupted in anxiety psychopathology.

### **Part 1: The Neurosteroids DHEA and Allopregnanolone**

The following paragraphs outline the evidence supporting a role for DHEA and allopregnanolone in emotion regulation processes, including the neurosteroid's mechanism of action, the behavioral and psychological effects of neurosteroid administration, the role of the neurosteroid in psychiatric disorder, and the potential underlying neurocircuitry of the observed effects.

#### **Dehydroepiandrosterone (DHEA)**

Gonadal steroids, once thought to act solely as endocrine messengers, have recently been found to act in a paracrine manner to impact neuronal activity (6). DHEA and DHEA sulfate (DHEAS), hereafter referred to as DHEA(S), are synthesized *de novo* in the adrenal glands and in the human brain, and central nervous system levels of DHEA are 6-8 times higher than blood levels (7). DHEA(S) is found throughout the brain, with highest concentrations in the prefrontal lobe (8) and hippocampus (9), where DHEA(S) administration increases glutamate, acetylcholine and norepinephrine release (10). DHEA(S) acts as a GABA(A) receptor noncompetitive antagonist and positive allosteric modulator at the NMDA receptor (for a review, see 11). DHEA(S) has neuroprotective, antioxidant, antihypertensive, anti-inflammatory, and antiglucocorticoid effects (11-14).

### *DHEA(S) administration ameliorates depressive-like behavior*

DHEA(S) has demonstrated robust antidepressant effects. Flinders sensitive line rats, a behavioral model of depression, exhibit lower baseline levels of DHEA in brain regions relevant to depression, and show behavioral improvements through DHEA administration (15). DHEA(S) administration also reduces immobility in the forced swim test (15-18). Furthermore, DHEA(S) reduces conditioned fear responding in rodents, indicating potential anxiolytic capacity (19). Animal neurochemical studies suggest multiple pathways mediating DHEA antidepressant effects including: 1) GABA(A) receptor modulation in the nucleus accumbens and ventral tegmental area (15); 2) reduction of monoamine oxidase activity in striatal regions (20); 3) sigma-receptor antagonism (21); and 4) reduced GABA-induced inhibition, for instance reversal of GABAergic blockade of serotonin firing in the raphe nucleus (22).

### *DHEA(S) is dysregulated in stress, anxiety, and depression*

Low serum DHEA(S) has been repeatedly linked to poor life satisfaction, psychosocial stress and functional limitations (11). Caregivers of Alzheimer's patients exhibit reduced DHEAS levels and increased cortisol/DHEAS ratios (23). Individuals with low serum DHEAS show increased vulnerability to social rejection (24). Other lines of evidence also support that natural variations are present in the general population under varying levels of stress. A general population sample of 22 to 55-year-olds from the general population found higher cortisol/DHEA ratios on weekdays than on weekends, as well as higher ratios on Mondays and Tuesdays than on other workdays (25). These differences suggest that everyday stressors can cause fluctuations of DHEA, even in the absence of psychopathology. Acute laboratory stress (Trier Social Stress Test) increases plasma DHEA(S) in healthy men and women (26), and lower levels of DHEA

during laboratory paradigms of psychosocial stress are associated with negative mood during recovery (27). DHEA administration in healthy individuals generally leads to improvements in mood, cognition and physical well-being (11). Together, these data evince a consistent pattern linking higher DHEA to lower stress and anxiety amongst healthy individuals.

Different types of psychopathology, however, may exhibit different patterns of DHEA(S) dysregulation. Plasma DHEA(S) levels are generally reported to be reduced in depression and elevated in PTSD. Three large-scale studies (N= 700, 1147, 3000) indicate reduced serum DHEA levels in depression (28-30). On the other hand, numerous studies have reported elevated DHEA(S) levels and DHEA(S) to cortisol ratios in PTSD (31-37). One such study reported that comorbid MDD lowered DHEA morning levels in the PTSD group (38). The authors hypothesized that MDD and PTSD might be associated with differential DHEA effects, leading to potentially conflicting research results in populations with comorbid MDD and PTSD. Of note, three double-blind, placebo-controlled studies of DHEA administration for MDD resulted in significantly reduced depressive symptoms (39-41). These antidepressant effects implicate a role for DHEA in the control of emotion regulation processes, a hypothesis that will be tested in Chapter 2.

#### *DHEA elevation may represent a compensatory response to stress*

Since anxiety disorders and PTSD in particular show consistently elevated levels of DHEA, Rasmusson and colleagues (42) have suggested that DHEA elevation occurs in response to chronic stress, and that greater elevations may be a mark of resiliency. Consistent with this hypothesis, plasma DHEA is positively associated with symptom improvement and coping in

PTSD (37). Also, peak change in DHEA response to ACTH stimulation is negatively correlated with PTSD symptoms, and peak DHEA/cortisol ratio is negatively correlated with negative mood symptoms, suggesting DHEA release is associated with adaptive responses to stress (34). Two studies of naval aviators and marines found DHEAS/cortisol ratios to be positively correlated with performance during stress and negatively correlated with symptoms of dissociation (43, 44). Furthermore, only PTSD patients who achieved remission after a 16-week psychotherapy course showed increases in DHEA; nonresponders exhibited reduced endpoint levels (45). This finding of greater DHEA induction in responders than nonresponders was recently replicated in a separate sample (46). This evidence of DHEA's association with resilience, along with evidence of DHEA's mood-boosting effects in various psychiatric disorders (40, 47, 48), suggests that DHEA elevation may serve as a compensatory mechanism in individuals under chronic stress. Finally, an open-label case study of 7-keto DHEA (a metabolite of DHEA) for five treatment-refractory women with PTSD induced "rapid and substantial" symptom reductions (49). As its administration has been associated with symptom improvement, positive mood and coping, DHEA is hypothesized in the current study to exert positive effects on emotion regulation processes.

#### *DHEA supports attentional mechanisms and related executive functions*

In addition to DHEA's antidepressant effects, numerous studies link DHEA to enhancement of attention and cognitive functions related to attention. DHEA augmentation in 75 elderly individuals improved performance on a selective attention test (50). In 62 schizophrenic patients, double-blind DHEA resulted in improved visual sustained attention (51). A recent study demonstrated that DHEAS improved visuospatial attention and response control in mice (52). In

large community studies, serum DHEAS independently contributes to performance in tasks of executive function, concentration and working memory (53). In 981 middle-aged and elderly men, higher DHEA(S) levels were associated with better working memory, processing speed and attention (54). A regression analysis of 55 schizophrenic patients treated with DHEA revealed that higher neurosteroid levels (DHEAS and androstenedione) contributed to 16.5% of the variance in sustained attention and 12% of the variance in executive function (55). In college students, higher DHEA-to-cortisol ratio is associated with better problem solving (56). DHEA administration also improves verbal fluency (57). In a low resolution brain electromagnetic tomography brain imaging study (a combination of EEG and MEG), DHEA was reported to enhance associative memory through modulation of anterior cingulate cortex and hippocampal activity (58), two regions that neuroimaging studies have consistently linked to attention control. This evidence of DHEA enhancing attention as well as executive processes closely connected with regulation of attention supports a role for DHEA in cognitive control, via attentional regulation of emotion. Given this evidence of DHEA's attention-enhancing effects, we further hypothesized it might impact memory for emotional stimuli.

#### *DHEA modulates attention and emotion regulation neurocircuitry*

There is limited but compelling evidence that DHEA impacts neurocircuits that support attention and emotion regulation. As detailed in Part 3 of this chapter, the amygdala, anterior cingulate cortex, and hippocampus are key regions in emotion regulation processes (59), and thus represent plausible targets through which DHEA could exert its anxiolytic and antidepressant effects. In rodents, DHEA(S) increases BDNF concentration (16) and 5-HT(2A) receptor expression (60) in the amygdala. In humans, DHEA administration increases activity in the

anterior cingulate cortex and may impact activity in the hippocampus (58). DHEAS also modulates glutamate (61), dopamine and serotonin (62) release in hippocampal neurons. Chapters 2 and 5 detail the influence of DHEA on regions within emotion regulation neurocircuits and on the intrinsic connectivity between these regions.

### **Allopregnanolone**

Allopregnanolone, a progesterone-derived pregnane steroid, is produced *de novo* in the human brain, independent of peripheral production (63). Allopregnanolone acts via nuclear steroid receptors, but also exhibits rapid, nongenomic effects via GABA(A) receptors (e.g. 64, 65). The metabolic pathway of allopregnanolone occurs as follows:

1. Cholesterol is oxidized to pregnenolone in mitochondria,
2. Pregnenolone is converted by 3 $\beta$ -hydroxysteroid dehydrogenase to progesterone (either peripherally, in adrenal glands or ovaries, or centrally, in neurons and glia),
3. Progesterone is metabolized irreversibly to dihydroprogesterone by 5- $\alpha$ -reductase (the rate-limiting enzyme),
4. Dihydroprogesterone metabolized by 3 $\alpha$ -hydroxysteroid dehydrogenase to allopregnanolone (66).

Allopregnanolone is a potent allosteric modulator of the GABA(A) receptor with anxiolytic properties (67). It lowers neuronal excitability with 20-fold higher efficacy than benzodiazepines and barbiturates (68), and modulates a broader range of GABA(A) receptors than either of these compounds (69). Thus, allopregnanolone shows great promise as an anxiolytic agent.



*Allopregnanolone administration ameliorates anxiety and depressive-like behavior*

Allopregnanolone has been consistently linked to stress and anxiety, such that chronic stress reduces allopregnanolone levels and correction of allopregnanolone deficiency alleviates anxiety.

Chronic social isolation decreases levels of allopregnanolone in cortex, hippocampus and plasma (70), increases aggressive behavior, and impairs contextual fear extinction (61, 71-73).

Conversely, social stimulation increases hippocampal levels of allopregnanolone-producing enzymes (74). These behavioral deficits are also reversed by a dose of S-norfluoxetine (the active metabolite of fluoxetine), which increases brain allopregnanolone but not serotonin levels, suggesting an anti-anxiety effect mediated by allopregnanolone (72, 75). Chemical depletion of allopregnanolone increases contextual freezing (72), reduces adolescent exploratory behaviors in novel environments (76), and reduces open arms time in elevated plus maze (77), indicating a dampening role of allopregnanolone in fear responding. Furthermore, allopregnanolone administration reduces stress and anxiety-like behavior in rodents (6, 64, 77-81). In particular, allopregnanolone administration reduces conditioned fear responding, facilitates fear extinction, and prevents the reinstatement of fear memory after extinction (72), and neonatal allopregnanolone enhancement increases novelty-directed locomotion in the open field test (76) and increases open arms time in the elevated plus maze (82).

Allopregnanolone also ameliorates depressive-like behavior, and may exert greater antidepressant effects than any of its pregnane steroid relatives. In mice, ovariectomy increases depressive-like behavior as measured by the forced swim test, and these effects are reversed by progesterone administration (83, 84). However, this manipulation has no effect if mice are co-

administered finasteride, which blocks the conversion of progesterone to allopregnanolone (83, 85, 86). Similarly, there is no effect in 5-alpha-reductase (allopregnanolone's rate-limiting enzyme) knockout mice (84). Notably, 5-alpha-reductase inhibitors administered for alopecia in humans induce anxiety and poorer social functioning (87). Blockade of progesterone to allopregnanolone metabolism impairs social and affective behavior in rats (68, 88), but is restored by allopregnanolone infusions to the ventral tegmental area, which catalyzes production of allopregnanolone from progesterone in hippocampus, cortex, and diencephalon (89). Finally, exploratory and anti-anxiety behavior is correlated with circulating and hippocampus levels of allopregnanolone, but not estrogen, progesterone, or corticosterone (88). Together, these findings demonstrate that allopregnanolone may show greater promise as an antidepressant and anxiolytic candidate than other pregnane neurosteroids.

#### *Allopregnanolone is dysregulated in stress, anxiety, and depression*

Allopregnanolone levels are reduced in depression and anxiety disorders, two conditions associated with poor emotion regulation (for a review see 4, 5). Plasma and CSF levels of allopregnanolone are reduced in women with major depression (90, 91), and these levels are normalized through SSRI administration (75). Indeed, several SSRIs, including fluoxetine, norfluoxetine, fluvoxamine and paroxetine, all elevate allopregnanolone brain levels (73, 90, 92-94). Furthermore, allopregnanolone changes pre- to post-treatment are positively correlated with improvement in depressive symptoms (90), suggesting that increases in allopregnanolone are associated with positive mood (95). CSF allopregnanolone is also reduced in women with posttraumatic stress disorder (PTSD), along with dihydroprogesterone (allopregnanolone precursor) to allopregnanolone ratio. After controlling for age and progesterone level,

allopregnanolone levels are 61% lower in women with PTSD than in healthy controls (96). In another study of women with PTSD, higher allopregnanolone was associated with less anxiety in response to trauma-related stimuli (97). More globally, PTSD patients exhibit decreased frontal lobe benzodiazepine receptor binding (98, 99) and decreased plasma GABA levels (100), indicating lower GABAergic tone that could potentially be ameliorated by positive GABA receptor modulation. Since depression and PTSD are associated with problems of emotion regulation, and effective treatments for these disorders raise allopregnanolone levels, allopregnanolone administration may improve processes involved in emotion regulation, a hypothesis that will be tested in this series of studies.

#### *Allopregnanolone modulates emotion regulation neurocircuitry*

One potential mechanism for allopregnanolone's effects in the central nervous system is its ability to directly impact emotion neurocircuitry. Allopregnanolone acts directly on GABA(A) receptors, which are present throughout the cortex and limbic system (101). A recent study reported a direct correlation between endogenous allopregnanolone level and amygdala response to negative stimuli (102). In a group of 28 women, greater allopregnanolone increase during the late luteal phase of the menstrual cycle was associated with decreased amygdala and medial prefrontal cortex response to aversive movie clips. Basic science research further supports the notion that allopregnanolone acts on threat and emotion regulation neural circuits. In rats, microinfusions of allopregnanolone directly into the amygdala produce anxiolytic (103), antidepressant (104), and anti-aggressive (105) effects. The central nucleus of the amygdala may be a particular target for allopregnanolone anxiolysis (106). Several studies have suggested that amygdala response to faces varies depending on allopregnanolone level. One study reported that

allopregnanolone reduced amygdala responses to faces (107). A second study reported that allopregnanolone enhanced amygdala activity, but also found increased functional coupling of the amygdala to the dorsal medial prefrontal cortex, an area implicated in emotion regulation (108). Therefore, allopregnanolone may moderate emotional response by modulating amygdala activity and/or altering functional coupling between amygdala and associated emotion regulation neurocircuitry. Chapters 3 and 5 detail the influence of allopregnanolone on regions within emotion regulation neurocircuits and on the intrinsic connectivity between these regions.

## **Part 2: Emotion regulation, the SEAT, and fMRI**

### *Emotion Regulation Neurocircuitry*

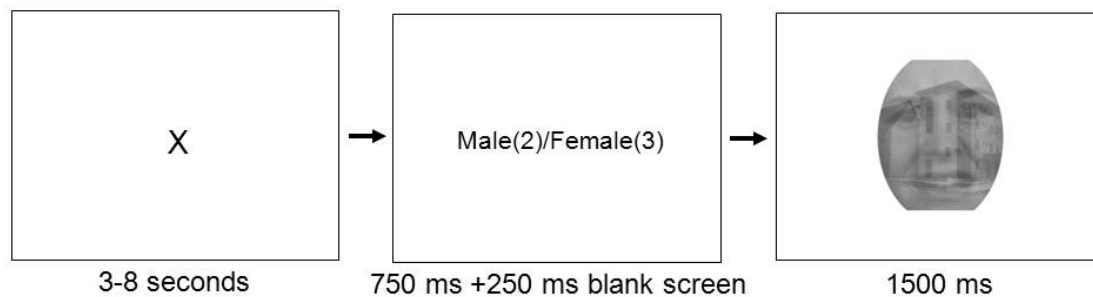
Converging evidence from lesion studies to affective neuroimaging work supports a central role for the amygdala and medial prefrontal cortex in emotion regulation and emotion induction. In rats, medial frontal cortical areas negatively modulate amygdala activity during extinction of conditioned fear responses (109, 110). In humans, dorsal medial prefrontal cortex is implicated in the regulation of emotional responses and autonomic arousal in healthy functioning and in psychiatric disorder (111, 112), and appears to provide modulation of emotional amygdala responses (113-115). Our laboratory has used paradigms of appraisal in [<sup>15</sup>O] PET and fMRI neuroimaging studies (116-118). We have shown that when healthy subjects shift their attention to become aware of and evaluate the intensity of their emotional responses to aversive stimuli (by rating the intensity of their emotional responses to IAPS pictures), the resultant processing leads to robust activation of dorsal medial prefrontal cortex and rostral anterior cingulate cortex, and dampening of amygdala responses.

*Shifted-Attention Emotional Appraisal Task (SEAT) paradigm engages appraisal and stimulus-related attentional shifts while viewing emotional faces*

In order to investigate the interaction of cognitive and emotional processes in emotion regulation, our laboratory has developed a novel emotional appraisal task modifying the task of Anderson and colleagues (119). The SEAT task presents compound stimuli that include both emotional faces and neutral scenes (see Figure 1.1). Stimuli include pictures of faces only, buildings only, and superimposed faces (foreground) on buildings (background). The face pictures depict neutral, angry, or fearful expressions, and the building pictures depict indoor or outdoor scenes. Each picture is presented for 1500 milliseconds, several times across cued questions in a random order. In three different conditions, participants are asked to respond to three different questions: (1) ‘Gender’: Whether the face in the foreground is male or female; (2) ‘Inside/Outside’: Whether the scene in the background is indoors or outdoors; or (3) ‘Like/Dislike’: Whether the face in the foreground is liked or disliked. This allows multiple components of emotion regulation processes to be probed, including (1) implicit emotional processing, (2) attentional modulation of emotion, and (3) appraisal modulation of emotion. The ‘Gender’ task reflects implicit emotional processing, as attention is directed to an emotional face, producing robust amygdala activation (120). The ‘Inside/Outside’ task reflects attentional modulation, as it requires shifting attention away from the emotional face, and leads to decreased amygdala activation with corresponding increases in regions associated with emotion regulation. The ‘Like/Dislike’ task requires appraisal of one’s emotional/evaluative state, and (similar to the ‘Inside/Outside’ task), has been found to reduce amygdala activation and increase activation of dorsal medial prefrontal cortex (117, 121) Chapters 2 and 3 investigate the neural correlates of

allopregnanolone and DHEA's impact on emotional response and regulation as assessed by the SEAT task, focusing on the brain regions mentioned above. Through their anxiolytic and antidepressant effects, we expected allopregnanolone and DHEA to enhance neural regulatory control of emotions as measured by the SEAT.

**Figure 1.1 Shifted-attention Emotional Appraisal Task**



### *The SEAT as an Emotion Regulation Task*

Converging evidence from two decades of research into the neurobiological basis of emotion and face processing suggests that the SEAT is a viable probe of emotion regulation. Emotion regulation can be defined as a change in the intensity and duration of negative affect (122). It has been well established that negative emotional faces induce corresponding negative emotions (123-125). Faces depicting threat-relevant emotions such as fear or anger also activate amygdala (126-130). Previous studies have shown that greater amygdala activation from the presentation of facial expressions is associated with greater emotional response (117, 124, 125, 131, 132). Therefore, the negative faces presented in the SEAT were expected to induce a negative emotional response, and the associated amygdala activation can be considered a reliable indicator of emotional arousal.

Modulation of attention away from negative faces is a viable method for regulating emotion and dampening amygdala response. Amygdala activity can be reduced by shifting attention away from negative emotional expressions (133) or by attending to less salient aspects of emotional faces, such as their age (134, 135). Since attention modulation is recognized as a core component of emotion regulation (136) and reduction in amygdala activity is associated with reduced negative affect, we hypothesized that shifting attention away from negative faces would reduce negative affect. In support of this theory, at least one previous study has demonstrated that attentional modulation away from threatening faces lowers frustration to a subsequent stressful task, supporting the notion that attentional modulation regulates emotion (137). Therefore, it is reasonable to conclude that the attention modulation condition ('inside/outside') in the SEAT is a valid measure of emotion regulation.

Modulation by appraisal is another form of emotion regulation skill that has been extensively studied by our laboratory and others. We have found that subjective rating of emotional stimuli, in particular the appraisal of personal association or relatedness to emotional stimuli, both reduces amygdala activation (118, 121) and increases activation in ventral and dorsal medial prefrontal cortex (117), areas key to cognitive control over emotion. Behavioral studies of emotional appraisal and labeling find that this strategy lowers distress (138) and facilitates habituation (139). Therefore, we believe that the "Like/dislike" condition can be conceptualized as another valid emotion regulation strategy.

### **Part 3: Posttraumatic Stress Disorder and Intrinsic Connectivity Networks**

One important representative of anxiety disorders is Posttraumatic Stress Disorder (PTSD). PTSD is a debilitating psychiatric disorder characterized by re-experiencing, avoidance and hyperarousal symptoms (140), with associated deficits in threat sensitivity (141-143) and fear extinction (144, 145). PTSD is highly comorbid with depression, and thus constitutes a prime candidate to test the therapeutic potential of neurosteroids like DHEA(S) and allopregnanolone. Converging findings indicate aberrations in emotion regulation neurocircuitry in PTSD, particularly in cortico-subcortical circuits involving amygdala, insula, mPFC and hippocampus (146, 147). The activation patterns of these regions have been examined extensively in the past, and the emerging picture suggests hyperactivation of the amygdala and insula and corresponding hypoactivation of mPFC (147). However, identifying dysregulated patterns of connectivity between these regions could shed additional light on the brain-basis of PTSD and on mechanisms of PTSD symptom development by revealing interactions between regions that are not discernible by viewing regions in isolation.

