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The Neurosteroids Allopregnanolone and Dehydroepiandrosterone Modulate Resting-State Amygdala Connectivity

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Abstract: The neurosteroids allopregnanolone and dehydroepiandrosterone (DHEA) are integral components of the stress response and exert positive modulatory effects on emotion in both human and animal studies. Although these antidepressant and anxiolytic effects have been well established, to date, little research 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 400 mg of pregnenolone ($N = 16$), 400 mg of DHEA ($N = 14$), or placebo ($N = 15$) and underwent 3T fMRI. Resting-state brain connectivity was measured using amygdala as a seed region. When compared with placebo, pregnenolone administration reduced connectivity between amygdala and dorsal medial prefrontal cortex, between amygdala and precuneus, and between amygdala and hippocampus. DHEA reduced connectivity between amygdala and periamygdala and between amygdala and insula. Reductions in amygdala to precuneus connectivity were associated with less self-reported 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. *Hum Brain Mapp 35:3249–3261, 2014.* © 2013 Wiley Periodicals, Inc.

Key words: dehydroepiandrosterone; pregnenolone; fMRI; pharmaco-fMRI; neuroactive steroid; anxiety

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INTRODUCTION

Allopregnanolone and dehydroepiandrosterone (DHEA) are endogenously produced neurosteroids with neuroprotective, anxiolytic, antidepressant, antioxidant, and antiglucocorticoid effects [Belelli and Lambert, 2005; Maninger et al., 2009; Patchev et al., 1994]. 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 [Genud et al., 2009; Pibiri et al., 2008]. DHEA and allopregnanolone administration reduce immobility in the forced swim test [Frye et al., 2004; Urani et al., 2001] and reduce stress and anxiety-like behavior in rodents [Frye and Rhodes, 2006; Melchior and Ritzmann, 1994]. In human studies, allopregnanolone and DHEA dysregulation is associated with anxiety and depression. Individuals with major depressive disorder exhibit reduced plasma and cerebrospinal fluid (CSF) levels of allopregnanolone [Romeo et al., 1998; Uzunova et al., 1998] and DHEA [Barrett-Connor et al., 1999; Morsink et al., 2007; Wong et al., 2011], and those with post traumatic stress disorder (PTSD) show reduced CSF allopregnanolone [Rasmusson et al., 2006]. Furthermore, increases in serum allopregnanolone and DHEA over a course of treatment predict symptomatic improvement [Amin et al., 2006; Olff et al., 2007; Uzunova et al., 1998; Yehuda et al., 2006]. 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 [Adolphs et al., 1999], fear conditioning [Armony and LeDoux, 1997], and emotional salience [Whalen et al., 2001], may be a particular locus of allopregnanolone and DHEA's effects. Our laboratory has recently demonstrated that single-dose DHEA [Sripada et al., 2013a] and the allopregnanolone precursor pregnenolone [Sripada et al., 2013b] decrease amygdala activity and increase activity in medial prefrontal regions during tasks engaging cognition–emotion interactions. It had been reported that progesterone administration (which in turn increases allopregnanolone levels) modulates amygdala responsivity to emotional faces [van Wingen et al., 2007] and increases functional connectivity between amygdala and dorsal medial prefrontal cortex (dmPFC) [van Wingen et al., 2008]. Allopregnanolone modulates GABA(A) receptor-mediated inhibitory postsynaptic currents in the central nucleus of the amygdala [Wang et al., 2007], and microinfusions of allopregnanolone directly into the amygdala produce anxiolytic [Engin and Treit, 2007] and antidepressant [Shirayama et al., 2011] effects. The anxiolytic and antidepressant effects of DHEA may be mediated by the amygdala as well. In animal models, DHEA administration increases BDNF concentration [Naert et al., 2007] and 5-HT(2A) receptor expression [Cyr et al., 2000] in the amygdala, and DHEA infusions into the amygdala modulate stress responsivity [Singh et al., 1994]. Thus, the amygdala might be a key region in the anxiolytic and emotion modulatory effects of neurosteroids.