Regions that are implicated in PTSD are key nodes in several major intrinsic connectivity networks (ICNs), and have also demonstrated modulation by neurosteroids. ICNs are large-scale networks identified by connectivity methods that are associated with characteristic functions (148, 149), are stable across tasks (150, 151) and over time (152, 153), correspond to anatomical white matter tracts (154), demonstrate direct behavioral correlates (155-157), and are linked to important functions such as processing speed (157) and cognitive flexibility (158) in health and in disease. Functional connectivity offers a uniquely powerful way to interrogate ICNs (148, 159, 160). Regions such as insula, amygdala, vmPFC, and hippocampus, which have been reported to



be dysregulated in PTSD (146, 147), and modulated by neurosteroids (58, 107, 108) are also involved in the amygdala functional connectivity network. In healthy subjects, during rest, the amygdala shows positive coupling with ventral medial prefrontal regions, insula, thalamus and striatum, and anti-correlations with superior frontal gyrus, bilateral middle frontal gyrus, posterior cingulate cortex and precuneus (161-163). These anticorrelations have been interpreted as dissociations between the emotion production network and the cognitive or affect regulation network (161).

Recently, functional connectivity analyses have begun to be used to probe network-level function in PTSD. During task-based studies, PTSD patients show exaggerated connectivity within the amygdala network (164). These relationships might potentially be better assessed, however, through connectivity analyses at rest, without the confounds of tasks that may be biased to elicit amygdala activity or provoke PTSD symptoms. Resting-state amygdala connectivity is reportedly altered in GAD (165), social phobia (166), MDD (167, 168), and bipolar disorder (169), however, amygdala connectivity at rest in PTSD has not been studied. Identifying abnormal patterns of connectivity within the amygdala resting-state network could illuminate abnormalities underlying heightened emotional reactivity in PTSD. Furthermore, identifying the influence of neurosteroids on this ICN could suggest potential avenues for future therapeutic interventions.

### **Summary and Synopsis of Findings**

Due to DHEA and allopregnanolone's positive impact on mood, emotion, and attention, we expect these neurosteroids to exert a positive modulatory influence on emotion regulation

neurocircuitry. In Chapter 2, we examine the impact of DHEA on emotion regulation neurocircuitry as measured by the SEAT task. In Chapter 3, we examine the impact of allopregnanolone on emotion regulation neurocircuitry as measured by the SEAT task. In Chapter 4, we report on altered intrinsic amygdala connectivity in PTSD. And in Chapter 5, we investigate how DHEA and allopregnanolone may modulate this network in a direction opposite to that observed in PTSD. DHEA and allopregnanolone's modulation of emotion regulation neurocircuits both during task and at rest demonstrate that these neurosteroids may directly impact the neurocircuitry that is dysregulated in anxiety disorders and may potentially represent a viable anxiolytic treatment.

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## **Chapter 2 : DHEA Enhances Emotion Regulation Neurocircuits and Modulates Memory for Emotional Stimuli**

### **Abstract**

Dehydroepiandrosterone (DHEA) is a neuroactive steroid with anxiolytic, antidepressant, and antiglucocorticoid properties. It is endogenously released in response to stress, and may reduce negative affect when administered exogenously. Although there have been multiple reports of DHEA's antidepressant and anxiolytic effects, no research to date has examined the neural pathways involved. In particular, brain imaging has not been used to link neurosteroid effects to emotion regulation neurocircuitry. To investigate the brain basis of DHEA's impact on emotion modulation, subjects were administered 400mg of DHEA (N=14) or placebo (N=15) and underwent 3T fMRI while performing the Shifted-Attention Emotional Appraisal Task (SEAT), a test of emotional processing and regulation. Compared to placebo, DHEA reduced activity in the amygdala and hippocampus, enhanced connectivity between the amygdala and hippocampus, and enhanced activity in the rACC. These activation changes were associated with reduced negative affect. DHEA also reduced memory accuracy for emotional stimuli, and reduced activity in regions associated with conjunctive memory encoding. These results demonstrate that DHEA reduces activity in regions associated with generation of negative emotion and enhances activity in regions linked to regulatory processes. Considering that activity in these regions is altered in mood and anxiety disorders, our results provide initial neuroimaging evidence that DHEA may be useful as a pharmacological intervention for these conditions and invite further investigation into the brain basis of neurosteroid emotion regulatory effects.

## Introduction

Dehydroepiandrosterone (DHEA) and its sulfated derivative, dehydroepiandrosterone sulfate [DHEAS, hereafter referred to together as DHEA(S)], are cholesterol-derived steroids synthesized in the adrenal glands (1) and *de novo* in neurons and glia (2). DHEA(S) acts as a GABA(A) receptor noncompetitive antagonist and positive allosteric modulator at the NMDA receptor (for a review, see 3). DHEA(S) has neuroprotective, antioxidant, antihypertensive, and anti-inflammatory effects (3, 4), and it reduces conditioned fear responding in rodents (5) and in humans (6). Furthermore, DHEA administration reduces circulating levels of cortisol (7, 8), and DHEAS is inversely related to peak cortisol in response to CRH infusion (9). Due to this profile of effects, DHEA shows promise as an anxiolytic and antidepressant target in psychiatric disorders.

DHEA(S) is dysregulated in mood and anxiety disorders, particularly in major depressive disorder and posttraumatic stress disorder (PTSD). Several large-scale studies (N= 700, 1147, 3000) indicate reduced serum DHEA levels in depression (10-12). Conversely, numerous studies report normal or elevated DHEA(S) in PTSD (13-17), though low levels have also been reported (18). Accumulating evidence suggests that increasing DHEA(S) attenuates anxious and depressive symptomatology, and Rasmusson and colleagues (19) have suggested that DHEA elevations in PTSD may represent a compensatory response to stress. In animal models, DHEA(S) administration reduces immobility in the forced swim test (20) and anxiety in the elevated plus maze (21). In healthy individuals, several studies indicate that DHEA administration improves mood (3, 8). In clinical populations, three double-blind, placebo-controlled studies of DHEA administration in MDD patients reported a significant reduction in

depressive symptomatology (22-24). Thus, elevating DHEA(S) levels may be a viable treatment strategy for the reduction of negative affect. No research to date, however, has investigated the mechanism by which DHEA exerts these anxiolytic and antidepressant effects. In particular, no studies have investigated a potential role for DHEA in emotion regulation. This question could be paramount to understanding why DHEA dysregulation is associated with distress and psychopathology.

In addition to DHEA's antidepressant effects, numerous studies link DHEA(S) to enhancement of attention, memory, and executive functioning. In large community samples, serum DHEAS is positively associated with performance in attention, concentration, processing speed and working memory tasks (25, 26). DHEA improves verbal fluency (27) and is associated with better problem solving (28) in healthy individuals, and contributes to sustained attention and executive function in schizophrenic patients (29). It has been suggested that DHEA's effects on memory may be exerted through modulation of the anterior cingulate cortex (ACC) and hippocampus (8). In conjunction with DHEA's antidepressant and anxiolytic effects, this evidence of DHEA's ability to enhance attention supports a role for DHEA in cognitive control via attentional modulation of emotion.

Growing evidence suggests that DHEA might have a direct impact on emotion neurocircuitry, including the amygdala, hippocampus, and ACC. DHEA(S) increases BDNF concentration (30) and 5-HT(2A) receptor expression (31) in the amygdala, a key region in threat detection (32), fear conditioning (33), and emotional salience (34). In humans, DHEA administration increases activity in the ACC (8), a modulatory region interconnected with limbic structures (35) and



central to emotional regulation (36), and may impact activity in the hippocampus, a region key to contextual memory (37) and fear conditioning (38). DHEAS also modulates glutamate (39), dopamine, and serotonin release in hippocampal neurons (40). As these three regions are key to emotion regulation processes (41), they represent plausible targets through which DHEA could exert its antidepressant effects.

In the current study, we used a novel probe of emotion induction, modulation, and regulation to delineate the brain mechanisms through which DHEA impacts emotional responses and emotion regulation. Given previous behavioral evidence of its anxiolytic, antidepressant and attention-enhancing effects, we expected DHEA to enhance neural regulatory control of emotions, and further hypothesized it might impact memory for emotional stimuli.

## **Methods**

### *Participants*

Study participants were 29 right-handed healthy male volunteers aged 18-34 years (mean  $\pm$  SD =  $23 \pm 3.62$ ) recruited from the community via advertisement. Our investigation was restricted to males because baseline levels and metabolism of DHEA vary by gender (42), and it was not feasible within the limited scope of this project to recruit enough participants of each gender to analyze each group separately. Exclusion criteria were a history of head injury, recent steroid use, or current or past psychiatric disorder, as assessed via the Mini-International Neuropsychiatric Interview (M.I.N.I., 43). Participants were given full details of the study and provided written informed consent. The study was approved by the Institutional Review Board of the University of Michigan Medical School.

### Procedure

All participants completed tests of working memory (Digit Span), and visual attention and task switching (Trail-Making Test), both prior to and one hour after drug administration. Change scores were calculated as post-administration score minus pre-administration score. This provided an ancillary test for the effects of DHEA on neurocognitive function. At one hour post drug administration, participants completed the Positive and Negative Affect Scale (PANAS-X), the Drug Effects Questionnaire, and a 20-item Visual Analogue Scale measuring anxiety and sedation. The Visual Analogue Scale measures consisted of 4 inch bars with 'Not At All' and 'Extremely' indicated at the extremities of each variable. Participants also completed an in-scan survey both immediately prior and after the emotion regulation task. They viewed a list of 22 emotions and rated them on a 5-item Likert-type scale (1 = not at all, 5 = extremely). Anxiety score was calculated as the sum of the following items: Nervous, Anxious, Fearful, and Stressed. All measures were compared between groups via independent-sample T-tests.

### Drug Administration

Study drug (DHEA) and matching placebo identical in appearance were obtained from Bell Pharmacy (Lakewood, CO), which provided certificates of analysis. Participants were randomly assigned to receive a single oral dose of 400 mg DHEA (n=14), or placebo (n=15). Participants and investigators were blind to condition. DHEA serum concentrations peak 60 to 480 minutes after DHEA administration, though the half-life of DHEA is 20-25 hours (44, 45). Drug and placebo administration occurred 120 minutes before neuroimaging to ensure elevated levels during the scan.

### *Steroid measurements*

We used circulating levels of DHEA, DHEAS, and cortisol as indicators of central neurosteroid levels. Serum DHEA levels are closely related to levels in cerebrospinal fluid (46), which in turn are closely related to levels in brain tissue (47). Serum samples for assay were collected once prior to drug administration and once after the scanning session. Saliva samples were collected four times throughout the experiment. Serum DHEA levels were determined via enzyme immunoassay (ALPCO Diagnostics, Salem, NH), and serum and salivary levels of cortisol and DHEAS were determined by chemiluminescent enzyme immunoassay (IMMULITE) according to the manufacturer's directions (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). Cortisol and DHEA(S) values were natural log transformed prior to analyses.

### *Shifted-Attention Emotional Appraisal (SEAT) Paradigm*

In order to investigate the interaction of cognitive and emotional processes in emotion regulation, our laboratory has developed a novel emotional appraisal task modifying the task of Anderson and colleagues (48). The SEAT task presents compound stimuli that include both emotional faces and neutral scenes. Stimuli included pictures of faces only, buildings only, and superimposed faces (foreground) and buildings (background). The face pictures depict neutral, angry, or fearful expressions, and the building pictures depict indoor or outdoor scenes. Each picture is presented for 1500 milliseconds, several times across cued questions in a random order. In three different conditions, participants are asked to respond to three different questions: (1) 'Gender': Whether the face in the foreground is male or female; (2) 'Inside/Outside': Whether the scene in the background is indoors or outdoors; or (3) 'Like/Dislike': Whether the

face in the foreground is liked or disliked. This allows multiple components of emotion regulation to be probed including (1) implicit emotional processing, (2) attentional modulation of emotion, and (3) appraisal modulation of emotion. The ‘Gender’ task reflects implicit emotional processing, as attention is directed to an emotional face, producing robust amygdala activation (49). The ‘Inside/Outside’ task reflects attentional modulation, as it requires shifting attention away from the emotional face, and leads to decreased amygdala activation with corresponding increases in regions associated with emotion regulation. The ‘Like/Dislike’ task requires appraisal of one’s emotional/evaluative state, and (similar to the ‘Inside/Outside’ task), has been found to reduce amygdala activation and increase activation of dorsal medial prefrontal cortex (dmPFC) (50, 51). Stimuli were presented using E-Prime software (E-Prime, Inc). Each image was presented three times, once for each task, with task type presented in random order. There were four runs and 55 trials per run. Trials consisted of a centered fixation crosshair presented for 3-8 sec, a task cue presented for 750 ms (and followed by a 250 ms blank screen), and a composite image presented for 1500 ms. Ten images of faces only and ten images of places only were interspersed throughout the task. Parahippocampal place area was computed as the contrast of face localizers minus place localizers. Prior to experimental trials, participants completed a practice session with images not used in the experiment. The entire task took approximately 26 minutes.

### Memory Task

Participants returned 24 hours later for computerized memory testing outside the scanner. First, item-specific memory was tested with faces or places that were previously presented as part of composite images. The conjunctive memory test required participants to make “match” (previously viewed faces and buildings presented in a combinations seen during encoding) or

“mismatch” (items in combinations not previously seen together) judgments. Participants were encouraged to respond quickly and accurately, but without imposed time limits. Accuracy and reaction time were recorded. Memory sensitivity was determined using signal detection theory, where  $d'$  (memory sensitivity) was calculated for conjunctive memory (“match vs. mismatch”) and item-specific memory (“yes vs. no”) for faces and buildings. Group effects (DHEA vs. placebo) on  $d'$  measures (conjunctive, item-specific) were examined using two-tailed independent t-tests. The C (bias) Criterion component of signal detection theory was computed to assess whether participants displayed a response bias (i.e. a tendency to endorse or reject items as having been previously presented). Additionally,  $d'$  values for conjunctive memory were entered as regressors in between-subject analyses.

## **Magnetic Resonance Imaging**

### *Image Acquisition*

In order to control for potential diurnal variations in DHEA (52, 53), all scans were conducted in the afternoon. Scanning was performed with blood-oxygen-level-dependent (BOLD) sensitive whole-brain fMRI on a Philips 3.0 Tesla Achieva X-series MRI (Philips Medical Systems) using a standard radiofrequency coil. After participants were positioned in the scanner, a T1-weighted low resolution structural image was acquired approximately parallel to the AC-PC line which was identical to the prescription of the functional acquisitions [gradient recall echo sequence (GRE), repetition time (TR) = 250 ms, echo time (TE)=5.7 ms, flip angle (FA)=90°, 2 averages, field of view (FOV)=22 cm, matrix=256×256, slice thickness=3 mm, 42 axial slices to cover the whole brain]. The intermediate template and fMRI images were acquired using an 8-channel SENSE head coil. Functional images were acquired with a T2\*-weighted, EPI acquisition

sequence (gradient recall echo, TR=2000 ms, TE=25 ms, FA=90°, FOV=22 cm, matrix=64×64, slice thickness=3 mm with no gap, 42 axial slices to cover the whole brain), followed by a high-resolution anatomical image. Three initial volumes were discarded from each run to allow for equilibration of the scanner signal. A high-quality T1-weighted structural image was obtained with the following parameters: TR=9.8 ms, TE=4.6 ms, FA=8°, TI=650 ms, FOV=26 cm, matrix=256×250 for in-plane resolution of 1 mm; slice thickness=1 mm with no gap, 160 contiguous axial slices to cover the whole brain.

### Preprocessing

A standard series of processing steps was performed using statistical parametric mapping (SPM8; [www.fil.ion.ucl.ac.uk/spm](http://www.fil.ion.ucl.ac.uk/spm)). Scans were reconstructed, motion-corrected, slice-time corrected, realigned to the first scan in the experiment to correct for head motion, co-registered with the high-resolution sagittal images, anatomically normalized to the Montreal Neurological Institute (MNI) 152 template brain, resampled to 3×3×3 mm<sup>3</sup> voxels, and smoothed with an 8×8×8-mm<sup>3</sup> kernel. Motion parameters (mean displacement, mean angle) were compared across drug conditions via Independent-Samples Kruskal-Wallis tests, and runs with any movement greater than 3 mm were excluded.

### Data Analysis

Maps of activation in each condition, as well as reaction time and on-line accuracy judgments were analyzed via a 2 (Drug Type: DHEA or Placebo) x 3 (Face Type: Angry, Fearful, Neutral) x 3 (Condition: Male/Female, Inside/Outside, Like/Dislike) repeated measures ANOVA to assess for main effects and interaction effects. Follow-up simple effects analyses were performed with

two-tailed  $t$ -tests, with significance threshold set to .05, corrected for multiple comparisons. Parahippocampal place area was determined by contrasting place localizers to face localizers. Levels of delta DHEA and cortisol (endpoint minus baseline) and self-report measures showing significant differences between drug groups were entered as regressors in between-subject analyses.

#### Whole Brain and Region of Interest Analysis

Z-score images from the individual activation maps were entered into second-level random-effects analyses implemented in SPM8. Second-level maps were corrected for multiple comparisons using whole-brain family-wise error correction,  $p < .05$ . Region of interest (ROI) analysis with small volume correction (SVC) was conducted with a priori brain areas identified in previous neuroimaging studies of DHEA(8). Activation threshold and cluster size were determined using AlphaSim (54) to correspond to a false positive rate of  $p < 0.05$ , corrected for multiple comparisons within ROIs. A priori ROIs of anatomical anterior cingulate cortex ( $k=819$ ), hippocampus ( $k=259$ ), and amygdala ( $k=63$ ) were used as masks. Images were thresholded using a voxelwise threshold of  $p < 0.005$  uncorrected with a minimum cluster size of 19 connected voxels for ACC, 6 voxels for hippocampus, and 3 voxels for amygdala. The corrected voxel-wise probabilities were: ACC  $p < 0.002$ , hippocampus  $p < 0.003$ , and amygdala  $p < 0.0036$ . Only the activations within the ROIs that survived the volume and voxel correction criteria were extracted and used for further analysis. Activation foci were labeled by comparison with the neuroanatomical atlas by Talairach and Tournoux (55). Reported voxel coordinates correspond to standardized Montreal Neurologic Institute (MNI) space.

The time series from significant clusters within regions of group difference were used in a psychophysiological interaction (PPI) analysis. Deconvolved time series in the anatomical right amygdala was extracted for each participant as the first regressor in the PPI analysis (physiological variable). The second regressor represented the experimental condition (emotion induction; psychological variable). The regressor of interest was the interaction between the time series of the seed region and the experimental condition.

## Results

Fourteen participants were administered DHEA and 15 were administered placebo. Groups did not differ by age ( $t(27)=1.11, p=.28$ ) or ethnicity ( $\chi^2(2)=4.03, p=.13$ ). DHEA levels typically decrease with age (56), however age was not significantly correlated with baseline DHEA or DHEAS in our sample (in all cases  $p>0.4$ ), therefore age was not included as a covariate in subsequent analyses. DHEA administration produced 3-4 fold increases in DHEA and DHEAS serum and salivary levels (all  $p$  values  $< 0.001$ ), and did not affect circulating cortisol. Repeated measures ANOVA (with treatment as a between subjects factor and DHEA(S) over time as a within subjects factor) showed that DHEA significantly raised serum DHEAS levels [main effect of treatment:  $[F(1,27)=13.70, p<.001$  and treatment-by-time interaction:  $[F(3,81)=15.81, p<.001]$ , serum DHEA levels [main effect of treatment:  $[F(1,27)=4.06, p=.05$  and treatment-by-time interaction:  $[F(1,27)=26.35, p<.001]$ , and salivary DHEAS levels [main effect of treatment:  $[F(1,27)=25.20, p<.001]$  and treatment-by-time interaction:  $[F(1,27)=106.09, p<.001]$ . Serum and salivary cortisol were not increased by DHEA treatment (in all cases  $p>0.4$ ). Baseline DHEA levels did not differ between placebo and DHEA groups [ $t(27)=0.81, p=.43$ ]. DHEAS was significantly elevated in the DHEA group (vs. placebo) beginning 60 minutes after

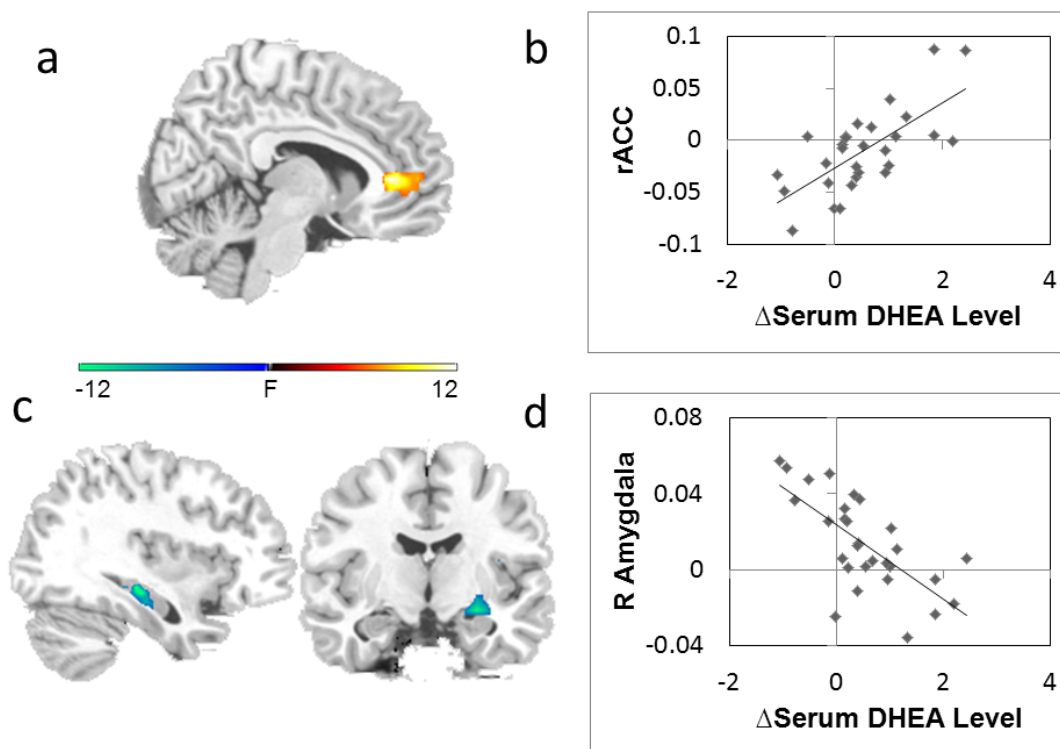


administration [ $t(27)=2.89, p=.008$ ] and sustained throughout the course of the experiment (time point 3 [ $t(27)=-3.67, p=.001$ ] and 4 [ $t(27)=-6.27, p<.001$ ]). DHEA administration increased serum DHEA 3-fold, from  $24.07 \pm 13.96$  ng/mL to  $80.60 \pm 57.72$  ng/mL,  $t(13)=3.73, p=.003$ . Similarly, serum DHEAS levels increased from  $284.04 \pm 105.62$   $\mu$ g/dL to  $1286.68 \pm 481.16$   $\mu$ g/dL and salivary DHEAS levels increased from  $0.76 \pm 1.13$   $\mu$ g/dL to  $2.70 \pm 2.41$   $\mu$ g/dL. Inter-assay and intra-assay variability was 6.96% and 4.1% (DHEA), 3.96% and 1.5% (DHEAS) and 6.84% and 3.46% (cortisol). There were no significant differences between DHEA and placebo groups in self-reported anxiety, sedation, or neurocognitive function (in all cases  $p>.1$ ). There were no significant differences in subjective drug effects ( $p>.4$ ). Participants' guesses of which drug they received did not deviate from chance ( $\chi^2(3)=1.81, p=.61$ ).