Although mounting evidence suggests that neurosteroids modulate amygdala activity, the reported direction of amygdala modulation is somewhat inconsistent. Activation of amygdala has been reported as increased [van Wingen et al., 2008] or decreased [Sripada et al., 2013b; van Wingen et al., 2007] by allopregnanolone depending on the task used. To fully understand the impact of neurosteroids on amygdala function, it may be necessary to examine their effects on amygdala connectivity at rest. Resting-state connectivity offers a powerful way to assess intrinsic connections between brain networks [Fox and Raichle, 2007; Fox et al., 2005; Raichle et al., 2001] 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 [Fulwiler et al., 2012; Kim et al., 2011; Roy et al., 2009]. Thus, resting-state analyses may provide a fuller understanding of neurosteroid modulation of the amygdala and its associated emotion production network. Previous investigations demonstrate that the amygdala is strongly positively correlated with contralateral amygdala and insula at rest and strongly anti-correlated with precuneus [Kim et al., 2011; Roy et al., 2009]. Because of their anxiolytic and antidepressant properties, we predicted that allopregnanolone and DHEA would modulate both positive (amygdala and insula) and negative (precuneus) connectivity within the amygdala functional connectivity network during rest [Roy et al., 2009] and that this effect would be associated with reduced self-reported negative affect. Furthermore, given the impact of allopregnanolone and DHEA on functional connectivity between amygdala and mPFC [Alhaj et al., 2006; Sripada et al., 2013a,b; van Wingen et al., 2008], we hypothesized that these neurosteroids would also modulate resting-state amygdala connectivity with mPFC.

MATERIALS AND 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 [Sheehan et al., 1998]. No participant was taking over-the-counter or prescription medication at the time of the experiment. 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

To control for any potential diurnal variation in endogenous DHEA [Stanczyk, 2006], all functional magnetic resonance imaging (fMRI) scans were conducted in the afternoon, and blood collection times were standardized across subjects. Baseline blood samples were drawn between 11:00 and 11:30 a.m., and postscan blood samples were drawn between 3:00 and 4:00 p.m. At 1-h postdrug administration, participants completed the Positive and Negative Affect Scale Expanded (PANAS-X) [Watson and Clark, 1994], the Drug Effects Questionnaire [Morean et al., 2013], and a 20-item Visual Analog Scale. The Drug Effects Questionnaire assessed the extent to which participants (1) felt any substance effects, (2) liked the effects, (3) felt high, and (4) wanted more of the substance. The Visual Analog Scale included the following items: Clearheaded, Anxious, Stimulated, Tired, Calm, Drowsy, Peaceful, Nervous, Hungry, Energetic, Uneasy, Relaxed, Alert, Contented, Focused, Dreamy, Restless, Nauseous, Worn Out, and Jittery. Each item consisted of a 4-inch bar with "Not At All" and "Extremely" indicated at the extremities.

Drug Administration

Study drugs (pregnenolone and DHEA) and matching placebo identical in appearance were obtained from Belmar 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. Although data are limited, allopregnanolone serum levels appear to reach three times baseline levels in 2 h after oral administration of 400 mg pregnenolone (Marx, unpublished data), and DHEA serum concentrations peak 60 to 480 min after DHEA administration [Arlt et al., 1998]. Thus, drug administration occurred 2 h before neuroimaging to ensure elevated levels during the scan.