Compared to placebo, DHEA increased activity in the rostral ACC (rACC), an emotion regulation region, across all conditions and face types (see Table 2.1, Figure 2.1). Across groups, serum DHEA level (endpoint minus baseline) was positively associated with rACC activity ( $[-3,41,4]$ ;  $r=.698, z=4.35, k=215, p<.05, SVC$ ; see Figure 2.1), indicating that greater peripheral increase in DHEA was associated with greater activation in this regulatory region. Furthermore, within the DHEA group, activity in this rACC region of group difference was positively correlated with increase in serum DHEA level ( $r=.633, p=.015$ ). To examine the relationship between this effect and negative mood, PANAS negative affect score was added as a regressor in a separate whole brain analysis. Results showed that a nearby region of ACC was negatively correlated with PANAS negative affect score, across groups ( $[-30,-28,-8]$ ;  $Z=3.48, k=15, p<.05, SVC$ ), suggesting that increased activity in this region is associated with less negative affect.

Additionally, DHEA administration reduced negative affect at trend level as measured by the PANAS-X [ $t(27)=1.87, p=.07$ ].

Compared to placebo, DHEA significantly reduced activity in emotion generation regions including the right amygdala/hippocampus and left hippocampus (see Table 2.1, Figure 2.1). Left hippocampus activity was reduced across all conditions and face types ( $p<.001$ ), whereas right amygdala/hippocampus activity was reduced specifically in the implicit emotion induction condition ( $p=.007$ ). Activity in this amygdala/hippocampus during implicit induction was negatively correlated with endpoint serum ( $r=-.42, p=.02$ ) and salivary ( $r=-.53, p=.003$ ) DHEA-to-cortisol ratio. Across all conditions and face types, change in serum DHEA level was negatively associated with right amygdala activity ( $[30,-1,-14]$ ;  $r=-.691, z=3.30, k=9, p<.05$ , SVC; see Figure 2.1), indicating that peripheral increase in DHEA was associated with reduced activation in this emotion generation region.

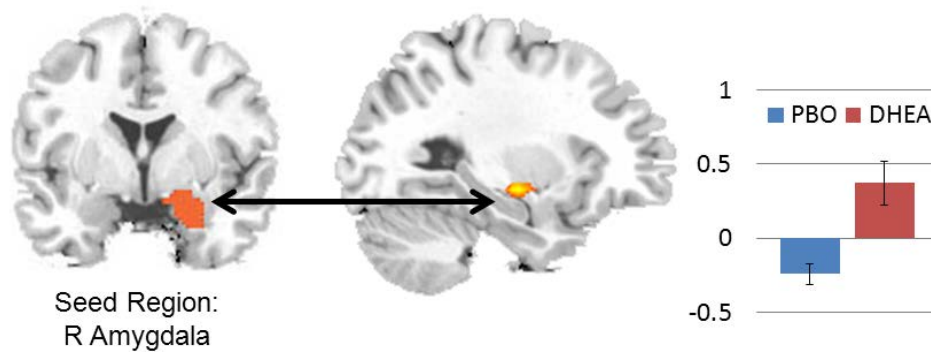


**Figure 2.1 (A) Compared to placebo, DHEA increased rACC activation across conditions and face types ( $x=8$ ). (B) Serum DHEA predicted increased rACC activation across groups. (C) Compared to placebo, DHEA decreased left hippocampus activation across conditions and face types ( $x=-35$ ) and decreased right amygdala/hippocampus activation during implicit emotion induction ( $y=-9$ ). (D) Delta serum DHEA (ng/mL) predicted decreased right amygdala activation across groups. Y Axis = Beta estimate.**

Additionally, during implicit emotion induction, a region of right amygdala and hippocampus that overlapped with the region observed in the group difference map was positively correlated with PANAS negative affect score ( $[24,-16,-8]$ ;  $Z=2.87$ ,  $k=15$ ;  $p<.05$ , SVC). This finding indicates that decreased activation in right amygdala/hippocampus is associated with reduced negative emotional response.

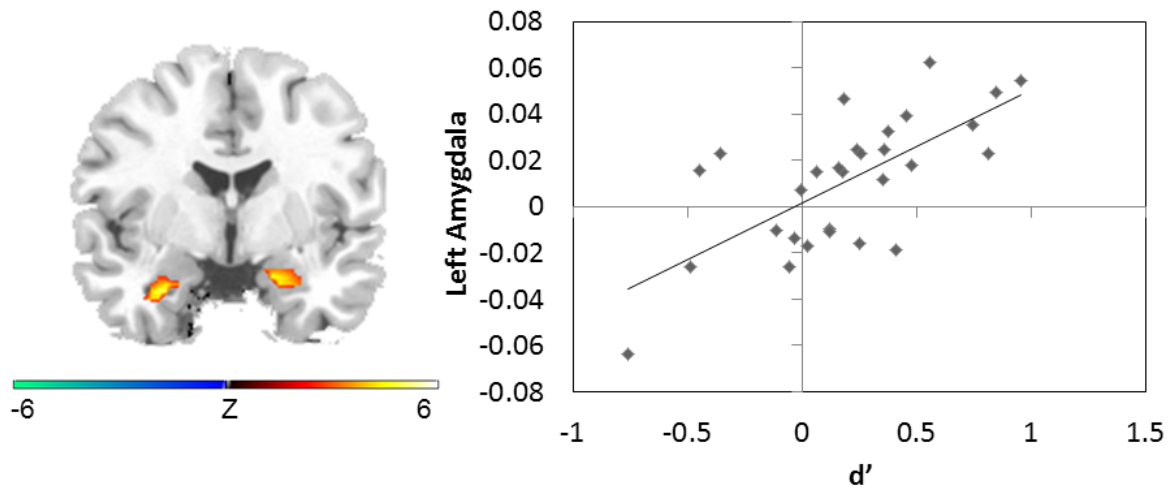
Since right amygdala showed differential between-group activation during implicit emotion induction, subsequent PPI analysis was restricted to the implicit emotion induction condition

with right amygdala as the seed region. Compared to placebo, the DHEA group showed significantly greater functional connectivity between right anatomical amygdala and right anterior hippocampus ([30,-13,-11];  $z=3.40$ ,  $k=33$ ,  $p<.05$ , SVC) and right posterior hippocampus ([24,-40,7];  $z=3.76$ ,  $k=14$ ,  $p<.05$ , SVC; see Figure 2.2) during the implicit emotion induction condition as compared to implicit baseline.



**Figure 2.2 DHEA increased functional connectivity between right amygdala ( $y=-18$ ) and right hippocampus ( $x=28$ ) during implicit emotional processing. Y Axis = Beta estimate.**

In the conjunctive memory task, DHEA reduced memory accuracy for emotional stimuli, and reduced activity in regions associated with conjunctive memory encoding. Conjunctive memory accuracy was reduced in the DHEA group ( $d'$ ;  $t(27)=2.31$ ,  $p=.029$ ), but DHEA did not impact item specific memory accuracy or response bias (in all cases  $p>0.3$ ). Across all conditions and face types, conjunctive memory accuracy was positively correlated with right amygdala/hippocampus ([18,-4,-23];  $r=.627$ ,  $z=3.31$ ,  $k=48$ ,  $p<.05$ , SVC) and left amygdala activity ([-27,-4,-29];  $r=.693$ ,  $z=4.24$ ,  $k=33$ ,  $p<.05$ , SVC; see Figure 2.3), suggesting that activation increases in these regions during encoding predicted successful memory retrieval. Interestingly, salivary cortisol was positively associated with left hippocampal activity ([-24,-31,-5];  $z=3.44$ ,  $k=32$ ,  $p<.05$ , SVC), indicating that increases in cortisol predicted enhanced activity in this region.



**Figure 2.3** Conjunctive memory accuracy ( $d'$ ) was positively correlated with right hippocampus and bilateral amygdala activation ( $y=-2$ ). Y Axis = Beta estimate.

There were no significant differences between DHEA and placebo groups in reaction time [ $F(1, 27)=.13, p=.72$ ] or accuracy [ $F(1, 27)=.22, p=.64$ ]. There was a significant main effect of task driven by increased accuracy ( $.81 \pm .024$  vs  $.68 \pm .015$ ) and decreased reaction time ( $1.32 \pm .061$  vs  $1.40 \pm .054$ ) in the attention modulation task as compared to the implicit emotion induction task [reaction time  $F(2, 54)=3.54, p=.04$ ]; accuracy  $F(2, 54)=31.39, p<.001$ ]. There was a significant interaction of task by face type driven by reduced accuracy in response to fearful ( $.65 \pm .020$  vs  $.79 \pm .025$ ) and angry faces ( $.64 \pm .016$  vs  $.81 \pm .025$ ) in the emotion induction task as compared to the attention modulation task [ $F(4, 108)=5.61, p<.001$ ].

Examination of motion parameter summary statistics revealed there were no differences between groups in mean displacement or mean angle (in all cases  $p>0.2$ ).

There was a main effect of condition in the dmPFC [ $(-12,59,28)$ ;  $F(2, 216)=49.96; k=400$ ;  $p<.001$ ], left anterior insula [ $(-45,26,1)$ ;  $F=32.01; Z=7.09; k=229; p<.001$ ], left parietal cortex ( $[-$

36,-82,31];  $F=46.02$ ;  $k=245$ ;  $p<.001$ ), left precuneus ([-15,-58,16];  $F=49.97$ ;  $k=192$ ;  $p<.001$ ), and bilateral parahippocampal gyrus (Right: [30,-40,71];  $F=58.59$ ;  $k=392$ ;  $p<.001$ ; Left: [-27,-43,-11];  $F=62.78$ ;  $k=183$ ;  $p<.001$ ; see Table 2.1). Follow-up simple effects tests revealed that, compared to the implicit emotion induction condition, dmPFC and anterior insula activity was increased in the appraisal condition ( $p<.001$ ), and left parietal cortex, precuneus, and parahippocampal gyrus activity was increased in the attention modulation condition ( $p<.001$ ). Parahippocampal activity showed significant overlap with Parahippocampal Place Area (Right: [30,-40,-11];  $k=91$ ; Left: [-27,-43,-11];  $k=40$ ), indicating heightened attention to location during this condition.

**Table 2.1 ANOVA of fMRI results for SEAT**

| <b>Contrast Map and Brain Region</b>                                                                                                                                                                                                                                                              | <b>Cluster Size</b> | <b>MNI Coordinates (x,y,z)</b> | <b>Analysis (F/t)</b> |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|--------------------------------|-----------------------|
| <i>Main Effect of Condition</i>                                                                                                                                                                                                                                                                   |                     |                                |                       |
| Superior Medial Frontal Gyrus                                                                                                                                                                                                                                                                     | 400                 | -12, 59, 28                    | 49.96                 |
| Left Insula                                                                                                                                                                                                                                                                                       | 229                 | -45, 26, 1                     | 32.01                 |
| Left Parietal Cortex                                                                                                                                                                                                                                                                              | 245                 | -36, -82, 31                   | 46.02                 |
| Left Precuneus                                                                                                                                                                                                                                                                                    | 192                 | -15, -58, 16                   | 49.97                 |
| Right Parahippocampal Gyrus                                                                                                                                                                                                                                                                       | 392                 | 30, -40, -11                   | 58.59                 |
| Left Parahippocampal Gyrus                                                                                                                                                                                                                                                                        | 183                 | -27, -43, -11                  | 62.78                 |
| <i>Main Effect of Drug</i>                                                                                                                                                                                                                                                                        |                     |                                |                       |
| <b>Anterior Cingulate Gyrus</b>                                                                                                                                                                                                                                                                   | 43                  | 3, 41, -2                      | 13.30                 |
| <b>Left Hippocampus</b>                                                                                                                                                                                                                                                                           | 11                  | -33, -28, -11                  | 22.07                 |
| <i>Drug by Condition Interaction</i>                                                                                                                                                                                                                                                              |                     |                                |                       |
| <b>Right Amygdala/Hippocampus</b>                                                                                                                                                                                                                                                                 | 10                  | 27, -10, -14                   | 6.9                   |
| Simple Effect: Emotion Induction: PBO>DHEA                                                                                                                                                                                                                                                        |                     |                                | 2.98                  |
| Simple Effect: PBO: Emotion Induction>Appraisal                                                                                                                                                                                                                                                   |                     |                                | 4.24                  |
| * <i>a priori</i> regions of interest (ROIs) in <b>bold</b> ; significant at $p < .05$ , corrected for multiple comparisons across the ROI. All other activations are presented at $p < .05$ , FWE corrected for multiple comparisons across the whole brain. MNI, Montreal Neurologic Institute. |                     |                                |                       |

## Discussion

In this study, we used a novel probe of emotion induction, modulation and regulation to assess the neural correlates of DHEA’s impact on emotional responses and their regulation. We demonstrated that DHEA reduces activity in regions associated with the generation of negative emotion and enhances activity in regions linked to regulatory control of emotion. These patterns of activation were associated with reduction in self-reported negative affect. Additionally, DHEA reduced conjunctive memory accuracy for emotional stimuli, suggesting a reduction in emotional reactivity during encoding. To our knowledge, this is the first neuroimaging study of

acute effects of DHEA administration, and the first to examine DHEA's impact on emotion neurocircuitry. These findings suggest a potential role for DHEA as anxiolytic and antidepressant agent, and invite further investigation into DHEA enhancement as a novel pharmacological intervention for the treatment of mood and anxiety disorders.

In our sample, single dose DHEA administration reduced amygdala and hippocampus activity and increased connectivity between these two regions during emotion induction. Additionally, the increase in serum DHEA was correlated with decreased amygdala activity, supporting the hypothesis that DHEA reduces emotional reactivity. The amygdala is a key region in threat detection (32), fear conditioning (33), and emotional salience (34) and the hippocampus is implicated in declarative memory (57), contextual memory (37) and fear conditioning (38). Both regions are associated with the detection of threat and production of negative emotional response (41), and previous research suggests that greater amygdala activation in response to the presentation of facial expressions is associated with greater emotional response (51). Thus, DHEA's reduction of activity in amygdala and hippocampus suggests that DHEA may aid in successful down-regulation of negative emotions induced by fearful and angry facial expressions, and, by extension, to aversive stimuli in the environment. In support of this notion, DHEA reduced negative affect in our subjects as assessed by the PANAS-X negative affect subscale (ten items assessing constructs of fear, hostility, guilt, and sadness, 58), albeit at trend level. Our results are consistent with those of large placebo-controlled studies that demonstrated DHEA's antidepressant effects (22-24), and with preclinical studies suggesting that DHEAS is inversely correlated with negative affect (59) and that DHEA decreases depressive-like behavior in animals (60). Here, we extend these findings by identifying the neural correlates of this effect.



DHEA administration was also associated with greater rACC activity across conditions and emotions, supporting that DHEA increases activity in regions linked to regulatory control over emotion. Animal studies of fear conditioning implicate mPFC in the extinction of conditioned fear (61), and in humans a recent Granger causality analysis demonstrated an inhibitory influence of the rACC on the amygdala (62). Increased rACC activity and decreased amygdala activity following DHEA administration is thus consistent with the notion of “top-down” regulation of amygdala by emotional regulatory circuits, a function that is likely diminished in some anxiety disorders (63). Postmortem data confirm that DHEA is present in high concentrations in the prefrontal lobe (64) and one previous neuroimaging study reported that DHEA modulated rACC activity (8). In our sample, serum increase in DHEA was associated with greater rACC activity, further supporting the hypothesis that DHEA is associated with increased activity in this region. Thus, DHEA may lead to increases in cognitive regulation, as well as suppression of negative emotional reactivity. Since these capabilities are impaired in some anxiety disorders, our data prompt future investigations into the role of DHEA and androsterone as anxiolytic agents.

In addition to modulating activity in brain regions associated with emotion generation and regulation, DHEA also decreased conjunctive memory for emotional stimuli, without affecting other aspects of memory (such as item specific recognition). This finding might seem counterintuitive given previous reports of DHEA’s role in attentional enhancement. DHEA augmentation improved performance on a selective attention test in elderly individuals (65), and improved sustained visual attention in schizophrenic patients (66). Similarly, DHEAS improves visuospatial attention and performance in humans (67) and mice (68). However, other studies

have suggested that DHEA may not benefit memory in healthy individuals (69), or might not affect all types of memory in the same way. In our sample, the accuracy of conjunctive memory, specifically, (conjunctive d') was positively associated with bilateral amygdala and right hippocampus activity. This replicates our earlier findings of neurocircuitry involved in conjunctive memory (70). DHEA reduced activity in these regions, suggesting a plausible mechanism by which DHEA might impact associative memory. Specifically, reduced emotional reactivity when viewing the images may have detrimentally affected memory for those images. This is consistent with findings that greater limbic activity in response to greater salience contributes to stronger encoding of emotional stimuli (71). A reduction in saliency leading to a reduction in emotional memory strength could be relevant to stress-related disorders such as major depression and posttraumatic stress disorder, in which enhanced strength of emotional memories may contribute to pathophysiology. It is important to note that reaction time and accuracy were not impacted by DHEA. Thus, DHEA did not reduce attention to, or impede performance on, the task. Rather, it reduced activity in specific regions related to negative emotion production, and reduced memory for the stimuli. Therefore, DHEA induction may be a plausible mechanism for modulation of emotional reactivity and memory for emotional stimuli. Furthermore, these modulatory effects may be beneficial for individuals with mood and anxiety disorders.

DHEA's reduction of hippocampal activity and its detrimental impact on conjunctive memory may both relate to its role as an antigluocorticoid. DHEA administration reduces circulating levels of cortisol (7, 8), and DHEAS is inversely related to peak cortisol in response to CRH infusion (9). DHEA antagonizes neurotoxic effects of cortisol on the hippocampus (72), and high

DHEA/cortisol ratio in depressed adults is associated with worsened contextual memory (73). These findings may indicate a stress-buffering effect of DHEA. In our data, DHEA did not reduce circulating cortisol levels. However, greater DHEA-to-cortisol ratio was associated with reduced amygdala/hippocampal activity. Furthermore, salivary cortisol was positively associated with left hippocampus activity. We have previously reported that cortisol administration increases hippocampal activity and enhances conjunctive memory(70). If DHEA antagonized cortisol in our sample (perhaps through inhibition of glucocorticoid receptor translocation, 74), this may have played a role in DHEA's reduction of hippocampal activity and detrimental impact on conjunctive memory. Thus, it is possible that DHEA attenuates cortisol's enhancement of hippocampally-mediated emotional memory formation.

Our finding of reduced memory for emotional stimuli supports the notion that DHEA elevation is a compensatory response to stress. Since anxiety disorders and PTSD in particular show consistently elevated levels of DHEA, Rasmusson and colleagues (19) have suggested that DHEA elevations occur in response to extreme stress, and that greater elevations may be a mark of resiliency. Consistent with this hypothesis, DHEA is positively associated with adaptive responses to stress (14, 75) and symptom improvement and coping in PTSD (76, 77). In addition, 7-keto DHEA reduced PTSD symptoms in a case-series of five treatment-refractory patients (78). Our findings that DHEA (1) decreased activity of brain regions associated with emotion generation, (2) increased activity in regions linked to effortful and automatic emotion regulation, and (3) reduced associative memory for emotional stimuli, provide novel evidence linking DHEA to processes relevant to resiliency and recovery.