Steroid Measurements

We used circulating levels of DHEA and allopregnanolone as indicators of central neurosteroid levels. In animal models, serum pregnenolone levels are closely related to levels in the hippocampus [Marx et al., 2006a], and serum DHEA levels are closely related to levels in CSF [Kancheva et al., 2011], although Kancheva and colleagues did not find a correlation between CSF and serum pregnenolone levels. CSF DHEA levels are also correlated with temporal cortex DHEA levels, as are CSF and temporal cortex pregnenolone levels [Naylor et al., 2008]. We collected serum samples for assay 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, Tarrytown, NY). Pregnenolone, pregnanolone, allopregnanolone, and androsterone levels in serum were determined by a highly sensitive and specific gas chromatography–mass spectrometry (GC/MS) method, as previously described [Marx et al., 2006b,c], with modifications (the electron impact mode was used rather than negative ion chemical ionization). One milliliter of serum was extracted three times in ethyl acetate before high-performance liquid chromatography 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. 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 [Sripada et al., 2013a,b] 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 min. 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, 2,000 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 × 64] was collected. After discarding three initial volumes to permit thermal equilibration of the MRI signal, 240 volumes were acquired over 8 min. 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 a fixation cross 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). Scans were reconstructed, motion-

corrected, slice-time corrected, realigned to the first scan in the experiment to correct for head motion, and coregistered 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). Scans were normalized to standard space, segmented into tissue types, bias-corrected, and iteratively realigned using DARTEL. Smoothing was performed with an $8 \times 8 \times 8$ mm^3 kernel. Motion parameters (mean displacement and 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 regions of interest (ROI) were constructed from cytoarchitectonically determined probabilistic maps of the human basolateral and centromedial amygdala, developed by Amunts et al. [2005] within the SPM Anatomy Toolbox [Eickhoff et al., 2005]. These subregions were combined to form a single, whole amygdala seed. We extracted the spatially averaged time series from right and left amygdala ROI for each participant. As resting-state functional connectivity measures low-frequency spontaneous BOLD oscillations (0.01- to 0.10-Hz band) [Fox et al., 2005], the time course for each voxel was band-passed filtered in this range. Next, motion scrubbing and nuisance regression were performed. During motion scrubbing, individual frames with excessive head motion were censored from the time series, following Power et al. [2012]. The target high-motion frame, but not those flanking it, was removed in accordance with the findings of Satterthwaite et al. [2013]. Nuisance 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 CSF masks, which has been demonstrated to effectively remove signals derived from the cardiac and respiratory cycle [Chang and Glover, 2009]. This method is comparable with the COMPCOR method and is complementary to the RETROICOR method [Behzadi et al., 2007]. In generating the white matter and CSF 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 anticorrelation with orthogonal networks that increase in proportion to the size of those networks [Anderson et al., 2011]. Pearson's product–moment correlation coefficients were calculated between average time courses in the seed ROI and all other voxels of the brain resulting in a three-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 ROI Analysis

The 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. At the second level, for between-group comparisons, we set a whole-brain voxel-wise significance threshold at $P < 0.05$, cluster-level corrected for multiple comparisons across the entire brain (cluster volume > 1,220 voxels); these cluster-level thresholds were calculated using Monte-Carlo simulations (AFNI AlphaSim; http://afni.nimh.nih.gov/afni/doc/manual/AlphaSim). To exclude a general effect of drug administration on the BOLD response [van Wingen et al., 2007], the primary visual cortex (V1) was used as the control region [Amunts et al., 2000]. Reported voxel coordinates correspond to standardized Montreal Neurologic Institute space. To assess for correlations with mood, PANAS-X scores were correlated with data extracted from regions of group difference (averaged across the entire cluster).

RESULTS

Participants

Sixteen participants were administered pregnenolone, 14 were administered DHEA, and 15 were administered placebo. Groups did not differ by age [$F(2,44) = 0.71$, $P = 0.5$]. Pregnenolone administration significantly increased serum pregnenolone, pregnanolone, and allopregnanolone levels, and DHEA administration increased serum DHEA, DHEAS, and androsterone levels ($P < 0.001$; details can be found in Sripada et al. [2013a,b]). There were no significant differences in subjective drug effects ($P > 0.4$), and participants' guesses of which drug they received did not deviate from chance [$\chi^2(6) = 3.80$, $P = 0.7$]. There were no significant differences between drug administration and placebo groups in self-reported sedation ($P > 0.5$).