This study has several limitations. First, we measured serum levels of DHEA, and not cerebrospinal fluid or brain levels. However, in animals, these levels are highly correlated (46, 79). Second, our sample size was modest, and thus our results should be considered preliminary. Our event-related fMRI design is not well-suited to assess DHEA's potential impact on overall brain perfusion and cerebral blood flow. Future studies should employ PET or arterial spin labeling to examine this issue. Although only conjunctive memory and item-specific memory for emotional faces was measured, it is possible that DHEA could adversely affect normal memory processes. However, previous evidence suggests that higher DHEA is associated with better working memory, episodic memory, and visuospatial memory (3). Thus, it is more likely that our findings represent a specific rather than a general effect. Our sample consisted of healthy male individuals without mood or anxiety disorder diagnoses. Though we report a trend-level improvement of negative affect after DHEA administration, it is a limitation that baseline measures of negative affect were not assessed. Thus, extrapolations to women or to clinical populations should be made with caution. Finally, though we have suggested DHEA as a potential therapeutic approach for anxiety disorders, results from this is a single dose study should be interpreted cautiously. DHEA's safety and efficacy in suppressing neural circuitry of negative emotions beyond a single dose has not been demonstrated.

In conclusion, we demonstrated that DHEA reduces activity in regions associated with the production of negative emotion, enhances activity in regions linked to top-down regulatory control over emotion, and reduces memory accuracy for emotional stimuli. These findings illuminate the neurocircuitry underlying DHEA's effects, and support further exploration of

DHEA's value as a novel pharmacological intervention for the treatment of mood and anxiety disorders.

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## **Chapter 3 : The Neurosteroid Allopregnanolone Enhances Activation of Emotion Regulation Neurocircuits**

### **Abstract**

The neuroactive steroid allopregnanolone is a potent allosteric modulator of the GABA(A) receptor with anxiolytic properties. Exogenous administration of allopregnanolone reduces anxiety, and allopregnanolone blockade impairs social and affective functioning. However, the neural mechanism whereby allopregnanolone improves mood and reduces anxiety is unknown. In particular, brain imaging has not been used to link neurosteroid effects to emotion regulation neurocircuitry. To investigate the brain basis of allopregnanolone's impact on emotion regulation, participants were administered 400mg of pregnenolone (N=16) or placebo (N=15) and underwent 3T fMRI while performing the Shifted-Attention Emotional Appraisal Task (SEAT), which probes emotional processing and regulation. Compared to placebo, allopregnanolone reduced activity in the amygdala and insula across all conditions. During the appraisal condition, allopregnanolone increased activity in the dorsal medial prefrontal cortex and enhanced connectivity between the amygdala and dorsal medial prefrontal cortex, an effect that was associated with reduced self-reported anxiety. These results demonstrate that in response to emotional stimuli, allopregnanolone reduces activity in regions associated with generation of negative emotion. Furthermore, allopregnanolone enhances activity in regions linked to regulatory processes. Aberrant activity in these regions has been linked to anxiety psychopathology. These results thus provide initial neuroimaging evidence that allopregnanolone

may be a target for pharmacological intervention in the treatment of anxiety disorders, and suggest potential future directions for research into neurosteroid effects on emotion regulation neurocircuitry.

## Introduction

Allopregnanolone (ALLO) is a progesterone-derived steroid with potent anxiolytic properties (1) that acts as a positive allosteric modulator at the GABA(A) receptor (2-4). Allopregnanolone is produced *de novo* in neurons and glia (2), in addition to synthesis in peripheral glands including the ovaries and adrenal glands (5). In tissue culture, it lowers neuronal excitability with 20-fold higher potency than benzodiazepines and barbiturates (6), and modulates a broader range of GABA(A) receptors than either of these compounds (7). Due to its potency and range, allopregnanolone shows promise as a mechanism for anxiolysis in psychiatric disorders.

Convergent evidence from animal studies and human clinical research implicates allopregnanolone dysregulation in mood and anxiety symptomatology. In rats, blockade of metabolism from progesterone to allopregnanolone impairs social and affective (anxiety-related) behavior in rats (8, 9), which is in turn restored by allopregnanolone infusion (6). Consistent with these findings, allopregnanolone infusions have reliably been shown to reduce stress and anxiety-like behavior in rodents (2, 3, 10-15). Furthermore, exploratory and low-anxiety behavior in rats is correlated with circulating and hippocampal levels of allopregnanolone, but not with levels of estrogen, progesterone, or corticosterone (9), suggesting that allopregnanolone may show greater promise as an anxiolytic candidate than other pregnane neurosteroids. Allopregnanolone also reduces conditioned fear responding, facilitates fear extinction, and prevents the reinstatement of fear memory after extinction (16). Studies in humans are less abundant, but broadly consistent with animal findings. Cerebrospinal fluid levels of allopregnanolone are reduced in women with major depressive disorder and PTSD, and increase with successful psychiatric treatment (17-20). Multiple antidepressant agents (including

fluoxetine, norfluoxetine, fluvoxamine and paroxetine) elevate allopregnanolone brain levels (19, 21-24), leading to the suggestion that allopregnanolone might be an important mechanism for the antidepressant effects of SSRIs (16, 18). Based on these observations, it has been suggested that allopregnanolone dysregulation may contribute to the development of neuropsychiatric disorders, and that restoration of allopregnanolone may be a potential pathway for symptomatic improvement (1, 7, 22). However, the specific neural mechanisms whereby allopregnanolone improves mood and anxiety symptomatology are unknown.

One potential mechanism for allopregnanolone's effects in the central nervous system is its ability to directly impact emotion neurocircuitry. Allopregnanolone acts directly on GABA(A) receptors, which are present throughout the cortex and limbic system (25). Allopregnanolone has particular impact on GABA(A) receptors in the amygdala (26). In rats, microinfusions of allopregnanolone directly into the amygdala produce rapid anxiolytic (27), antidepressant (28), and anti-aggressive (29) effects. In humans, progesterone administration (which increases downstream allopregnanolone) modulates amygdala responses to emotional faces (30), and increases functional connectivity between amygdala and dorsal medial prefrontal cortex (dmPFC) (31), a regulatory region interconnected with limbic structures (32) and crucial to emotional regulation (33). However, no studies have yet examined the impact of allopregnanolone on emotion regulation, a function subserved by these neurocircuits and postulated to be disrupted in anxiety disorders (for reviews, see 34, 35). Since deficits in emotion regulation may contribute to or maintain anxiety (35), understanding allopregnanolone's impact on emotion regulation neurocircuits may be central to understanding why allopregnanolone dysregulation is associated with distress and psychopathology.

In the current study, we used a probe of emotion induction, modulation, and regulation (36, 37) to examine the neural basis of allopregnanolone's effects on emotion processing. Given previous behavioral and preclinical evidence of its anxiolytic and antidepressant effects, we expected allopregnanolone to diminish responses in emotion generation neurocircuits, and enhance activation in regions subserving regulatory control of emotions.

## **Methods**

### *Participants*

Study participants were 31 right-handed healthy male volunteers aged 18-32 years (mean  $\pm$  SD=22  $\pm$  3.38) recruited from the community via advertisement. Our investigation was restricted to males because allopregnanolone levels fluctuate over the course of the menstrual cycle and may have differential impact on mood depending on menstrual cycle phase (38), and it was not feasible within the limited scope of this project to recruit women in all phases of the menstrual cycle. Exclusion criteria were history of head injury, recent steroid use, or current or past psychiatric disorder, as assessed via the Mini-International Neuropsychiatric Interview (M.I.N.I.; 39). Participants were given full details of the study and provided written informed consent. The study was approved by the Institutional Review Board of the University of Michigan Medical School.

### Procedure

All participants completed tests of working memory (Digit Span), visual attention and task switching (Trail-Making Test), both prior to and one hour after drug administration. Change scores were calculated as post-administration score minus pre-administration score. This provided an ancillary test for the effects of allopregnanolone on neurocognitive function. At one hour post-drug administration, participants completed the Positive and Negative Affect Scale (PANAS-X), the Drug Effects Questionnaire, and a 20-item Visual Analogue Scale measuring anxiety and sedation. The Visual Analogue Scale measures consisted of 4-inch bars with 'Not At All' and 'Extremely' indicated at the extremities of each variable. Participants also completed an in-scan survey both immediately prior and after the emotion regulation task. They viewed a list of 22 emotions and rated them on a 5-item Likert-type scale (1 = not at all, 5 = extremely). Anxiety score was calculated as the sum of the following items: Nervous, Anxious, Fearful, and Stressed. All measures were compared between groups via independent-sample T-tests.

### Drug Administration

Study drug (pregnenolone) and matching placebo identical in appearance were obtained from Bell Pharmacy (Lakewood, CO), which provided certificates of analysis. Participants were randomly assigned to receive a single oral dose of 400 mg pregnenolone (n=16), or placebo (n=15). Participants and investigators were blind to condition. Pregnenolone was administered as a precursor loading strategy to significantly increase downstream allopregnanolone levels. Pregnenolone is lipophilic and readily crosses the blood brain barrier. We have previously found that pregnenolone is preferentially metabolized to allopregnanolone, rather than other compounds such as cortisol or DHEA (40, 41); however these metabolites were also assayed.



Allopregnanolone serum levels have been reported to triple two hours after oral administration of 400 mg pregnenolone (42). Thus, drug administration occurred two hours before neuroimaging to ensure elevated levels during the scan.

### *Steroid measurements*

We used circulating levels of allopregnanolone and pregnenolone as indicators of central neurosteroid levels. In animal models, serum allopregnanolone levels are closely related to levels in the hippocampus (21). Serum samples for assay were collected once prior to drug administration and once after the scanning session. Pregnenolone and allopregnanolone levels in serum were determined by a highly sensitive and specific gas chromatography-mass spectrometry method in the negative ion chemical ionization mode, as described previously (43, 44). One ml of serum was extracted three times in ethyl acetate before high performance liquid chromatography (HPLC) purification using tetrahydrofuran, ethanol, and hexane in the mobile phase. All samples were injected in duplicate. Mean intra-assay coefficients of variation for pregnenolone and allopregnanolone were 0.9% and 2.9%, respectively. The limit of detection with this method was 1 pg for both pregnenolone and allopregnanolone. Serum DHEA levels were determined via enzyme immunoassay (ALPCO Diagnostics, Salem, NH), and serum levels of cortisol and DHEAS were determined by chemiluminescent enzyme immunoassay (IMMULITE) according to the manufacturer's directions (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). All neurosteroid values were natural log transformed prior to analyses.

### Shifted-Attention Emotional Appraisal (SEAT) Paradigm

In order to investigate the brain basis of emotional response and regulation, our laboratory has developed an emotional appraisal task (36, 37) modifying the task of Anderson and colleagues (45). We have previously demonstrated that the SEAT is an effective probe of neurosteroid regulatory effects (36). The SEAT task presents compound stimuli that include both emotional faces and neutral scenes. Stimuli include composite pictures of superimposed faces (foreground) and buildings (background), as well as 20 pictures of faces or buildings only. The face pictures depict neutral, angry, or fearful expressions, and the building pictures depict indoor or outdoor scenes. In three different conditions, participants are asked to respond to three different questions: (1) ‘Gender’: Whether the face in the foreground is male or female; (2) ‘Inside/Outside’: Whether the scene in the background is indoors or outdoors; or (3) ‘Like/Dislike’: Whether the face in the foreground is liked or disliked. This probes multiple components of emotion regulation, including (1) implicit emotional processing, (2) attentional modulation of emotion, and (3) modulation of emotion by appraisal. The ‘Gender’ condition probes implicit emotional processing, as attention is focused on an emotional face while one is identifying its gender. The ‘Inside/Outside’ condition engages attentional modulation, as it requires focusing attention on the building components superimposed on the emotional face. The ‘Like/Dislike’ condition engages cognitive appraisal of one’s emotional/evaluative state while processing the emotional stimulus, and engaging appraisal has been found to increase activation of dmPFC (46, 47).

Stimuli were presented using E-Prime software (E-Prime, Inc). Each image was presented three times, once for each condition, with condition type presented in random order. There were four

runs and 55 trials per run. Trials consisted of a centered fixation crosshair presented for 3-8 sec, a condition cue presented for 750 ms (and followed by a 250 ms blank screen), and a composite image presented for 1500 ms. Ten images of faces only and ten images of places only were interspersed throughout the task and used as localizers only. Prior to experimental trials, participants completed a practice session with images not used in the experiment. The entire task took approximately 26 minutes.

## **Magnetic Resonance Imaging**

### *Image Acquisition*

MRI scanning occurred on a Philips 3.0 Tesla Achieva X-series MRI (Philips Medical Systems). After a T1 image (T1-overlay) was obtained, a T2\*-weighted, echoplanar acquisition sequence [GRE; repetition time, 2000 ms; echo time, 25 ms; flip angle, 90°; field of view (FOV), 22 cm; 42 slice; thickness/skip, 3.0/0 mm matrix size equivalent to 64 x 64] was collected. After discarding three initial volumes to permit thermal equilibration of the MRI signal, 185 volumes were acquired per run. After acquiring the functional volumes, a high-resolution T1 scan was obtained for anatomic normalization [26 FOV; thickness/skip, 1.0/0 mm]. E-prime was used to present stimuli and record responses (Psychology Software Tools, Pittsburgh, PA). Participants viewed the stimuli through MR-compatible liquid crystal display goggles (NordicNeuroLabs <http://www.nordicneurolab.com>) and responded to those stimuli using an MRI-compatible button box.

### Preprocessing

A standard series of processing steps was performed using statistical parametric mapping (SPM8; [www.fil.ion.ucl.ac.uk/spm](http://www.fil.ion.ucl.ac.uk/spm)). Scans were reconstructed, motion-corrected, slice-time corrected, realigned to the first scan in the experiment to correct for head motion, co-registered with the high-resolution sagittal images, anatomically normalized to the Montreal Neurological Institute (MNI) 152 template brain, resampled to  $3 \times 3 \times 3 \text{ mm}^3$  voxels, and smoothed with an  $8 \times 8 \times 8 \text{ mm}^3$  kernel. Motion parameters (mean displacement, mean angle) were compared across drug conditions via Independent-Samples Kruskal-Wallis tests, and runs with any movement greater than 3 mm were excluded.

### Data Analysis

Maps of activation in each condition, as well as reaction time and on-line accuracy judgments were analyzed via a 2 (Drug Type: Pregnenolone or Placebo) x 3 (Face Type: Angry, Fearful, Neutral) x 3 (Condition: Male/Female, Inside/Outside, Like/Dislike) repeated measures ANOVA to assess for main effects and interaction effects. Follow-up simple effects analyses were performed with two-tailed t-tests, with significance threshold set to .05, corrected for multiple comparisons. Parahippocampal place area was determined by contrasting place localizers to face localizers. Levels of allopregnanolone and pregnenolone (endpoint minus baseline) were entered as regressors in between-subject analyses.

### Whole Brain and Region of Interest Analysis

Z-score images from the individual activation maps were entered into second-level random-effects analyses implemented in SPM8. Second-level maps were corrected for multiple

comparisons using whole-brain family-wise error correction,  $p < .05$ . In addition, region of interest (ROI) analysis with small volume correction (SVC) was conducted with a priori brain areas identified in previous neuroimaging studies of allopregnanolone (30, 31) and studies using the SEAT task (37). Activation threshold and cluster size were determined using AlphaSim (48) to correspond to a false positive rate of  $p < 0.05$ , corrected for multiple comparisons within ROIs. A priori ROIs of anatomical dmPFC ( $k=1473$ ), insula ( $k=536$ ), and amygdala ( $k=69$ ) were used as masks. Images were thresholded using a voxelwise threshold of  $p < 0.05$  uncorrected with a minimum cluster size of 30 voxels for amygdala, 81 connected voxels for insula, and 170 voxels for dmPFC. Only the activations within the ROIs that survived the volume and voxel correction criteria were extracted and used for further analysis. Activation foci were labeled by comparison with the neuroanatomical atlas by Talairach and Tournoux (49). Reported voxel coordinates correspond to standardized Montreal Neurologic Institute (MNI) space.

The time series from significant clusters within regions of group difference were used in a psychophysiological interaction (PPI) analysis. Deconvolved time series in the anatomical dmPFC was extracted for each participant as the first regressor in the PPI analysis (physiological variable). The second regressor represented the experimental condition (appraisal; psychological variable). The regressor of interest was the interaction between the time series of the seed region and the experimental condition.

## **Results**

Sixteen participants were administered pregnenolone and 15 were administered placebo. Groups did not differ by age ( $t(29) = .94$ ,  $p = .36$ ) or ethnicity ( $\chi^2(2) = 4.26$ ,  $p = .12$ ). Pregnenolone

administration resulted in threefold elevations in serum levels of pregnenolone (paired  $t(15)=10.89, p<0.001$ ), and increased allopregnanolone sevenfold (paired  $t(15)=13.59, p<0.001$ ). Pregnenolone administration also increased levels of pregnanolone (allopregnanolone's stereoisomer) by approximately 60% and reduced DHEAS levels by approximately 5%. Compared to placebo, pregnenolone administration did not differentially alter serum cortisol, DHEA, or androsterone levels (in all cases  $p>0.3$ ). There were no significant differences between pregnenolone and placebo groups in self-reported anxiety, sedation, or neurocognitive function (in all cases  $p>0.2$ ). There were no significant differences in subjective drug effects ( $p>.5$ ). Participants' guesses of which drug they received did not deviate from chance ( $\chi^2(3)=1.34, p=.71$ ). Since pregnenolone administration was utilized as a precursor loading strategy to enhance allopregnanolone levels, we will use the term "ALLO group" to refer to the participants who were administered pregnenolone. This issue is further detailed in the discussion.

## **SEAT Task**

### *Behavioral Results*

There were no significant differences between ALLO and placebo groups in reaction time [ $F(1, 29)=1.72, p=.20$ ] or accuracy [ $F(1, 29)=.031, p=.86$ ]. There was a significant main effect of task driven by increased accuracy ( $.81 \pm .028$  vs  $.70 \pm .017$ ) and decreased reaction time ( $1.34 \pm .055$  vs  $1.44 \pm .05$ ) in the attention modulation task as compared to the implicit emotion induction task [reaction time  $F(2, 58)=6.57, p=.003$ ; accuracy  $F(2, 58)=41.11, p<.001$ ].

### fMRI Results

Examination of motion parameter summary statistics revealed there were no differences between groups in mean displacement or mean angle (in all cases  $p > 0.07$ ). Maximum displacement did not exceed 3 mm.

### Main Effect of Drug

There was a main effect of drug in the amygdala ([27,-1,-17];  $F(1,29)=9.97, p < .05, SVC$ ) and the insula ([42,8,4];  $F(1,29)=10.97, p < .05, SVC$ ) such that pregnenolone decreased amygdala and insula activity across all conditions and face types (see Figure 3.1). Across all conditions and face types, change in serum allopregnanolone level was negatively associated with right amygdala activity ([27,-1,-17];  $r = -.66, t = 3.79, p < .001$ ), and change in serum pregnenolone level was negatively associated with right insula activity ([42,11,4];  $r = -.65, t = 3.81, p < .001$ ), indicating that peripheral increase in allopregnanolone and pregnenolone was associated with reduced activation in these emotion generation regions.

### Main Effect of Condition: Effects of Appraisal

There was a main effect of condition in the dmPFC [(-12,32,58);  $F(2, 232)=6.71; k=255; p < .001$ ], and left anterior insula [(-48,26,-2);  $F=6.72; k=160; p < .001$ ] such that activity in these regions was increased during the appraisal condition as compared to the implicit emotion induction condition ( $p < .001$ ).