Motion and Physiological Variables

There were no movements greater than 3 mm and no motion differences between DHEA and placebo groups ($P > 0.2$). In the pregnenolone administration group, mean displacement was reduced (mean = 0.066 mm) when compared with the placebo group (mean = 0.099 mm, $P = 0.006$). To correct for this discrepancy, we implemented motion scrubbing procedures. There were no group differences in heart rate or respiration ($P > 0.7$).

TABLE I. Functional connectivity under Placebo

Contrast map and brain region	Cluster size	MNI coordinates			Analysis (z)
		x	y	z	
Left amygdala positive correlations under PBO					
Superior temporal gyrus/left insula	8,153	−27	−7	−29	7.28
Medial frontal gyrus	59	−6	41	−23	4.45
Right lingual gyrus	13	15	−40	−2	4.29
Right Heschl's gyrus	16	39	−25	7	3.92
Inferior frontal gyrus	178	−48	11	28	3.83
Right insula	14	30	−7	10	3.71
Left angular gyrus	51	−57	−70	25	3.48
Left amygdala negative correlations under PBO					
Right middle frontal gyrus	52	36	47	13	4.12
Posterior cingulate cortex	13	6	−43	22	3.61
Right amygdala positive correlations under PBO					
Right parahippocampal gyrus	1,935	24	−10	−23	7.66
Superior temporal gyrus	1,766	−33	−4	−29	6.31
Right inferior temporal gyrus	13	48	−49	−26	5.37
Left inferior temporal gyrus	436	−54	−67	−5	4.71
Right precentral gyrus	1,015	60	−4	46	4.09
Left precentral gyrus	19	−48	−7	25	3.99
Inferior parietal lobule	23	−48	−34	49	3.84
Supplementary motor area	58	12	−22	52	3.79
Right paracentral lobule	14	6	−40	67	3.64
Left paracentral lobule	35	−9	−31	73	3.61
Right amygdala negative correlations under PBO					
Cingulate gyrus	71	−3	−19	25	4.27
Precuneus	111	12	−64	31	3.98
Left precuneus	12	−9	−70	34	3.38

MNI, Montreal Neurologic Institute; PBO, placebo.
Data thresholded at $P < 0.001$, uncorrected, extent threshold $k = 10$.

fMRI Findings

Under the placebo condition, the left amygdala seed showed positive connectivity with right amygdala, periamygdala, bilateral hippocampus and superior temporal gyrus, and anti-correlations with posterior cingulate cortex (see Table I). The right amygdala seed showed positive connectivity with left amygdala, bilateral periamygdala and bilateral hippocampus, and anti-correlation with precuneus (see Table I).

When compared with placebo, pregnenolone administration reduced connectivity between the left amygdala seed and a cluster encompassing dmPFC and precuneus (see Table II and Fig. 1). Pregnenolone also reduced connectivity between the right amygdala seed and left hippocampus (see Fig. 1).

When compared with placebo, DHEA reduced connectivity between the left amygdala seed and a cluster encompassing left amygdala, left periamygdala, and left insula (see Table II and Fig. 2). There were no significant differences between DHEA and placebo groups in connectivity with the right amygdala seed.

We also conducted an exploratory analysis of differences in amygdala functional connectivity between pregnenolone and DHEA administration groups. When compared with DHEA, pregnenolone reduced functional connectivity between left amygdala and dmPFC (see Table II).

Correlations with PANAS-X

When compared with placebo, DHEA administration reduced PANAS-X negative affect score at trend level [$t(27) = 1.87$, $P = 0.07$], as reported in Sripada et al. [2013a]. To probe the neural underpinnings of this effect, correlations were computed between PANAS-X negative affect score and the values extracted from regions of group difference. These included (1) the dmPFC and precuneus-encompassing cluster that exhibited reduced connectivity with left amygdala in the pregnenolone group, (2) the left hippocampus-encompassing cluster that exhibited reduced connectivity with right amygdala in the pregnenolone group, and (3) the left insula-encompassing cluster that exhibited reduced connectivity with left amygdala in the DHEA group. Across all three groups (pregnenolone, DHEA, and placebo), there was a positive correlation between PANAS-X negative affect score and functional connectivity between right amygdala and the

TABLE II. Group comparisons

Contrast map and brain region	Cluster size	MNI coordinates x	y	z	Analysis (z)
Left amygdala ALLO > placebo					
Left superior frontal gyrus	2,687	−9	−28	76	3.84
Superior medial frontal gyrus (dmPFC)	**196**	**−12**	**41**	**37**	**2.82**
Precuneus	**199**	**−9**	**−40**	**55**	**2.86**
Right amygdala ALLO > placebo					
Left middle temporal gyrus	1,522	−45	−19	−11	3.91
Left hippocampus	**18**	**−36**	**−16**	**−11**	**2.84**
Left amygdala DHEA > placebo					
Left supramarginal gyrus	2,038	−66	2	7	3.62
Left insula	**165**	**−36**	**−1**	**−8**	**2.81**
Left amygdala	**8**	**−24**	**−7**	**−14**	**2.40**
Left amygdala DHEA > ALLO					
Medial frontal gyrus	2,049	−15	50	10	3.88
dmPFC	**396**	**−12**	**53**	**10**	**3.58**

ALLO, allopregnanolone; dmPFC, dorsal medial prefrontal cortex; MNI, Montreal Neurologic Institute.
Cluster-level significance set at $P < 0.05$, whole brain corrected for multiple comparisons. Within each significant cluster, *Z*-score and associated a priori region are noted along with MNI atlas coordinates of peaks in boldface.

hippocampus-encompassing cluster ($r = 0.294$, $P = 0.05$), indicating that reduced connectivity with hippocampus was associated with less negative affect. Across the groups, there was also a positive correlation between PANAS-X negative affect score and functional connectivity between left amygdala and the insula-encompassing

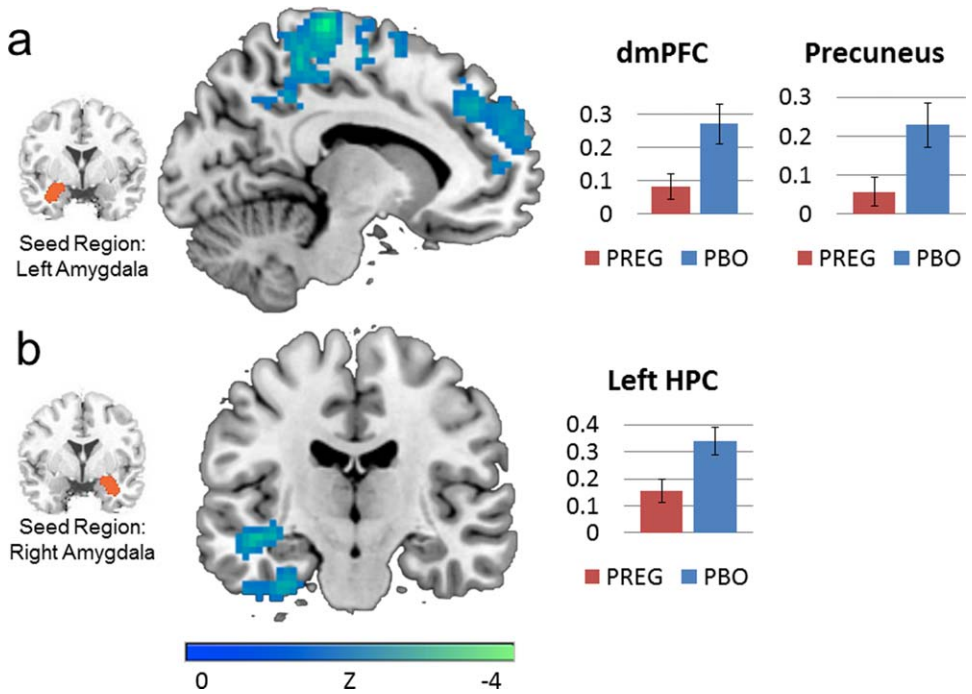

Figure 1.