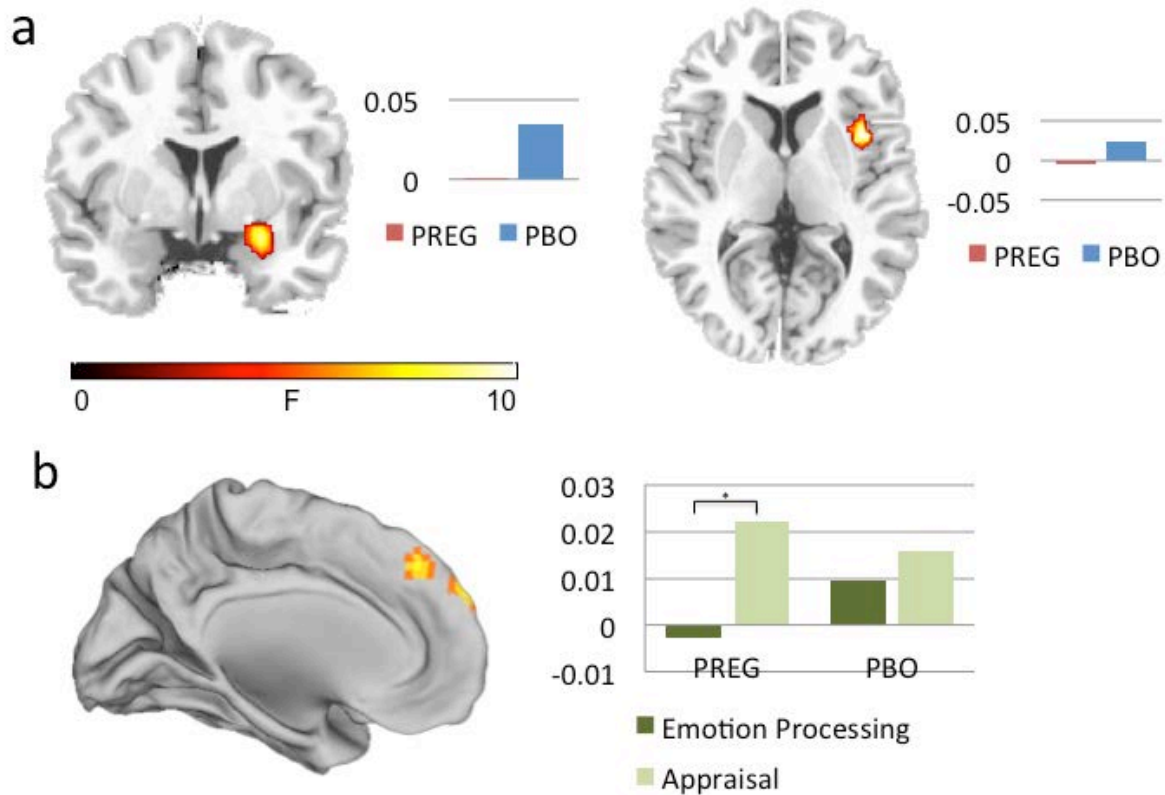
### Main Effect of Condition: Effects of Attention Modulation

There was a main effect of condition in the left parietal cortex ( $[-36,-82,28]$ ;  $F=54.26$ ;  $k=341$ ;  $p<.001$ ), left precuneus ( $[-15,-55,13]$ ;  $F=45.02$ ;  $k=173$ ;  $p<.001$ ), and bilateral parahippocampal gyrus (Right:  $[30,-40,-11]$ ;  $F=56.94$ ;  $k=169$ ;  $p<.001$ ; Left:  $[-27,-43,-11]$ ;  $F=64.07$ ;  $k=225$ ;  $p<.001$ ) such that activity in these regions was increased in the attention modulation condition as compared to the implicit emotion induction condition ( $p<.001$ ). Parahippocampal activity showed significant overlap with Parahippocampal Place Area (Right:  $[30,-40,-14]$ ;  $k=158$ ; Left:  $[-27,-37,-14]$ ;  $k=131$ ), indicating heightened attention to location during this condition.

### Drug by Condition Interaction

There was a significant drug by condition interaction in the dmPFC ( $[3,56,37]$ ;  $F(2,232)=6.41$ ,  $p<.05$ , SVC), such that pregnenolone increased dmPFC activity in the appraisal condition as compared to the implicit emotion induction condition ( $p=.002$ ; see Figure 3.1). The placebo group showed no significant differences between conditions in this dmPFC region. DMPFC activation in the ALLO group was positively correlated with self-reported anxiety ( $r=.50$ ,  $p=.047$ ). Since this region showed differential activation due to drug (ALLO>PBO) during appraisal, subsequent PPI analysis was performed with this dmPFC region as a seed during the appraisal condition to identify differential patterns of connectivity across the drug versus placebo groups.

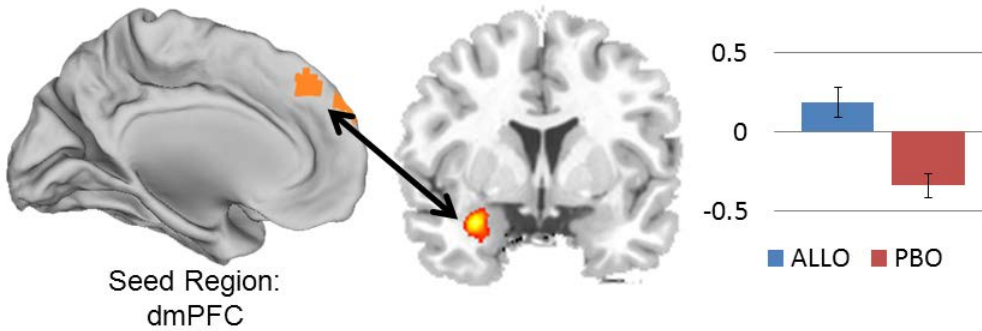




**Figure 3.1 (A) Compared to placebo, allopregnanolone decreased activation in right amygdala ( $y=2$ ) and right insula ( $z=-6$ ) across conditions and face types. (B) Compared to placebo, allopregnanolone increased dorsal medial prefrontal cortex activation during appraisal ( $x=0$ ). Y Axis = Beta estimate.**

### Psychophysiological Interaction (PPI)

Compared to placebo, the ALLO group showed significantly greater functional connectivity between the dmPFC and left amygdala ( $[-30,-1,-23]$ ;  $t=4.8$ ,  $p<.001$ ; see Figure 3.2) during the appraisal condition as compared to implicit baseline. No other region showed differential connectivity with the dmPFC. Functional connectivity between dmPFC and amygdala in the ALLO group was inversely correlated with self-reported anxiety ( $r=-.52$ ,  $p=.046$ ).



**Figure 3.2 Allopregnanolone increased functional connectivity between dorsal medial prefrontal cortex (dmPFC) and left amygdala ( $y=3$ ) during appraisal. Y Axis = Beta estimate.**

## Discussion

We used a novel probe of emotion induction, modulation and regulation to assess the neural basis of allopregnanolone's impact on emotion processing. We demonstrate that allopregnanolone reduces activity in regions associated with the generation of negative emotion, increases activity in regions linked to regulatory processes, and enhances the functional connectivity between the two; an effect that was associated with less self-reported anxiety. To our knowledge, this is the first neuroimaging study to demonstrate an effect of allopregnanolone on emotion regulation, a function likely dysregulated in anxiety disorders. These findings add to the current knowledge regarding the effects of allopregnanolone on emotion neurocircuits, and provide neuroimaging evidence that allopregnanolone may modulate neurocircuits in directions counter to those observed in anxiety psychopathology.

Pregnenolone administration reduced activity in neural circuits associated with the generation of negative emotions. Across all conditions and all face types, pregnenolone decreased right amygdala and right insula activity, and serum levels of pregnenolone and allopregnanolone were negatively correlated with amygdala and insula activation levels. The amygdala is a key region in threat detection (50), fear conditioning (51), and emotional salience (52). The insula is

responsible for interoception (53), disgust (54), emotion processing (55), emotional recall (33), and anticipation of aversive stimuli (56). Amygdala and insula are structurally interconnected (57), and recent fMRI studies report enhanced coupling between insula and amygdala during negative emotion induction in healthy volunteers (58) and during symptom provocation in recently traumatized individuals (59). Both regions are associated with negative emotional response (55), and greater amygdala activation in response to the presentation of facial expressions is associated with greater magnitude of emotional response (47, 60-63).

Additionally, activation reductions in amygdala and insula are associated with down-regulation of negative emotions (64). Thus, allopregnanolone's reduction of activity in amygdala and insula suggests that allopregnanolone may reduce emotional reactivity to aversive stimuli.

Pregnenolone also increased activity in the dmPFC, a region linked to regulatory control over emotion. This finding was specific to the appraisal condition. We have previously demonstrated that shifting attention to become aware of and evaluate the intensity of one's emotional response to aversive stimuli leads to robust activation of dmPFC and rostral ACC (46, 47, 65, 66).

Behavioral studies of emotional appraisal and labeling report that this strategy lowers distress (67) and facilitates habituation (68). Thus, allopregnanolone's enhancement of dmPFC activity during appraisal suggests that allopregnanolone may facilitate the evaluation of one's own emotional response and aid in successful down-regulation of negative emotions. Interestingly, greater dmPFC activity during appraisal was associated with greater self-reported anxiety. As the dmPFC is central to conscious threat appraisal (69), greater dmPFC activity in individuals with higher self-reported anxiety could reflect greater levels of threat processing. Alternatively, higher activity in this region could reflect greater task engagement in certain individuals.

Finally, pregnenolone increased connectivity between amygdala and dmPFC during appraisal, with greater connectivity associated with reduced self-reported anxiety. Psychophysiological interaction (PPI) reflects the connectivity between one region and another during a particular context, controlling for the baseline relationship between regions. Thus, the current results suggest that allopregnanolone is associated with greater functional coupling between amygdala and dmPFC during appraisal specifically. However, PPI does not assess the impact of third-party regions on the two regions of interest, and does not reflect causal relationships. Thus, while these correlational findings do not necessarily provide evidence of an inhibitory or excitatory relationship, or indicate the sequence of amygdala activation and dmPFC activation, they may suggest potential neural mechanisms of emotional regulation given known structural connections and reciprocal feedback loops between amygdala and mPFC (70-72). Previous research indicates that emotion regulation depends on interactions between dmPFC and amygdala (73). At least one previous study has demonstrated that enhanced connectivity between dmPFC and amygdala is associated with successful emotion regulation and less negative affect (74). Of note, some evidence suggests that successful regulation is associated with an inverse relationship (anti-correlation) between dmPFC and amygdala (64, 75). However, in our sample, greater connectivity between amygdala and dmPFC was associated with less self-reported anxiety, suggesting allopregnanolone's modulatory effects on connectivity may aid dmPFC-mediated appraisal and/or reduce amygdala-mediated negative emotional responding. Our findings extend those of Van Wingen and colleagues (31) by demonstrating that allopregnanolone's selective enhancement of dmPFC to amygdala connectivity is associated with reduced anxiety. Since several anxiety disorders are characterized by a lack of neural regulatory control (76) and

impaired emotion regulation (35), future studies should examine allopregnanolone's potential as a neurosteroid target for pharmacologic intervention for these individuals.

Allopregnanolone likely impacts emotion regulation neurocircuitry through GABAergic mechanisms, though it may also impact this circuitry through its enhancement of neurogenesis (77) myelination (78) or neuroprotection (79-82). Amygdala and mPFC are rich in GABA(A) receptors (25) and endogenous allopregnanolone (44), suggesting that allopregnanolone could feasibly have a direct impact on activity in these regions. Indeed, in our sample, allopregnanolone serum level was more strongly correlated to amygdala activity than activity in any other brain region. Preclinical research suggests that the amygdala may be a particular target of allopregnanolone's anxiolytic effects (27). In rats, microinfusions of allopregnanolone directly into the amygdala produce anxiolytic (27) antidepressant (28) and anti-aggressive (29) effects. In humans, individuals with PTSD exhibit decreased frontal lobe benzodiazepine receptor binding (83, 84) and decreased plasma GABA levels (85), suggesting decreased GABAergic tone. In previous neuroimaging studies, greater endogenous allopregnanolone has been reported to be associated with lower amygdala reactivity (30, 38) and greater coupling between amygdala and dmPFC (31). Though we did not directly test the GABAergic effect of our intervention, our findings illuminate potential neural pathways through which pregnenolone administration and resulting increases in allopregnanolone levels could feasibly impact GABAergic transmission in a manner that is relevant to pathological anxiety.

There are several limitations to this study. There are several limitations to this study. Limitations of our intervention include the fact that we measured serum levels of allopregnanolone, and not

CSF or brain levels. However, in animals, neurosteroid levels appear to be highly correlated (86). Secondly, as steroid levels were only measured twice (once at baseline and once at the 3-hour endpoint), it is possible that steroids showing no change (including cortisol and DHEA) were in fact acutely changed but had returned to baseline by the endpoint of our experiment. Third, our event-related fMRI design is not well-suited to assess allopregnanolone's potential impact on overall brain perfusion (81) or neurovascular coupling. Future studies should employ PET or arterial spin labeling to examine these issues. Sample limitations include the fact that our sample size was modest, thus our results should be considered preliminary. In particular, our power to detect differences between groups was limited by our small sample size; therefore, our study requires replication. Additionally, our sample consisted of healthy male individuals without mood or anxiety disorder diagnoses. Thus, extrapolations to women or to clinical populations should be made with caution. Potential behavioral data limitations include the fact that pregnenolone administration did not reduce overall self-reported anxiety, thus it is possible that the observed correlations between anxiety and amygdala-dmPFC coupling might be related to normal variations in anxiety levels rather than a drug induced effect per se. However, it may not be anticipated that participants without baseline anxiety symptoms would necessarily report decreases in self-reported anxiety. Future investigations in participants meeting criteria for anxiety disorders at study entry may help to clarify this issue. Finally, our drug manipulation involved the administration of pregnenolone, not allopregnanolone. Since allopregnanolone is not currently commercially available for clinical use, it was necessary to administer pregnenolone as a precursor loading strategy to increase downstream allopregnanolone levels. As our results demonstrate, oral administration of pregnenolone increases allopregnanolone levels sevenfold. We have framed our results in terms of an allopregnanolone manipulation, but our

results may also be attributable to increases in pregnenolone. Pregnenolone levels are low in individuals with major depression (87) and anxiety disorders (88, 89), and are increased by fluoxetine administration in rats (21) and in humans (90). Therefore pregnenolone may also be relevant to anxiety symptomatology, and may influence relevant neurocircuits. Thus, future studies should attempt to disentangle the emotion regulatory effects of pregnenolone versus its metabolite allopregnanolone.

In conclusion, we demonstrate that pregnenolone administration (leading to increased downstream allopregnanolone levels) reduces activity in regions associated with the generation of negative emotion and enhances activity in regions linked to regulatory control over emotion, as well as increasing connectivity between two of these regions (dmPFC and amygdala). Considering the wealth of evidence that neurocircuits involving these regions are altered in anxiety disorders, our results invite further investigation into the brain basis for allopregnanolone's use as an anxiolytic pharmacological intervention.

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## **Chapter 4 : Altered Resting-State Amygdala Functional Connectivity in Men with Posttraumatic Stress Disorder**

### **Abstract**

Converging neuroimaging research suggests altered emotion neurocircuitry in posttraumatic stress disorder. Emotion activation studies in PTSD have shown hyperactivation in emotion related regions including amygdala and insula, and hypoactivation in emotion regulation regions including medial prefrontal cortex (mPFC) and anterior cingulate cortex (ACC). However, few studies have examined patterns of connectivity at rest in PTSD, a potentially powerful method for illuminating brain network structure. Using amygdala as a seed region, “resting state” brain connectivity was measured using 3T fMRI in returning male Afghanistan (OEF) or Iraq (OIF) veterans with PTSD (n=15) and without PTSD (n=14). Compared to combat controls, PTSD patients showed greater positive connectivity between amygdala and insula, reduced positive connectivity between amygdala and hippocampus, and reduced anti-correlation between amygdala and dorsal ACC and rostral ACC. These results demonstrate that studies of functional connectivity during resting-state can discern aberrant patterns of coupling within emotion circuits and suggest a possible brain basis for emotion processing/ regulation deficits in PTSD.

## **Introduction**

Posttraumatic stress disorder (PTSD) is a debilitating psychiatric disorder characterized by symptoms of reexperiencing, hyperarousal, emotional numbing and avoidance (1), however, exact brain mechanisms involved in the generation of PTSD symptoms or in PTSD pathophysiology have yet to be elucidated. Converging neuroimaging research points to a potentially critical role for disrupted emotion neurocircuitry in PTSD, and while many studies have delineated patterns of activations during face viewing or symptom provocation (for a review, see 2), relatively few have examined patterns of connectivity in the brain of PTSD patients at rest, a potentially powerful method for illuminating brain network structure (3, 4). The majority of PTSD neuroimaging studies to date have described abnormalities in emotion “generation” regions such as amygdala or insula, and emotional regulation regions including anterior cingulate cortex (ACC) and medial prefrontal cortex (mPFC). This is consistent with the known role of amygdala as a key region in threat detection (5), fear conditioning (6), and emotional salience (7), and of the mPFC as a modulatory region interconnected with limbic structures (8) and involved in emotional regulation (9). Taken together, fMRI studies of PTSD suggest patterns of hyperactivation of the amygdala and insula to emotion related stimuli and corresponding hypoactivation of ventromedial prefrontal and rostral anterior cingulate cortices (2). This pattern of amygdala hyperactivity and medial prefrontal cortex hypoactivity was recently confirmed by a meta-analysis of 15 PTSD neuroimaging studies (10) and is generally understood to reflect a lack of regulatory control over emotion in PTSD.

Studies of functional connectivity, however, can provide additional and potentially more direct information about regulatory relationships between mPFC and amygdala. The amygdala has tight

structural connections and reciprocal feedback loops with mPFC and orbitofrontal cortex (11) as well as with dorsolateral PFC (12) and ACC (13). As amygdala hyperactivity tends to coincide with mPFC hypoactivity in healthy individuals (9, 14), recent studies have begun to investigate task-related functional connectivity between these regions. Roy and colleagues (15) reported functional connectivity of amygdala with ventral medial prefrontal regions (including medial frontal gyrus and rostral ACC), insula, thalamus and striatum at rest, and anti-correlations with dorsal ACC, superior frontal gyrus, bilateral middle frontal gyrus and PCC, interpreted as dissociations between the emotion production network and the cognitive or affect regulation network. In PTSD, amygdala connectivity during task-based studies has yielded inconsistent findings. For instance, one [<sup>15</sup>O]-H<sub>2</sub>O positron emission (PET) study of recently traumatized individuals found positive functional connectivity between amygdala and ACC in response to trauma scripts (16). In contrast, a different PET study reported anti-correlation between amygdala and ACC during neutral (but not trauma) scripts, and reductions in the strength of this connectivity in PTSD (17). It is noteworthy, however, that regardless of the direction of the connectivity identified (positive connectivity versus anti-correlation), the majority of studies have found diminished strength of connectivity in PTSD than in comparison subjects (2, 16, 18, 19). These relationships might potentially be better assessed, however, through connectivity analyses at rest, without the confounds of tasks that may be biased to elicit amygdala activity or provoke PTSD symptoms.

PTSD fMRI studies have also demonstrated aberrant activity in the insula, an area responsible for interoception (20), disgust (21), emotion processing (22), emotional recall (9), and anticipation of aversive stimuli (19). Amygdala and insula are structurally interconnected (23),



and early PET studies reported increased insula activity in response to trauma script-driven imagery in PTSD (24), though in some studies no more than in combat-exposed control subjects (25). Recent fMRI studies have reported greater insula activation in anticipation of negative images (19) and negative emotional faces (18) in PTSD, and enhanced coupling between insula and amygdala during negative emotion induction in healthy volunteers (26) and during symptom provocation in recently traumatized individuals (16). Etkin and Wager's meta-analysis (10) also suggests co-activation of right amygdala and insula across studies, and collectively these studies offer evidence of strong anatomical and functional links between amygdala and insula during emotion processing.

Finally, the hippocampus, a medial temporal lobe region adjacent to the amygdala that is implicated in declarative memory (27), contextual memory (28) and fear conditioning (29), has been an important area of interest in PTSD research. Functional neuroimaging studies of the hippocampus in PTSD have yielded conflicting findings, with some showing hyperactivity and some revealing hypoactivity (2). A quantitative meta-analysis, however, suggested overall reduced hippocampal activity in PTSD (10). Additionally, it has been hypothesized that hippocampus integrates context into emotional memories and modulates amygdala activity according to context (30), a function that might be disrupted in PTSD.

Recent studies have begun to utilize resting-state connectivity in PTSD and have reported alterations in subcortical (31) and default network connectivity (32-35). Resting-state connectivity offers a powerful way to assess intrinsic connections between brain networks (3, 4, 36), which in turn have been linked to important functions such as processing speed (37) and

cognitive flexibility (38) in health and in disease. Resting-state amygdala connectivity may have particular relevance for the study of mood and anxiety disorders, as it has been reported as altered in GAD (39), social phobia (40), MDD (41, 42), and bipolar disorder (43), however, amygdala connectivity at rest in PTSD has not been studied. Given hypotheses that medial PFC exerts regulatory control over the amygdala, we hypothesized anti-correlations between mPFC and amygdala, and positive connectivity between amygdala and insula. Based on heightened emotion reactivity and diminished emotion control in PTSD, we hypothesized enhanced positive connectivity between amygdala and insula, and reduced anti-correlation between PFC and amygdala in PTSD patients. Finally, we have recently proposed that PTSD is associated with failure to contextualize emotional memory (30, 44), and given the hippocampal role in contextual processing (28), we hypothesized reduced positive connectivity between amygdala and hippocampus in PTSD patients as compared to combat controls.

## **Methods**

### *Participants*

We scanned 30 veterans returning from deployments to Afghanistan (OEF) and Iraq (OIF), with current PTSD diagnosis and seeking treatment for PTSD at the VA Ann Arbor (n = 15), or without PTSD (n = 15). Imaging data from one control participant was lost due to scanner malfunction, leaving a final sample of 15 PTSD patients and 14 Combat Controls. All participants in the PTSD group met DSM-IV criteria for current (past month) PTSD as assessed via the Clinician Administered PTSD Scale (CAPS; 45); all CAPS scores in the PTSD group were greater than or equal to 50 (mean  $\pm$  SD = 75.9  $\pm$ 17.2). No psychiatric diagnoses were allowed in the control group, and CAPS scores in the control group (mean  $\pm$  SD = 10.9  $\pm$ 7.7)

were significantly lower than in the PTSD group ( $t(26) = 12.9, p < .001$ ). Seven patients in the PTSD group met diagnostic criteria for depression and one had comorbid panic disorder, assessed by the Mini-International Neuropsychiatric Interview (M.I.N.I.; 46). There were no other current Axis I or Axis II disorders in the PTSD group. Two PTSD patients were using trazodone as a sleep aid; no other psychiatric medications were permitted. All subjects were right-handed males between 21 and 37 years old. Mean age  $\pm$  SD of the subjects included was  $27.3 \pm 4.5$  years for PTSD patients and  $26.6 \pm 3.3$  for Combat Controls ( $t(26) = .477, p = .637, n.s.$ ). Groups did not differ by race ( $\chi^2(5, N = 28) = 4.18, p = .52$ ), marital status ( $\chi^2(3, N = 28) = 6.27, p = .099$ ), or level of education ( $\chi^2(3, N = 26) = 5.39, p = .15$ ).