(a) Pregnenolone administration reduced functional connectivity between left amygdala and a cluster encompassing dmPFC and precuneus ($x = -11$). (**b**) Pregnenolone administration reduced functional connectivity between right amygdala and left hippocampus ($y = -16$). Bar graphs depict percent signal change. Error bars depict standard error of the mean. dmPFC, dorsal medial prefrontal cortex; HPC, hippocampus. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

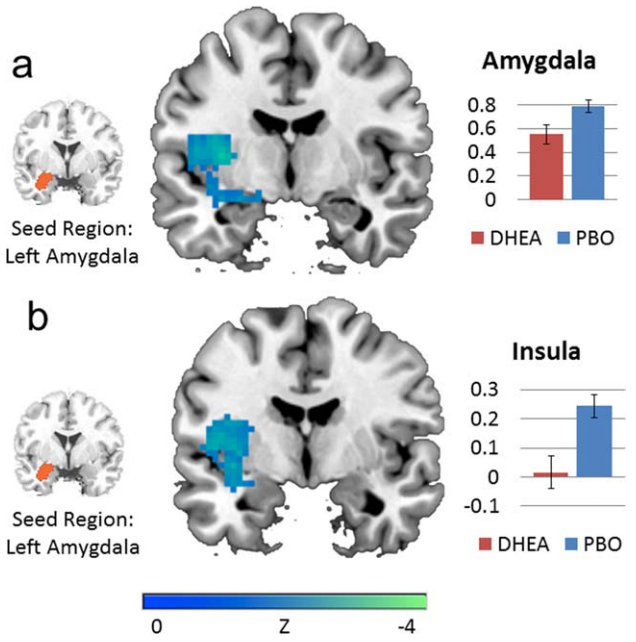

Figure 2.

DHEA reduced functional connectivity between left amygdala and (**a**) periamygdala ($y = -7$) and (**b**) left insula ($y = 4$). Bar graphs depict percent signal change. Error bars depict standard error of the mean. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cluster ($r = 0.291$, $P = 0.05$). Additional correlations were computed between PANAS-X negative affect score and the values extracted from anatomical insula, dmPFC, hippocampus, and precuneus. Across all three groups (pregnenolone, DHEA, and placebo), there was a positive correlation between PANAS-X negative affect score and left amygdala functional connectivity with anatomical precuneus ($r = 0.319$, $P = 0.035$).

Correlations with Neurosteroid Levels

To further probe relationships between neurosteroids and connectivity patterns, correlations were computed between delta serum neurosteroid levels (endpoint minus baseline) and the values extracted from regions of group difference. Across all three groups, functional connectivity between the right amygdala seed and left hippocampus-encompassing cluster was negatively associated with delta serum pregnenolone ($r = -0.513$, $P < 0.001$), delta serum pregnanolone ($r = -0.326$, $P = 0.031$), and delta serum allopregnanolone ($r = -0.553$, $P < 0.001$). Across all three groups, functional connectivity between the left amygdala seed and left insula-encompassing cluster was negatively associated with delta serum DHEAS ($r = -0.436$, $P = 0.003$) and delta serum androsterone ($r = -0.390$, $P = 0.009$). Additionally, functional connectivity between left amygdala and anatomical left insula was negatively correlated with delta serum DHEAS ($r = -0.302$, $P = 0.047$), and functional connectivity between right amygdala and anatomical precuneus was negatively correlated with delta serum pregnanolone ($r = -0.301$, $P = 0.047$). Neither pregnenolone nor DHEA significantly modulated connectivity between amygdala and primary visual cortex (left amygdala, $P > 0.4$; right amygdala, $P > 0.7$), indicating that drug effects on other brain regions were not attributable to general changes in the BOLD response.

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. When compared with placebo, pregnenolone administration reduced connectivity between amygdala and dmPFC, between amygdala and precuneus, and between amygdala and hippocampus. When compared with placebo, DHEA reduced amygdala connectivity with insula and with periamygdaloid regions. These connectivity patterns 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 that DHEA reduced functional connectivity within left amygdala and between left amygdala and left periamygdala, 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 [Bishop et al., 2004] and trait [Etkin et al., 2004] anxiety, and a meta-analysis of neuroimaging studies in various anxiety disorders concluded that elevated amygdala activity is present across anxiety disorders [Etkin and Wager, 2007]. Furthermore, both animal and human connectivity data suggest that amygdala and extended amygdala function as a coordinated unit to process threat [Liberzon et al., 2003; Oler et al., 2012]. It is still unclear, however, if greater interamygdala connectivity is associated with higher levels of anxiety. For example, Pantazatos et al. [2012] have argued that right and left amygdala are more coupled while viewing neutral faces than fearful faces. However, given the strong link between amygdala activation and negative emotional response, it is reasonable to interpret reduced interamygdala connectivity as potentially contributing to reduced emotional reactivity. This

reduction of connectivity within amygdala and between amygdala and periamygdala may thus be relevant DHEA's anxiolytic effects [Maninger et al., 2009; Melchior and Ritzmann, 1994].

DHEA administration also reduced functional connectivity between left amygdala and left insula. The insula is implicated in interoception [Oppenheimer et al., 1992], disgust [Britton et al., 2006], emotion processing [Stein et al., 2007], emotional recall [Phan et al., 2002], and anticipation of aversive stimuli [Simmons et al., 2008]. Amygdala and insula are structurally interconnected [Aggleton et al., 1980], and negative emotion induction enhances coupling between insula and amygdala in healthy volunteers [van Marle et al., 2010]. We have previously reported that amygdala exhibits greater resting-state connectivity with insula in patients with PTSD compared with healthy and combat-exposed control participants [Sripada et al., 2012a,b]. This finding has been replicated by others [Rabinak et al., 2011]. Thus, reduced connectivity between amygdala and insula could represent a protective/resilience factor in the development of pathological anxiety states. In support of this hypothesis, functional connectivity between amygdala and insula was positively associated with negative affect in our sample. DHEA reduced connectivity between amygdala and insula and also reduced PANAS-X at trend level. However, given the lack of a statistically significant decrease in PANAS-X scores, it is possible that the association between negative affect and amygdala–insula connectivity may be due to spontaneous variations in mood rather than a drug-induced effect.

During rest, the amygdala shows positive functional connectivity with ventral medial prefrontal regions, insula, thalamus, and striatum, and anticorrelations with superior frontal gyrus, bilateral middle frontal gyrus, posterior cingulate cortex, and precuneus [Fulwiler et al., 2012; Kim et al., 2011; Roy et al., 2009]. 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, and implicated in homeostatic regulation, interoceptive, autonomic, and reward processing [Cauda et al., 2011; Dosenbach et al., 2007; Seeley et al., 2007; Sridharan et al., 2008]. 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. DHEA's reduction of amygdala to insula connectivity and interamygdala connectivity suggests that this neurosteroid may modulate the intrinsic connectivity of the salience network. During task-based studies, there is preliminary evidence to suggest that DHEA modulates anterior cingulate cortex [Alhaj et al., 2006; Sripada et al., 2013a] and insula activity [Alhaj et al., 2006] and that allopregnanolone influences activity in amygdala [Ossewaarde et al., 2010a; van Wingen et al., 2007, 2008] and ventral striatum [Ossewaarde et al., 2010b], all components of the salience network. We have previously suggested that stronger within-salience network connectivity is associated with greater anxiety [Sripada et al., 2012a,b]. In addition, both PTSD and major depressive disorder were found to be associated with greater salience network activity in recent meta-analyses [Hamilton et al., 2012; Patel et al., 2012]. As elevations in neurosteroids have been associated with symptomatic improvement in anxiety disorders [Rasmusson et al., 2006], the observed reduction of intrasalience network connectivity could suggest a potential mechanism for this anxiolytic effect.