PTSD patients were notified at their initial VA visit of the opportunity to participate in research, and all interested eligible participants were included in the study. Control participants were recruited from the community via advertisement. After a complete description of the study was provided to the participants, written informed consent was obtained. Participants in both groups were exposed to the same conditions. All procedures were carried out between August 2008 and July 2010. The study was approved by the Institutional Review Boards of the University of Michigan Medical School and the Ann Arbor Veterans Affairs Healthcare System. All procedures were carried out in accordance with the Declaration of Helsinki as adopted and promulgated by the National Institutes of Health.

### Resting State Paradigm

Participants underwent structural (sMRI) and functional (fMRI) scanning that included both emotion regulation and conditioning tasks (reports forthcoming) and resting state procedures.

Resting-state scans always occurred prior to tasks. Subjects were positioned in the MR scanner and their heads comfortably restrained to reduce head movement. Participants lay supine in the fMRI scanner and wore glasses with built-in mirrors (NordicNeuro Labs) in order to view the projected stimuli inside the scanner. A black fixation cross on a white background was displayed in the center of the screen for 10 minutes (300 volumes). Participants were instructed to relax and keep their eyes open and fixed on the cross. A pulse oximeter was attached to the participant's finger in order to obtain their cardiac activity. In addition, a pressure belt was worn around the participant's abdomen in order to obtain their respiratory activity.

### Image Acquisition

Scans were collected on a 3.0 Tesla General Electric Signa® Excite™ scanner (Milwaukee, WI). After subjects were positioned in the scanner, a T1-weighted low resolution structural image was prescribed approximately parallel to the AC-PC line [gradient recall echo sequence (GRE), repetition time (TR) = 250 ms, echo time (TE) = 5.7 ms, flip angle (FA) = 90°, 2 averages, field of view (FOV) = 22 cm, matrix = 256 × 256, slice thickness = 3 mm, 40 axial slices to cover the whole brain], which was similar to the prescription of the functional acquisitions. Functional images were acquired with a T2\*-weighted, reverse spiral acquisition sequence (gradient recall echo, TR = 2000 ms, TE = 30 ms, FA = 90°, FOV = 22 cm, matrix = 64 × 64, slice thickness = 3 mm with no gap, 40 axial slices to cover the whole brain) which has been shown (47) to minimize signal drop-out in regions such as ventral striatum and orbitofrontal cortex that are vulnerable to susceptibility artifact. The intermediate template and fMRI images were acquired using a GE Quadrature sending and receiving head coil. The four initial volumes were discarded from each run to allow for equilibration of the scanner signal. A high-quality T1-weighted

structural image (sMRI) was obtained using a 3-D Volume Inversion Recovery Fast Spoiled Gradient Recall Echo (IR-FSPGR) protocol (TR = 12.3 ms, TE = 5.2 ms, FA = 9°, TI = 650 ms, FOV = 26 cm, matrix = 256 × 256 for in-plane resolution of 1 mm; slice thickness = 1 mm with no gap, 160 contiguous axial slices to cover the whole brain). The sMRI images were acquired with an eight-channel GE Phase Array receiving head coil.

### *Preprocessing*

An initial series of pre-processing steps was carried out. First, we removed k-space outliers in raw data that were two standard deviations away from the mean and substituted them with the average value from neighboring voxels. Next, a  $B_0$  field map was used in the reconstruction of the images to remove the distortions that resulted from magnetic field inhomogeneity [IEEE-TIME, 10:629-637, 1991]. The variance due to physiological responses (i.e., cardiac and respiratory sources) was removed using regression (48). The data were then slice-time corrected using local sinc interpolation (49) and realigned using MCFLIRT in FSL (50). Additional pre-processing and image analysis were performed in SPM5 (Wellcome Department of Cognitive Neurology, London, UK; <http://www.fil.ion.ucl.ac.uk>). First we co-registered the high-resolution T1 images to the functional images. Second, T1 images were normalized to the scalped T1 template and the functional volumes were normalized to the Montreal Neurological Institute (MNI) template using a similar transformation matrix. Third, images were smoothed using an isotropic 5 mm full-width-half maximum (FWHM) Gaussian kernel.

### Data Analysis

Resting state functional connectivity measures low-frequency spontaneous BOLD oscillations (0.01 – 0.10 Hz band) (36), thus, the time-course for each voxel was band-passed filtered in this range. Amygdala seed region ROIs were constructed from cytoarchitectonically determined probabilistic maps of the human basolateral and centromedial amygdala, adapted from Etkin and colleagues (39). These subregions were combined to form a single, whole amygdala seed. We extracted the spatially averaged time series from right and left amygdala ROIs for each participant. A global-signal regressor was added to the single-group model to remove non-specific global sources of noise associated with BOLD fMRI scanning, consistent with a number of recent resting state studies that also used global-signal regression (15, 36). Note that global-signal regression raises certain methodological issues, which are discussed in the Discussion section. Pearson product-moment correlation coefficients were calculated between average time courses in the amygdala “seed” ROIs and all other voxels of the brain resulting in a 3-dimensional correlation coefficient image (r-image). Both positive correlations and anti-correlations were computed. These r-images were then transformed to z-scores using a Fisher r-to-z transformation.

### Whole Brain and Region of Interest Analysis

Z-score images from the individual functional connectivity analyses were entered into second-level random-effects analyses (one-sample and two-sample t-tests) implemented in SPM5. Second-level maps were thresholded at  $p < 0.005$ , uncorrected, extent threshold  $k=20$ . In addition, region of interest analysis with small volume correction was conducted. A priori regions of interest including the hippocampus, anterior cingulate cortex, and insula were used as

masks, as these regions are of interest in PTSD (2, 10). Images were thresholded using a voxelwise threshold of  $p < 0.005$  uncorrected with a minimum cluster size of four connected voxels (for hippocampus clusters), and six connected voxels (for ACC or insula clusters). These combinations of activation threshold and cluster size were determined using AlphaSim (51) to correspond to a false positive rate of  $p < 0.05$ , corrected for multiple comparisons within regions of interest.

Using the thresholds and cluster sizes defined above, the corrected voxel-wise probabilities are as follows: hippocampus  $p < 0.00097$ , ACC  $p < 0.0003$ , and insular cortex  $p < 0.00045$ . Only the activations within the regions of interest that survived the volume and voxel correction criteria were extracted and used for further analysis. Connectivity foci were labeled by comparison with the neuroanatomical atlas by Talairach and Tournoux (52). Reported voxel coordinates correspond to standardized Montreal Neurologic Institute (MNI) space. To assess for correlations with symptom severity, CAPS scores were added as regressors in a separate whole brain analysis of connectivity between amygdala and ROIs.

## **Results**

### **Combat Controls**

#### *Right Amygdala*

The right amygdala seed showed positive connectivity with a number of regions, including left amygdala, bilateral peri-amygdala and bilateral hippocampus, and anti-correlation with dorsal

medial PFC (dmPFC), dorsal ACC (dACC), precuneus, lateral PFC and inferior parietal cortex (see Figure 4.1).

#### Left Amygdala

The left amygdala seed showed positive connectivity with right amygdala, peri-amygdala, bilateral hippocampus and middle temporal gyrus, and anti-correlation with dACC, rostral ACC (rACC), lateral PFC, inferior parietal cortex and precuneus (see Figure 4.1).

### **PTSD**

#### Right Amygdala

The right amygdala seed showed positive connectivity with a number of regions, including left amygdala, peri-amygdala, bilateral hippocampus and bilateral insula, and anti-correlation with dmPFC, rACC and inferior parietal cortex (see Figure 4.1).

#### Left Amygdala

The left amygdala seed showed positive connectivity with right amygdala, peri-amygdala, bilateral hippocampus and bilateral insula, and anti-correlation with dACC, lateral PFC, inferior parietal cortex and precuneus (see Figure 4.1).



## **PTSD versus Combat Controls**

### *Right Amygdala*

In direct comparison to healthy combat controls, PTSD patients showed greater positive connectivity between the right amygdala seed and right insula/STG ([48, -39, 21];  $k = 26$ ;  $Z = 3.38$ ;  $p < .005$ ), and reduced anti-correlation between right amygdala seed and dorsal ACC ([-12, 24, 30];  $k = 24$ ;  $Z = 3.32$ ;  $p < .005$ ; see Table 4.1, Figure 4.1). Compared to PTSD patients, combat controls showed greater positive connectivity between right amygdala seed and left hippocampus ([-30, -21, -9];  $k = 20$ ;  $Z = 3.48$ ;  $p < .005$ ), and left inferior orbital frontal gyrus ([-30, 36, -9];  $k = 22$ ;  $Z = 3.45$ ;  $p < .005$ ; see Table 4.1, Figure 4.1). No other group differences were observed.

### *Left Amygdala*

Compared to healthy combat controls, PTSD patients showed greater positive connectivity between the left amygdala seed and right insula ([54, 0, -3];  $k = 28$ ;  $Z = 3.56$ ;  $p < .005$ ) and reduced anti-correlation between right amygdala seed and rostral ACC ([6, 36, 12];  $k = 20$ ;  $Z = 3.64$ ,  $p < .005$ ; see Table 4.1, Figure 4.1). Combat controls did not show greater connectivity than PTSD patients in any significant clusters ( $k > 20$ ).

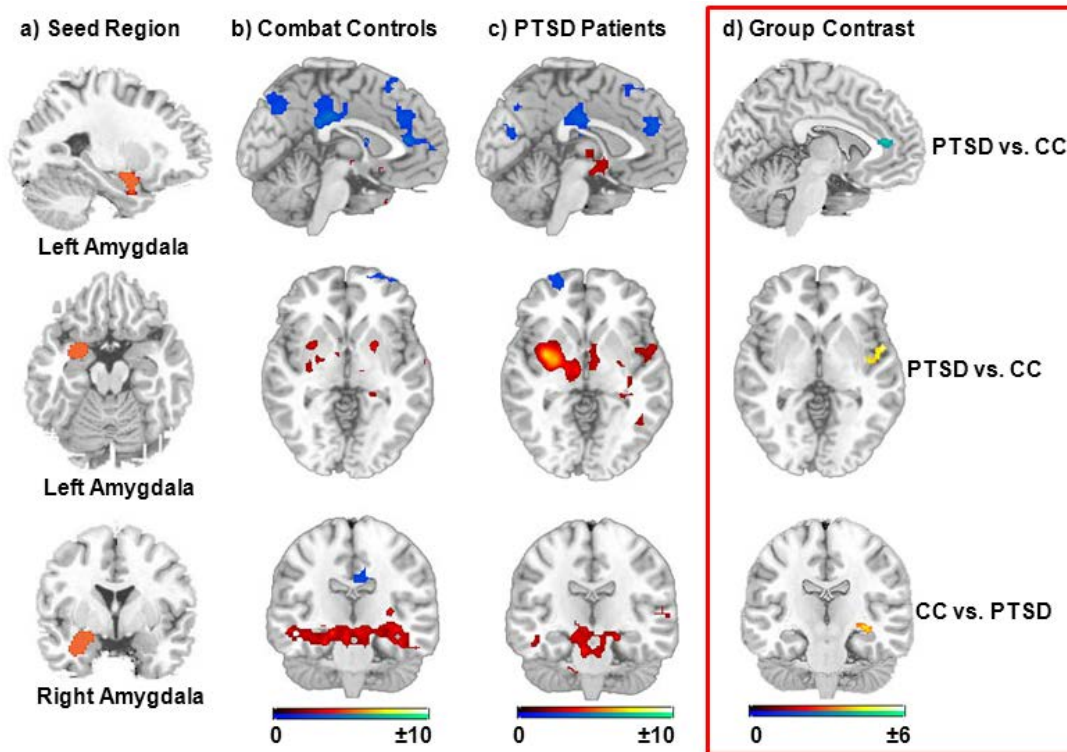
### *Correlations with Symptom Severity*

Within the PTSD group, symptom severity as measured by the CAPS was not significantly associated with connectivity between amygdala and ROIs.

**Table 4.1 Activation results from two-group comparison**

| Contrast Map and Brain Region        | Cluster Size | MNI Coordinates (x,y,z) | Analysis (z) |
|--------------------------------------|--------------|-------------------------|--------------|
| Right Amygdala PTSD>CC               |              |                         |              |
| Right Insula/Superior Temporal Gyrus | 26           | 48, -39, 21             | 3.38         |
| Dorsal Anterior Cingulate Cortex†    | 24           | -12, 24, 30             | 3.32         |
| Right Amygdala CC>PTSD               |              |                         |              |
| Left Hippocampus                     | 20           | -30, -21, -9            | 3.48         |
| Left Inferior Orbital Frontal Gyrus  | 22           | -30, 36, -9             | 3.45         |
| Left Amygdala PTSD>CC                |              |                         |              |
| Rostral Anterior Cingulate Cortex†   | 20           | 6, 36, 12               | 3.64         |
| Right Insula                         | 28           | 54, 0, -3               | 3.56         |
| Left Amygdala CC>PTSD                |              |                         |              |
| No clusters greater than 20 voxels   |              |                         |              |

†Indicates reduced anti-correlation in the PTSD group.



**Figure 4.1 Functional connectivity analysis.** PTSD patients (c) compared to combat controls (b) showed reduced anti-correlation between the left amygdala (=seed region shown in a) and rostral anterior cingulate cortex (d; top), increased positive connectivity between left amygdala and right insula (d; middle), and reduced positive connectivity between right amygdala and left hippocampus (d; bottom). Slices displayed at MNI-coordinates x = 3 (top row), z = 1 (middle row) and y = -18 (bottom row). Color bar depicts t-score. Activations are corrected for multiple comparisons within regions of interest.

## Discussion

In this study, we investigated patterns of resting state functional connectivity of the amygdala in whole brain analyses, comparing military combat veterans deployed to Iraq or Afghanistan (OEF/OIF) with PTSD versus OEF/OIF combat-exposed healthy veterans. To our knowledge, this is the first examination of resting-state amygdala connectivity in PTSD, and we found greater positive connectivity between the amygdala and insula, reduced positive connectivity between the amygdala and hippocampus, and reduced anti-correlation between the amygdala and dorsal and rostral ACC in PTSD subjects. These findings suggest abnormalities in emotion generation and regulation circuits that may contribute to the pathophysiology of PTSD, and demonstrate that studies of functional connectivity of the amygdala may be used to discern aberrant patterns of coupling within these circuits.

Consistent with previous resting state functional connectivity studies (14, 15) and effective connectivity studies (17, 53), we found anti-correlations between amygdala and medial prefrontal regions including dACC and rACC, consistent with our a priori hypothesis. While resting state functional connectivity analyses examine correlations (i.e. between activity in the seed ROI and other brain regions), and thus do not allow for inferences about causal relationships, other lines of evidence support an inhibitory role of dmPFC in relationship to amygdala activity. Animal studies of fear conditioning establish mPFC involvement in the extinction of conditioned fear (54), and in humans, a recent Granger causality analysis of a face-processing task indicated an inhibitory influence from the rACC to the amygdala (55), supporting the idea that rACC might be suppressing amygdala activity. Resting-state amygdala connectivity studies also reveal anti-correlations between dmPFC and amygdala in low-anxious

individuals and interestingly, a lack of coupling in high-anxious individuals (14). Similarly, Gilboa and colleagues reported that during neutral emotion conditions, the ACC exerts an inhibitory influence on the amygdala, and this relationship is diminished in PTSD (17). These studies provide accumulating albeit indirect support for interpreting anti-correlations between medial PFC and amygdala as an inhibitory relationship. If indeed ACC and mPFC play an important inhibitory role in modulating amygdala signal, our findings of a weaker anti-correlation between amygdala and ACC might reflect diminished capacity of medial prefrontal regions in PTSD to suppress amygdala activity. This is indeed consistent with a theory of diminished “top-down” regulation of amygdala by emotional regulatory circuits (10). Such a lack of inhibition from dorsal medial regions to the amygdala can be interpreted as a deficit in automatic emotion regulation (10) or a lack of cognitive control over emotion (53). It is noteworthy that individuals with PTSD demonstrate both of these deficits in clinical settings (56). Moreover, training PTSD patients in emotion regulation strategies has been shown to reduce negative emotional responses and normalize PFC responses to aversive stimuli (57).

We also found increased functional connectivity between bilateral amygdala and right insula. Similar findings of enhanced connectivity between insula and amygdala were previously reported in recently traumatized individuals during symptom provocation (16). In PTSD, insula activation has been associated with trauma recall (21) and viewing fearful faces (18), and co-occurring increases in insula and amygdala activity were found to be greater in PTSD than combat-exposed or healthy controls during trauma reminders (58) and fear acquisition (59). The amygdala and insula have strong structural connections (60), and exhibit high functional connectivity at rest (61). Hyperconnectivity of amygdala and anterior insula could indicate

stronger anticipation of negative events, as seen in the PTSD symptom of hyperarousal, and hyperconnectivity between posterior insula and amygdala could indicate a tighter functional link between visceral perception and emotional response, as seen in re-experiencing symptoms. Indeed, insula responsivity to negative images in PTSD has previously shown to be positively correlated with hyperarousal symptoms (19), re-experiencing symptoms (62) and flashback intensity (63). Given these findings, our finding of increased amygdala-insula functional connectivity at rest might suggest a role for maladaptive coupling of emotion and visceral sensation in PTSD symptoms.

We have also observed reduced positive connectivity between amygdala and left hippocampus in PTSD. This is in contrast to several recent studies showing increased amygdala hippocampus correlations during symptom provocation or induction of negative mood states. For example, PTSD patients show exaggerated activity in both amygdala and hippocampus while recollecting negative autobiographical memories (64) and while viewing negative pictures (65), and the degree of this hyperactivity correlated with symptom severity (65). On the other hand, studies using neutral tasks more often report decreased hippocampal activity in PTSD (66). One study found that amygdala hyperactivity during fear extinction and hippocampal hypoactivity during extinction recall was associated with a behavioral deficit in extinction recall in PTSD (67). Thus, existing findings suggest divergent patterns of amygdala-hippocampus connectivity in fear or safety contexts. A failure to properly contextualize threat and safety signals, or integrate corrective information into fear schemas, may be relevant to the development of PTSD symptomatology. Weakened connection strength between amygdala and hippocampus during times of safety, as suggested by the current study, could contribute to these deficits.

To our knowledge, there have been four previous studies of resting-state connectivity in PTSD, all using a thalamus or PCC/precuneus seed for connectivity analysis. Bluhm, Lanius and colleagues (32) reported reduced functional connectivity between PCC/precuneus and amygdala in PTSD; however, in a more recent study with acutely traumatized individuals, they found PCC/precuneus to amygdala connectivity to be positively correlated with PTSD symptoms (33). In our study, the amygdala was less anti-correlated with PCC/precuneus in PTSD than controls, though the extent of this activation was small (less than 10 voxels). A recent connectivity modeling analysis found a strong functional link between the PCC and amygdala in healthy individuals (15). Disruptions in this connection may point to default network disturbances in PTSD – a hypothesis that merits further research.

The patterns of amygdala connectivity at rest found in this study in PTSD patients are consistent with the idea that this disorder involves a functional dissociation between dorsal regions (including PCC, dACC, dmPFC and dlPFC) involved in effortful emotion regulation and ventral regions (amygdala, subgenual ACC, and insula) involved in emotion experience (68). Our findings of enhanced connectivity in emotion experience regions (amygdala and insula) and reduced connectivity between these regions and emotion regulation regions suggest plausible mechanisms involved in exaggerated emotional responses and apparent regulation dysfunction seen in PTSD.

## **Limitations**

Our study has several limitations. First, we tested male veterans with combat exposure, and thus generalizations to women or to non-combat related PTSD cannot be made. Second, our PTSD sample included seven participants with comorbid major depression. Though depression commonly co-occurs with PTSD in veteran populations (up to 80% by some estimations; 69), our inclusion of depressed participants may render some of our effects attributable to the presence of depression independently or in interaction with PTSD. Of note, our results were not affected by the removal of participants with current comorbid depression. Therefore, we retained depressed participants in our final analysis. Third, we have interpreted our findings under the assumption that both groups responded similarly to the scanning environment. It is possible, however, that PTSD patients experienced higher levels of anxiety during the scan, potentially contributing to differential patterns of connectivity. Fourth, our methods are essentially correlational and thus do not allow for inferences about causal relationships. Future studies should use methods designed to probe effective connectivity (e.g., dynamic causal modeling, path analytic methods such as mediation analysis) to further clarify causal interrelationships between amygdala and emotion production and regulation regions. Finally, we used global regression (GR) to remove global sources of noise and interpreted negative correlations to represent anti-correlated brain regions. GR averages whole brain activity at every time point and factors out this value from the time series of every voxel. GR is particularly helpful to interpret within group effects – if global signal is not removed, every region in the brain appears to be massively correlated with every other region (70). Thus, GR could be considered a strength of our study insofar as it allows analysis of within group effects as well as between group effects. However, our use of GR is a limitation in that there is continued controversy whether GR

introduces artifactual anti-correlation(70). Recently, however, it had been convincingly argued that GR does not introduce large-scale artifacts and globally regressed connectivity maps more accurately depict the known anti-correlations between functional networks in the brain (71, 72). In sum, recent resting-state functional connectivity studies of the amygdala have used GR and identified networks proposed to be anti-correlated with the amygdala (e.g. Roy and colleagues; 15), thus we have adopted an approach that is consistent with existing practices in the literature.

## **Conclusion**

In conclusion, enhanced amygdala coupling with emotion production regions including insula, and reduced amygdala coupling with emotion regulation and contextualization regions including hippocampus and dorsal/rostral ACC was observed in PTSD patients. These findings suggest abnormalities in emotion generation and regulation circuits may contribute to PTSD pathophysiology, and demonstrate that studies of functional connectivity of the amygdala during resting-state may be used to discern aberrant patterns of coupling within these circuits.



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## **Chapter 5 : The Neurosteroids Allopregnanolone and DHEA Modulate Resting-State Amygdala Connectivity**

### **Abstract**

The neuroactive steroids allopregnanolone and dehydroepiandrosterone (DHEA) are integral components of the stress response and exert positive modulatory effects on emotion in both human and animal studies. Though these antidepressant and anxiolytic effects have been well established, little research to date has examined their neural correlates, and no research has been conducted into the effects of neurosteroids on large-scale networks at rest. To investigate the neurosteroid impact on intrinsic connectivity networks, participants were administered 400mg of pregnenolone (N=16), 400mg of DHEA (N=14), or placebo (N=15) and underwent 3T fMRI. Resting-state brain connectivity was measured using amygdala as a seed region. Compared to placebo, both neurosteroids reduced connectivity between amygdala and precuneus, and pregnenolone reduced connectivity with contralateral amygdala and peri-amygdaloid regions. Reductions in amygdala connectivity with precuneus were associated with less negative affect. These results demonstrate that neurosteroids modulate amygdala functional connectivity during resting-state, and may be a target for pharmacological intervention. Additionally, allopregnanolone and DHEA may shift the balance between salience network and default network, a finding that could provide insight into the neurocircuitry of anxiety psychopathology.

## Introduction

Allopregnanolone and dehydroepiandrosterone (DHEA) are endogenously-produced neurosteroids with neuroprotective, anxiolytic, antidepressant, antioxidant, and antiglucocorticoid effects (1-3). Deficiencies in allopregnanolone and DHEA are related to anxiety and depressive-like behavior. For instance, endogenous levels of allopregnanolone and DHEA are decreased in animal models of depression, and these animals exhibit behavioral improvements after DHEA administration or allopregnanolone induction (4, 5). DHEA and allopregnanolone administration reduce immobility in the forced swim test (6, 7), and reduce stress and anxiety-like behavior in rodents (8, 9). In human studies, allopregnanolone and DHEA dysregulation is associated with anxiety and depression. Individuals with major depressive disorder exhibit reduced plasma and cerebrospinal fluid levels of allopregnanolone (10, 11) and DHEA (12-14), and those with posttraumatic stress disorder (PTSD) show reduced cerebrospinal fluid allopregnanolone (15). Furthermore, increases in serum allopregnanolone and DHEA over a course of treatment predict symptomatic improvement (10, 16-18). Therefore, these neurosteroids show promise as targets for antidepressant and anxiolytic effects in psychiatric disorders.

Converging preclinical and neuroimaging research suggests that the amygdala, a key region in threat detection (19), fear conditioning (20), and emotional salience (21), may be a particular locus of allopregnanolone and DHEA's effects. Our laboratory has recently demonstrated that single-dose DHEA (22) and the allopregnanolone precursor pregnenolone (23) decrease amygdala activity and increase activity in medial prefrontal regions during tasks engaging

cognition-emotion interactions. Recent reports suggest that progesterone administration (which in turn increases allopregnanolone levels) modulates amygdala responsivity to emotional faces (24), and increases functional connectivity between amygdala and dorsal medial prefrontal cortex (dmPFC) (25). Allopregnanolone reduces the magnitude of GABA(A) receptor-mediated inhibitory postsynaptic currents in the central nucleus of the amygdala (26), and microinfusions of allopregnanolone directly into the amygdala produce anxiolytic (27) and antidepressant (28) effects. The anxiolytic and antidepressant effects of DHEA may be mediated by the amygdala as well. In animal models, DHEA administration increases BDNF concentration (29) and 5-HT(2A) receptor expression (30) in the amygdala, and DHEA infusions into the amygdala modulate stress responsivity (31). Thus, amygdala might be a key region in the anxiolytic and emotion modulatory effects of neurosteroids.

Though mounting evidence suggests that neurosteroids modulate amygdala activity, the reported direction of amygdala modulation is somewhat inconsistent. Activation of amygdala has been variously reported to be increased (25), or decreased (23, 24), depending on the task employed. To fully understand the impact of neurosteroids on amygdala function, it may be necessary to examine their effect on amygdala connectivity at rest. Resting-state connectivity offers a powerful way to assess intrinsic connections between brain networks (32-34) without external demands or confounds imposed by specific tasks. The amygdala resting-state network encompasses regions associated with emotion generation, including ventral medial prefrontal cortex, insula, thalamus and striatum (35-37). Thus, resting-state analyses may provide a fuller understanding of neurosteroid modulation of the amygdala and its associated emotion production network. Due to their anxiolytic and antidepressant properties, we predicted that



allopregnanolone and DHEA would reduce coupling within the amygdala functional connectivity network during rest, and that this effect would be associated with reduced self-reported negative affect.

## **Methods**

### Participants

Study participants were 45 right-handed healthy male volunteers aged 18-34 years (mean  $\pm$  SD=22  $\pm$  3.6) recruited from the community via advertisement. Exclusion criteria were a history of head injury, recent steroid use, or current or past psychiatric disorder, as assessed via the Mini-International Neuropsychiatric Interview (M.I.N.I.; 38). Participants were given full details of the study and provided written informed consent. The study was approved by the Institutional Review Board of the University of Michigan Medical School.

### Drug Administration

Study drugs (pregnenolone and DHEA) and matching placebo identical in appearance were obtained from Bell Pharmacy (Lakewood, CO), which provided certificates of analysis. Participants were randomly assigned to receive a single oral dose of 400 mg pregnenolone, 400 mg DHEA, or placebo. Participants and investigators were blind to condition. Pregnenolone was administered as a precursor loading strategy to significantly increase downstream allopregnanolone levels. Allopregnanolone serum levels reach three times baseline levels two hours after oral administration of 400mg pregnenolone (39), and DHEA serum concentrations peak 60 to 480 minutes after DHEA administration (40). Thus, drug administration occurred two

hours before neuroimaging to ensure elevated levels during the scan. At one hour post drug administration, participants completed the Positive and Negative Affect Scale (PANAS-X) and the Drug Effects Questionnaire.

### *Steroid measurements*

We used circulating levels of DHEA and allopregnanolone as indicators of central neurosteroid levels. In animal models, serum allopregnanolone levels are closely related to levels in the hippocampus (41), and serum DHEA levels are closely related to levels in cerebrospinal fluid (42). Serum samples for assay were collected once prior to drug administration and once after the scanning session. Serum DHEA levels were determined via enzyme immunoassay (ALPCO Diagnostics, Salem, NH), and serum DHEAS levels were determined by chemiluminescent enzyme immunoassay (IMMULITE) according to the manufacturer's directions (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). Pregnenolone and allopregnanolone levels in serum were determined by a highly sensitive and specific gas chromatography-mass spectrometry method in the negative ion chemical ionization mode. One ml of serum was extracted three times in ethyl acetate before high performance liquid chromatography (HPLC) purification using tetrahydrofuran, ethanol, and hexane in the mobile phase. All samples were injected in duplicate. Mean intra-assay coefficients of variation for pregnenolone and allopregnanolone were 0.9% and 2.9%, respectively. The limit of detection with this method was 1 pg for both pregnenolone and allopregnanolone. All neurosteroid values were natural log transformed prior to analyses.

### Resting State Paradigm

Participants underwent structural (sMRI) and functional (fMRI) scanning that included both an emotion regulation task (22, 23) and resting state procedures. Resting-state scans always occurred prior to tasks. Participants were positioned in the MR scanner and their heads comfortably restrained to reduce head movement. Heart rate and respiration measurements were acquired for group comparisons (via Independent-Samples Kruskal-Wallis tests). A black fixation cross on a white background was displayed in the center of the screen for 8 minutes. Participants were instructed to relax and keep their eyes open and fixed on the cross.

### Image Acquisition

MRI scanning occurred on a Philips 3.0 Tesla Achieva X-series MRI (Philips Medical Systems). After a T1 image (T1-overlay) was obtained, a T2\*-weighted, echoplanar acquisition sequence [GRE; repetition time, 2000 ms; echo time, 25 ms; flip angle, 90°; field of view (FOV), 22 cm; 42 slice; thickness/skip, 3.0/0 mm matrix size equivalent to 64 x 64] was collected. After discarding three initial volumes to permit thermal equilibration of the MRI signal, 185 volumes were acquired per run. After acquiring the functional volumes, a high-resolution T1 scan was obtained for anatomic normalization [26 FOV; thickness/skip, 1.0/0 mm]. Participants viewed stimuli through MR-compatible liquid crystal display goggles (NordicNeuroLabs <http://www.nordicneurolab.com>).

### Preprocessing

A standard series of processing steps was performed using statistical parametric mapping (SPM8; [www.fil.ion.ucl.ac.uk/spm](http://www.fil.ion.ucl.ac.uk/spm)). Scans were reconstructed, motion-corrected, slice-time corrected, realigned to the first scan in the experiment to correct for head motion, and co-registered with the high-resolution sagittal images. Normalization was performed using the voxel-based morphometry toolbox implemented in SPM8 ([www.fil.ion.ucl.ac.uk/spm](http://www.fil.ion.ucl.ac.uk/spm)). Scans were normalized to standard space, segmented into tissue types, bias-corrected, and iteratively realigned using DARTEL. Smoothing was performed with an  $8 \times 8 \times 8 \text{ mm}^3$  kernel. Motion parameters (mean displacement, mean angle) were compared across drug conditions via Independent-Samples Kruskal-Wallis tests, and runs with any movement greater than 3 mm were excluded.

### Data Analysis

Right and left amygdala seed region ROIs were constructed from cytoarchitectonically determined probabilistic maps of the human basolateral and centromedial amygdala. These subregions were combined to form a single, whole amygdala seed. We extracted the spatially averaged time series from right and left amygdala ROIs for each participant. Next, regression was performed to remove the effects of nuisance variables unrelated to neuronal activity. Covariates of no interest included six motion regressors generated from the realignment step noted above. In addition, we included five principal components of the BOLD time series extracted from white matter and cerebrospinal fluid masks, which has been demonstrated to effectively remove signals derived from the cardiac and respiratory cycle (43). This method is comparable to the COMPCOR method and is complementary to the RETROICOR method (44).

In generating the white matter and cerebrospinal fluid regressors, subject-specific masks were first created using VBM-based segmentation implemented in SPM8. Masks were eroded using FSL-Erode to eliminate border regions of potentially ambiguous tissue-type. A spatially averaged time series was extracted from these masks, and the first five principal components were included in the regression. The residuals from this regression were then retained for further analysis. We did not perform global-signal regression, as it had been suggested that this may produce spurious anti-correlation with orthogonal networks that increase in proportion to the size of the those networks (45). Since resting-state functional connectivity measures low-frequency spontaneous BOLD oscillations (.01 – .10 Hz band) (32), the time-course for each voxel was band-passed filtered in this range. Pearson product-moment correlation coefficients were calculated between average time courses in the seed regions of interest (ROIs) and all other voxels of the brain resulting in a 3-dimensional correlation coefficient image (r-image). These r-images were then transformed to z-scores using a Fisher r-to-z transformation.

#### Whole Brain and Region of Interest Analysis

Z-score images from the individual functional connectivity analyses were entered into second-level random-effects analyses (one-sample and two-sample t-tests) implemented in SPM8. Second-level maps were thresholded at  $p < .005$ , extent threshold  $k=20$ , corrected for multiple comparisons within regions of interest via family-wise error correction. ROIs were selected based on regions that have been shown to exhibit strong positive coupling (amygdala and insula) and negative coupling (precuneus) with the amygdala during rest (35). Only the clusters within the regions of interest that survived the volume and voxel correction criteria were extracted and used for further analysis. However, for future hypothesis generation and potential metaanalyses,

we list all activations of  $p < .005$ , extent threshold  $k = 20$  in Table 1. Reported voxel coordinates correspond to standardized Montreal Neurologic Institute (MNI) space. To assess for correlations with mood, PANAS-X scores were entered as regressors in between-subject analyses.

## Results

### Participants

Sixteen participants were administered pregnenolone, 14 were administered DHEA, and 15 were administered placebo. Groups did not differ by age ( $F(2,44) = .71$ ,  $p = .5$ ). Pregnenolone administration significantly increased serum allopregnanolone levels, and DHEA increased serum DHEA and DHEAS levels [ $p < .001$ ; details can be found in (22, 23)]. There were no significant differences in subjective drug effects ( $p > .4$ ). Participants' guesses of which drug they received did not deviate from chance ( $\chi^2(6) = 3.80$ ,  $p = .7$ ).

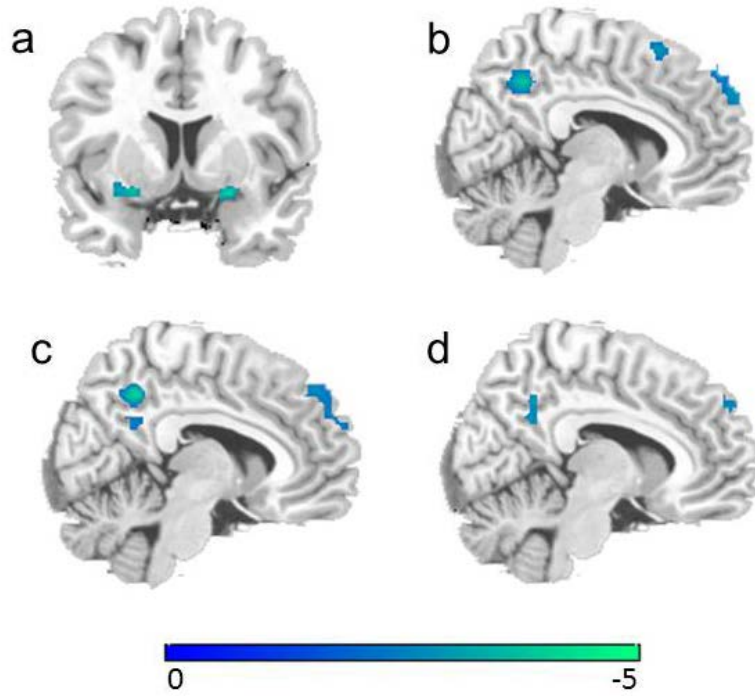
### Motion

There were no movements greater than 3 millimeters and no motion differences between DHEA and placebo groups ( $p > .2$ ). In the allopregnanolone group, mean displacement was reduced (mean = .066 mm) as compared to the placebo group (mean = .099 mm,  $p = .006$ ). Implications for interpretation of results are detailed in the Discussion section. There were no group differences in heart rate or respiration ( $p > .7$ ).

## fMRI Findings

Compared to placebo, pregnenolone reduced connectivity between the right amygdala seed and left amygdala ( $[-21,5,-17]$ ;  $k=43$ ;  $Z=3.57$ ;  $p=.026$ , FWE-corrected), between right amygdala and right peri-amygdala ( $[24,5,-14]$ ;  $k=27$ ;  $Z=3.45$ ;  $p=.024$ , FWE-corrected), and between right amygdala and left precuneus ( $[-3,-55,40]$ ;  $k=50$ ;  $Z=3.58$ ;  $p=.05$ , FWE-corrected, see Figure 5.1). Pregnenolone also reduced connectivity between the left amygdala seed and left precuneus ( $[-6,-52,40]$ ;  $k=54$ ;  $Z=3.74$ ;  $p=.034$ , FWE-corrected, see Figure 5.1). Whole-brain results can be found in Table 5.1.

Compared to placebo, DHEA reduced connectivity between the left amygdala seed and precuneus, albeit at a lower threshold ( $[6,-67,28]$ ;  $k=20$ ;  $Z=3.21$ ;  $p<.001$ , see Figure 5.1). There were no differences between groups in connectivity between amygdala and insula. Across all three groups (pregnenolone, DHEA, and placebo), there was a positive correlation between PANAS-X negative affect score and amygdala-precuneus coupling, from both right amygdala to precuneus ( $[3,-58,19]$ ;  $k=31$ ;  $r=.47$ ,  $Z=3.25$ ;  $p<.001$ ) and left amygdala to precuneus ( $[6,-55,16]$ ;  $k=113$ ;  $r=.51$ ,  $Z=3.64$ ;  $p=.046$ , FWE-corrected).



**Figure 5.1 Functional connectivity analysis. (a) Compared to placebo, allopregnanolone reduced functional connectivity between right amygdala and left amygdala and between right amygdala and right peri-amygdala ( $y=7$ ). (b) Allopregnanolone reduced functional connectivity between right amygdala and precuneus ( $x=-8$ ), and (c) between left amygdala and precuneus ( $x=-8$ ). (d) DHEA reduced functional connectivity between left amygdala and precuneus ( $x=-9$ ). Activations are corrected for multiple comparisons within regions of interest.**



**Table 5.1 Activation results from two-group comparison**

| Contrast Map and Brain Region            | Cluster Size | MNI Coordinates (x y z) | Analysis (z) |
|------------------------------------------|--------------|-------------------------|--------------|
| Right Amygdala ALLO > Placebo            |              |                         |              |
| Left Superior Parietal Gyrus             | 41           | -27 -61 64              | 3.02         |
| Right Amygdala Placebo > ALLO            |              |                         |              |
| Left Superior Temporal Gyrus             | 28           | -42 -34 4               | 4.24         |
| <b>Left Precuneus</b>                    | <b>50</b>    | <b>-3 -55 40</b>        | <b>3.58</b>  |
| <b>Left Amygdala/Peri-Amygdala</b>       | <b>43</b>    | <b>-21 5 -17</b>        | <b>3.57</b>  |
| <b>Right Amygdala/Peri-Amygdala</b>      | <b>27</b>    | <b>24 5 -14</b>         | <b>3.45</b>  |
| Left Inferior Temporal Gyrus             | 28           | -48 -19 -32             | 3.44         |
| Left Posterior Middle Temporal Gyrus     | 22           | -51 -40 -8              | 3.18         |
| Left Fusiform Gyrus                      | 25           | -30 -46 -17             | 3.09         |
| Left Middle Frontal Gyrus                | 32           | -27 17 34               | 3.08         |
| Right Fusiform Gyrus                     | 24           | 27 -40 -8               | 3.04         |
| Left Anterior Middle Temporal Gyrus      | 29           | -54 -22 -14             | 2.94         |
| Left Amygdala ALLO > Placebo             |              |                         |              |
| Right Inferior Temporal Gyrus            | 38           | 60 -43 -23              | 3.32         |
| Left Amygdala Placebo > ALLO             |              |                         |              |
| Right Parahippocampal Gyrus              | 60           | 36 -67 7                | 3.81         |
| <b>Left Precuneus</b>                    | <b>54</b>    | <b>-6 -52 40</b>        | <b>3.74</b>  |
| Left Middle Temporal Gyrus               | 60           | -54 -25 -11             | 3.68         |
| Right Precentral Gyrus                   | 28           | 63 8 16                 | 3.30         |
| Left Middle Temporal Gyrus/Angular Gyrus | 52           | -39 -64 25              | 3.20         |
| Left Superior Frontal Gyrus              | 28           | -12 53 25               | 3.08         |
| Cuneus                                   | 22           | 0 -97 -2                | 3.04         |
| Right Superior Frontal Gyrus             | 28           | 18 -10 76               | 3.00         |
| Right Lingual Gyrus                      | 41           | 6 -82 -14               | 2.97         |
| Right Amygdala DHEA > Placebo            |              |                         |              |
| No clusters greater than 20 voxels       |              |                         |              |
| Right Amygdala Placebo > DHEA            |              |                         |              |
| Left Superior Temporal Gyrus             | 24           | -42 -31 1               | 3.91         |
| Right Inferior Parietal Lobule           | 38           | 45 -37 34               | 3.89         |
| Right Superior Frontal Gyrus             | 26           | 18 65 1                 | 3.51         |
| Left Angular Gyrus                       | 70           | -39 -61 31              | 3.45         |
| Right Cerebellum                         | 35           | 15 -37 -23              | 3.04         |
| Left Amygdala DHEA > Placebo             |              |                         |              |
| No clusters greater than 20 voxels       |              |                         |              |
| Left Amygdala Placebo > DHEA             |              |                         |              |
| Left Angular Gyrus                       | 199          | -54 -64 25              | 4.14         |
| Left Precentral Gyrus                    | 26           | -48 -7 22               | 3.21         |
| Left Superior Frontal Gyrus              | 44           | -18 53 25               | 3.16         |
| Precuneus                                | 20           | 6 -67 28                | 2.89         |

\*regions of interest (ROIs) in **bold**; significant at  $p < .05$ , family-wise error corrected for multiple comparisons across the ROI. All other activations are presented at  $p < .005$  (uncorrected) with a cluster extent threshold of at least 20 contiguous voxels. MNI, Montreal Neurologic Institute.

## Discussion

To interrogate the neural underpinnings of anxiety- and mood-altering properties of neurosteroids, we investigated the impact of single-dose pregnenolone and DHEA on patterns of resting-state functional connectivity of the amygdala. Compared to placebo, both neurosteroids reduced connectivity between amygdala and precuneus, and, in addition, pregnenolone reduced connectivity with contralateral amygdala and peri-amygdaloid regions. Reductions in amygdala to precuneus connectivity were associated with less self-reported negative affect. To our knowledge, this is the first investigation of resting-state functional connectivity after neurosteroid administration, and the first to demonstrate that neurosteroids modulate intrinsic connectivity networks in a way that is relevant to negative affect. Our results demonstrate that allopregnanolone and DHEA reduce connectivity within the resting-state amygdala network, and may also shift the balance between intrinsic connectivity networks, a function that could provide insight into the neurocircuitry of anxiety psychopathology.

We found pregnenolone reduced functional connectivity between right amygdala and left amygdala, and between right amygdala and right peri-amygdaloid region, suggesting reduced connectivity within this emotion generation network. Converging evidence from multiple lines of investigation suggests that higher anxiety is associated with greater amygdala activity. Greater amygdala activation is associated with greater state (46) and trait (47) anxiety, and a metaanalysis of neuroimaging studies in various anxiety disorders concluded that elevated amygdala activity is present across anxiety disorders (48). Furthermore, both animal and human connectivity data suggest that amygdala and extended amygdala function as a coordinated unit to process threat (49, 50). It is still unclear, however, if greater inter-amygdala connectivity is

associated with higher levels of anxiety. For example, Pantzatos and colleagues (51) have argued that right and left amygdala are more coupled while viewing neutral faces than fearful faces. However, given (1) the strong link between amygdala activation and negative emotional response, and (2) the strong anatomical inter-amygdala connection, which is monosynaptic in rodents (52), it is reasonable to interpret reduced inter-amygdala connectivity as potentially contributing to reduced emotional reactivity. This reduction of connectivity between right and left amygdala thus may be relevant to allopregnanolone's anxiolytic effects (2, 5, 7, 8).

During rest, the amygdala shows positive coupling with ventral medial prefrontal regions, insula, thalamus and striatum, and anti-correlations with superior frontal gyrus, bilateral middle frontal gyrus, posterior cingulate cortex and precuneus (35-37). Notably, the observed amygdala resting-state connectivity map shows substantial overlap with the salience network, an intrinsic connectivity network responsible for detecting and orienting to salient stimuli, implicated in homeostatic regulation, interoceptive, autonomic, and reward processing (53-56). Amygdala, along with dorsal anterior cingulate cortex, anterior insula/inferior frontal gyrus, and ventral striatum, are key nodes in this network. Both allopregnanolone and DHEA have demonstrated effects on this circuit in neuroimaging studies. Pregnenolone's reduction of inter-amygdala connectivity suggests this neurosteroid may modulate the intrinsic connectivity of the salience network. During various task-based studies, DHEA modulates anterior cingulate cortex (22, 57) and insula activity (57), and allopregnanolone influences activity in amygdala (24, 25, 58) and ventral striatum (59), all components of the salience network. We have previously suggested that stronger within-salience network connectivity is associated with greater anxiety (60, 61). In concert, both PTSD and major depressive disorder were found to be associated with greater

salience network activity in recent metaanalyses (62, 63). Since elevations in allopregnanolone have been associated with symptomatic improvement in anxiety disorders (16), allopregnanolone's reduction of intrasaliency network connectivity could suggest a potential mechanism for this anxiolytic effect.

Finally, pregnenolone and DHEA reduced amygdala coupling with precuneus. Across groups, greater amygdala to precuneus connectivity was associated with greater PANAS-X negative affect [ten items assessing constructs of fear, hostility, guilt, and sadness (64)], suggesting that a reduction in this connectivity is associated with less negative affect. The amygdala is structurally connected with both dorsal (65) and ventral (66) precuneus in macaques, and shows negative functional connectivity with the precuneus at rest (67). This is consistent with the key role of the precuneus in the default network, a network that is anti-correlated with salience network at rest (32). The default network is associated with stimulus-independent, internally-focused thought, including spontaneous cognition, autobiographical memory, prospection and mind-wandering (68-71), thus this network is active during non-structured tasks such as resting-state. The current data suggest that allopregnanolone and DHEA might further decouple amygdala from the default network, contributing to enhanced segregation between default network and salience network.

Recently, our group has proposed that decreased segregation, i.e. inappropriate connectivity between salience network and default network, contributes to anxiety psychopathology (61). Among participants with PTSD, we found that greater functional connectivity between default network and salience network regions was correlated with elevated PTSD symptoms (61). Similarly, enhanced cross-network connectivity between default network and thalamus (a node

in salience network) has been observed in PTSD in a different study (72). Furthermore, enhanced connectivity between default network and amygdala predicted the development of PTSD symptoms in acutely traumatized individuals (73), and enhanced connectivity between default network and insula was associated with higher self-reported anxiety in a separate study (74). Precuneus exhibits greater connectivity with amygdala in generalized anxiety (75) and panic disorder (76), and with anterior cingulate cortex (a key node in salience network) in social anxiety disorder (77). Moreover, in healthy controls, acute laboratory stress increases amygdala resting-state connectivity with precuneus and posterior cingulate cortex (78), and viewing fearful as compared to neutral face enhances amygdala to precuneus coupling (51). Together, these data evince a consistent pattern in which anxiety disorders or induction of anxious symptoms is associated with reduced segregation between salience network and default network. Thus, allopregnanolone and DHEA's enhancement of segregation between default network and salience network highlights the potential contribution of these neurosteroids to the amelioration of anxiety.

Though both pregnenolone and DHEA reduced connectivity between amygdala and precuneus, only pregnenolone reduced inter-amygdala and peri-amygdala connectivity. These differences may be due to differing mechanisms of action. DHEA appears to have anxiolytic-like actions (1, 9), however it demonstrates modest negative modulation of GABA(A) receptors (79). Thus, its anxiolytic-like actions in rodents may involve other mechanisms. In contrast, allopregnanolone has very pronounced effects at the GABA(A) receptor, and lowers neuronal excitability with 20-fold higher potency than benzodiazepines and barbiturates (80). Allopregnanolone's greater GABAergic activity, in combination with the greater wealth of evidence linking

allopregnanolone to amygdala modulation, may help to explain the somewhat stronger modulation of amygdala connectivity under allopregnanolone versus DHEA.

There are several limitations to this study. First, we measured serum levels of allopregnanolone and DHEA, and not cerebrospinal fluid or brain levels. However, in animals, these levels are highly correlated (42, 81). Second, our sample consisted of healthy male individuals without mood or anxiety disorder diagnoses. Thus, extrapolations to women or to clinical populations should be made with caution. Third, in the allopregnanolone manipulation, we administered pregnenolone, allopregnanolone's precursor. Though we have framed our results in terms of an allopregnanolone manipulation, it is possible that our results may be attributable to increases in pregnenolone in addition to allopregnanolone. Since allopregnanolone is not commercially available for clinical use, it was necessary to administer pregnenolone as a precursor loading strategy to increase downstream allopregnanolone levels, and our results demonstrate that oral administration of pregnenolone increases allopregnanolone levels sevenfold (see 23). Fourth, we found that pregnenolone reduced average head displacement by .03 mm. While movement parameters were regressed out of our model, this difference may nonetheless have impacted our results. For instance, Van Dijk and colleagues have suggested that small head movements can inflate measures of local functional coupling and deflate measure of long-range functional coupling (82). Since the placebo group in our study demonstrated more average displacement than the allopregnanolone group, this may have partially accounted for the placebo group's greater levels of amygdala to peri-amygdala and contralateral amygdala connectivity. However, the observed group differences in long-range coupling (amygdala to precuneus) are unlikely to have been influenced by small differences in movement, since the direction of the observed

effect ran counter to the effect that movement may introduce. Finally, we have conceptualized decreased within-salience network connectivity as relevant to reduced anxiety; however decreased connectivity within this network may also have drawbacks. For instance, stronger salience network connectivity has been associated with better working memory (83). Future studies should investigate neurosteroid modulation of salience network in anxiety-disordered populations to further assess the benefits and disadvantages of these patterns.

In conclusion, allopregnanolone and DHEA reduced amygdala coupling with other regions of the amygdala/salience network, and reduced amygdala coupling with regions of default network in our participants. These findings suggest that neurosteroids modulate amygdala functional connectivity during the resting state, and may shift the balance between salience network and default network at rest. These findings provide insight into the neurocircuitry of anxiety, and suggest that allopregnanolone and DHEA may be potential targets for pharmacological intervention for anxiety psychopathology.

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## **Chapter 6 : Conclusion**

In this dissertation we present a series of experiments designed to elucidate the impact of the endogenous neurosteroids DHEA and allopregnanolone on emotion regulation neurocircuits and intrinsic connectivity networks. In Chapter 2, we examine the impact of single-dose DHEA on the Shifted-Attention Emotional Appraisal Task (SEAT), and demonstrate that DHEA reduces activity in the amygdala and hippocampus and increases activity in the rostral anterior cingulate cortex (rostral ACC). In Chapter 3, we report that during the SEAT, allopregnanolone reduces activity in the amygdala and insula and increases activity in the dorsal medial prefrontal cortex (dorsal mPFC). In Chapter 4, we examine resting-state amygdala connectivity in individuals with PTSD and demonstrate that this disorder involves increased connectivity between amygdala and insula and reduced negative coupling between amygdala and rostral and dorsal ACC. Finally, in Chapter 5, we demonstrate that allopregnanolone and DHEA administration reduce coupling within the amygdala functional connectivity network, both between amygdala and contralateral amygdala, and between amygdala and precuneus. Together, these results demonstrate that neurosteroids modulate the emotion neurocircuitry that is dysregulated in PTSD, and suggest that future studies are warranted to determine if DHEA and allopregnanolone enhancement might represent a potential therapeutic intervention for individuals with this disorder.

Since the circuits modulated by neurosteroid administration show aberrant activity in PTSD during emotion regulation tasks (1, 2), it is possible that DHEA and allopregnanolone may help

rectify disruptions in these circuits and thus improve emotion regulation capabilities in PTSD. This is evidenced by DHEA and allopregnanolone's reduction of amygdala and insula activity and enhancement of mPFC activity, modulations that run counter to the amygdala hyperactivity and mPFC hypoactivity observed in metaanalyses of PTSD (2-4). Research supports that training individuals with PTSD in emotion regulation strategies reduces negative emotional responding and normalizes PFC responses to aversive stimuli (1), suggesting that mPFC modulation is a key route to successful emotion regulation in PTSD. In this dissertation, we further demonstrate that individuals with PTSD show increased resting-state connectivity between amygdala and insula, both regions of the salience network. Allopregnanolone's demonstrated ability to reduce connectivity within this network further highlights its potential role to ameliorate disruptions in neural circuitry observed in PTSD.

Our findings suggest both similar and divergent mechanisms for the two neurosteroid interventions. Both DHEA and allopregnanolone reduced activity in amygdala, though under DHEA, this effect was specific to the emotion induction condition, and under allopregnanolone, this effect survived across conditions. Both DHEA and allopregnanolone increased activity in medial prefrontal regions, though again, the conditions under which this effect occurred varied by drug. Both neurosteroids decreased amygdala resting-state connectivity to precuneus, an effect that was associated with less negative affect. And finally, serum levels of both DHEA and allopregnanolone were negatively correlated with amygdala activity. These results suggest a consistent pattern of reduced activity in regions associated with the generation of negative emotion and enhanced activity in regions linked to regulatory control of emotion. These findings extend previous reports of DHEA's antidepressant effects (5-7), and allopregnanolone's

association with successful psychiatric treatment (8-11) by delineating the likely neural circuitry involved. These convergent effects may be attributable to a shared pharmacologic mechanism. Both allopregnanolone and androsterone (DHEA's metabolite) are positive allosteric modulators of the GABA(A) receptor, though androsterone may have lower potency than allopregnanolone (12). Furthermore, the amygdala has been identified as a particular locus of both allopregnanolone and DHEA's effects (13-18). Amygdala has reciprocal connections and feedback loops with medial prefrontal cortex, insula and hippocampus (19-21), thus, neurosteroid modulation of amygdala is a putative pathway to these wide-ranging effects.

Allopregnanolone and DHEA also demonstrated several discrepant effects. In addition to its reduction of amygdala activity, allopregnanolone also reduced activity in the insula, and reduced resting-state connectivity between right amygdala and left amygdala and between right amygdala and peri-amygdala. None of these effects were present in the DHEA group. This difference might be attributable to allopregnanolone's greater ability to modulate amygdala as compared to DHEA (22). The insula is structurally tightly linked to basolateral amygdala (20), suggesting that greater modulation of the amygdala could potentially lead to greater modulation of the insula as a result. Together, these findings of reduced activity and reduced connectivity of amygdala and adjacent regions demonstrate that allopregnanolone exerts robust effects on emotion generation neurocircuitry. DHEA, in contrast, exerted a robust reduction of hippocampal activity and enhancement of hippocampal-amygdala connectivity. Additionally, DHEA reduced performance on a conjunctive memory test, an effect that may have been associated with its reduction of hippocampal activity. These effects might be linked to DHEA's antiglucocorticoid profile (23-25). We have previously demonstrated that cortisol administration increases



hippocampal activity, and that this effect mediates cortisol's ability to selectively enhance conjunctive memory (26). Cortisol was also positively associated with hippocampal activity in the current study. Thus, DHEA's antiglucocorticoid effects might contribute to its reduction of hippocampal activity and subsequent reduction of emotional memory trace strength.

Differing effects of allopregnanolone and DHEA may also be due to different mechanisms of action at the molecular level. Allopregnanolone acts directly on GABA(A) receptors, and lowers neuronal excitability with 20-fold higher potency than benzodiazepines and barbiturates (27-31). Amygdala and mPFC are rich in GABA(A) receptors (32) and endogenous allopregnanolone (33), suggesting that allopregnanolone could feasibly have a direct impact on activity in these regions. Indeed, in our sample, allopregnanolone serum level was more strongly correlated to amygdala activity than activity in any other brain region. Allopregnanolone may also influence mood and affect through dopaminergic mechanisms (e.g. 34, 35, 36). Finally, allopregnanolone may impact emotion regulation neurocircuitry through other central nervous system effects, such as enhancement of neurogenesis (37) myelination (38) and neuroprotection (27-30). Given the rapidity of the observed effects, however, it is likely that allopregnanolone exerted its current effects by altering neuronal excitability at membrane-bound ligand-gated ion channel receptors. Allopregnanolone's potency at the GABA(A) receptor may have translated to a more specific anxiolytic profile, as compared to DHEA.

DHEA, conversely, has more pronounced actions at the NMDA receptor. It is a positive allosteric modulator at the NMDA receptor (39), an effect that is potentially mediated through sigma receptors (40). DHEA also exerts stress-buffering effects at mu-opioid receptors (41).

DHEA has been implicated in neuroprotection, neurite growth, and neurogenesis, and has antioxidant and anti-inflammatory effects (40). One notable difference between the DHEA and allopregnanolone manipulations was that only DHEA impacted conjunctive memory, by impairing memory for emotional stimuli. This ran somewhat counter to our hypotheses. In previous studies, allopregnanolone rather than DHEA has been demonstrated to impair learning and memory in rodents (42-44). DHEA is associated with improved working memory in humans (45) and retention of conditioned fear in mice (46) and one study of DHEA administration improved episodic memory (24). However, memory for emotional faces could rely on different mechanisms than the tasks that are reportedly improved by DHEA (47). DHEA's reduction of emotional memory may be due to its antiglucocorticoid effects, as discussed previously. Alternatively, this effect could be related to DHEA's antidepressant effects, since lessened negative affect may have contributed to a mood-congruent bias away from negative emotional expressions (48), resulting in poorer memory for those stimuli.

## **Caveats, Limitations and Methodological constraints**

### **Sample**

One limitation that applies for all the studies in this dissertation is that the sample size was modest, and thus our results require replication. Our samples (in Chapters 2, 3, and 5) consisted of healthy male individuals without mood or anxiety disorder diagnoses. Thus, extrapolations to women or to clinical populations should be made with caution. Additionally, our PTSD sample in Chapter 4 included participants with co-morbid major depression. Though depression commonly co-occurs with PTSD in veteran populations (up to 80% by some estimates, 49), inclusion of depressed participants may render some of the variance attributable to the presence

of depression. However, our results were not affected by the removal of participants with current comorbid depression. Therefore, we retained depressed participants in our final analysis.

## **Experimental Task**

### SEAT

In this dissertation, we used a novel paradigm of emotion regulation. Additional studies are needed to assess the reliability and replicability of these results. Despite this limitation, we believe the SEAT has strong a priori theoretical and empirical support. The results from this dissertation support the use of the SEAT as a useful measure of emotion modulation and regulation. The indoor/outdoor condition activated attention modulation circuits (50), including parietal cortex and precuneus. The Like/Dislike condition activated appraisal modulation circuits (51), including dmPFC, and IFG/anterior insula. These findings are consistent with previous findings from the SEAT (52), as well as other paradigms investigating the neurocircuitry of emotion (53), and establish the SEAT as a reliable probe of two commonly used emotion regulation strategies.

### Resting-State Connectivity

A limitation to our resting-state connectivity studies is that seed voxel-based resting state connectivity is essentially correlational and thus does not allow for inferences about causal relationships. Activation differences and connectivity differences cannot be disentangled using seed-based connectivity measures. Since we did not collect measures of mental activity during

the resting state scan, we cannot discern whether differential engagement in various cognitive processes could be driving the observed group differences.

## **Pharmacology**

There are also pharmacological limitations to these studies. We measured serum levels of allopregnanolone and DHEA, and not cerebrospinal fluid or brain levels. However, in animals, these levels are highly correlated (54, 55). Additionally our allopregnanolone drug manipulation involved the administration of pregnenolone. Since allopregnanolone is not currently commercially available for clinical use, it was necessary to administer pregnenolone as a precursor loading strategy to increase downstream allopregnanolone levels. As our results demonstrate, oral administration of pregnenolone increases allopregnanolone levels sevenfold. We have framed our results in terms of an allopregnanolone manipulation, but our results may also be attributable to increases in pregnenolone. Pregnenolone levels are low in individuals with major depression (56) and anxiety disorders (57, 58), and are increased by fluoxetine administration in rats (59) and in humans (60). Therefore pregnenolone may also be relevant to anxiety symptomatology, and may influence relevant neurocircuits. Thus, future studies should attempt to disentangle the emotion regulatory effects of pregnenolone versus its metabolite allopregnanolone.

One final limitation to these studies is that we have framed allopregnanolone as an anxiolytic and antidepressant agent, but there is some evidence to suggest that allopregnanolone has biphasic effects. In particular, high doses of allopregnanolone are associated with anxiolytic and antidepressant effects, whereas low doses may be associated with anxiogenic effects (61).

Several studies suggest that these paradoxical effects occur in animals (62, 63), postmenopausal women (64, 65), and women with premenstrual syndrome (66). Previous neuroimaging studies report that moderate levels of allopregnanolone (luteal phase levels) increases amygdala activity (67) but high levels of allopregnanolone decrease amygdala activity (16). However, it should be noted that healthy women (without premenstrual syndrome) show no adverse effects in response to physiological levels of allopregnanolone, and show anxiolytic effects in response to higher levels (68). Furthermore, it is significant that within the same women, menstrual cycles associated with higher luteal phase allopregnanolone induce less irritability, greater cheerfulness, greater well-being, and more energy, as compared to cycles with lower luteal phase allopregnanolone (69). These data collectively suggest that women with menstrual cycle alterations show varying responses to escalating levels of allopregnanolone, but that healthy women usually experience anxiolytic effects.

## **Future Directions**

Future investigations into the role of allopregnanolone and DHEA in emotion regulation neurocircuitry should include samples of varying age and gender, as well as populations with mood and anxiety psychopathology. Women, as previously mentioned, may have differing responses to allopregnanolone based on menstrual cycle phase and sensitivity to ovarian cyclicity. In particular, allopregnanolone supplementation might be a potential intervention for women with postpartum depression, since this disorder has been linked to rapid declines in progestins (70). Additionally, since DHEA declines with age and older adults show consistently lower levels of DHEA (71), investigations into the impact of DHEA administration on emotion regulation processes in older adults could highlight a potential corrective role for this

neurosteroid. Finally, investigations into the impact of neurosteroids on emotion regulation neurocircuits in anxiety disorders such as PTSD are warranted. Allopregnanolone and DHEA may correct for emotion regulation skill deficits and heightened emotional reactivity observed in PTSD.

## **Summary**

DHEA and allopregnanolone are endogenously produced steroids with anxiolytic and antidepressant effects. Dysregulated release of these neurosteroids has been linked to various forms of psychopathology. Recently, novel techniques have been developed to probe the etiology of anxiety disorders, including resting-state fMRI and pharmacofMRI. However, few studies to date have deployed these new methods to investigate neurosteroid modulation of emotion, an issue that is paramount to understanding why neurosteroid dysregulation is associated with distress and psychopathology. In this dissertation, we investigated the impact of DHEA and allopregnanolone administration on (1) a novel paradigm that probes emotional response and regulation, and (2) resting-state amygdala functional connectivity, to identify the influence of neurosteroids on emotion regulation neurocircuits and intrinsic connectivity networks that are disrupted in anxiety psychopathology. We demonstrate that during emotion regulation tasks, DHEA and allopregnanolone reduce activity in regions associated with the generation of negative emotion and enhance activity in regions linked to regulatory processes. Further, we demonstrate that these neurosteroids modulate amygdala intrinsic connectivity in ways that may correct for deficiencies observed in posttraumatic stress disorder. Thus, our results provide initial neuroimaging evidence that DHEA and allopregnanolone may be useful as pharmacological

interventions for anxiety disorders and invite further investigation into the brain basis of neurosteroid emotion regulatory effects.

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