Finally, pregnenolone reduced amygdala connectivity with dmPFC, precuneus, and hippocampus. Across groups, greater amygdala to precuneus connectivity was associated with greater PANAS-X negative affect (10 items assessing constructs of fear, hostility, guilt, and sadness) [Watson and Clark, 1994], suggesting that a reduction in this connectivity is associated with less negative affect. In monkeys, the amygdala is structurally connected with dorsal precuneus [Leichnetz, 2001], ventral precuneus [Parvizi et al., 2006], and dmPFC/dorsal anterior cingulate cortex [Ghashghaei et al., 2007] and in humans, amygdala typically shows negative functional connectivity with the precuneus and dmPFC at rest [Roy et al., 2009; Zhang and Li, 2012]. This is consistent with the key roles of the dmPFC and precuneus in the default network, a network that is anticorrelated with salience network at rest [Fox et al., 2005]. The default network is associated with stimulus-independent, internally focused thought, including spontaneous cognition, autobiographical memory, prospection, and mind wandering [Menon, 2011; Qin and Northoff, 2011; Spreng et al., 2009; Toro et al., 2008], thus this network is active during nonstructured tasks such as resting state. The current data suggest that allopregnanolone might further reduce functional connectivity between amygdala and default network, contributing to enhanced segregation between default network and salience network.

Recently, our group has proposed that decreased segregation, that is, inappropriate connectivity between salience network and default network, contributes to anxiety psychopathology [Sripada et al., 2012b]. Among participants with PTSD, we found that greater functional connectivity between default network and salience network regions was correlated with elevated PTSD symptoms [Sripada et al., 2012b]. Similarly, enhanced cross-network connectivity between default network and thalamus (a node in salience network) has been observed in PTSD in a different study [Yin et al., 2011]. Furthermore, enhanced connectivity between default network and amygdala predicted the development of PTSD symptoms in acutely traumatized individuals [Lanius et al., 2010], and enhanced connectivity between default network and insula was associated with higher self-reported anxiety in a separate study [Dennis et al., 2011]. Precuneus exhibits greater connectivity with amygdala in generalized anxiety [Strawn et al., 2012] and panic disorder [Pannekoek et al., 2013a] and with

anterior cingulate cortex (a key node in salience network) in social anxiety disorder [Pannekoek et al., 2013b]. Moreover, in healthy controls, acute laboratory stress increases amygdala resting-state connectivity with precuneus and posterior cingulate cortex [Veer et al., 2011], although the authors note that this connectivity pattern might indicate healthy rather than pathologic recovery and adaptation in the poststressor period. Another study in healthy individuals demonstrated that viewing fearful when compared with neutral faces enhanced amygdala to precuneus coupling [Pantazatos et al., 2012]. Together, these data evince a consistent pattern in which anxiety disorders or induction of anxiety is associated with reduced segregation between salience network and default network. Thus, allopregnanolone's enhancement of segregation between default network and salience network highlights the potential contribution of this neurosteroid to the amelioration of anxiety.

Although both pregnenolone and DHEA reduced amygdala intrinsic connectivity, pregnenolone exerted a greater effect than DHEA on connectivity with medial prefrontal regions. These differences may be due to differing mechanisms of action. DHEA appears to have anxiolytic-like actions [Maninger et al., 2009; Melchior and Ritzmann, 1994]; however, it demonstrates modest negative modulation of GABA(A) receptors [Imamura and Prasad, 1998]. Thus, its anxiolytic-like actions in rodents may involve other mechanisms. In contrast, allopregnanolone has very pronounced effects at the GABA(A) receptor and decreases GABA(A) receptor responses with 20-fold higher potency than benzodiazepines and barbiturates [Majewska et al., 1986]. 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. Limitations of our sample include the fact that the number of participants in each group was relatively small, thus, our results should be considered preliminary. 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. The between-group design of our study does not allow for a baseline comparison of functional connectivity. Thus, it is difficult to determine whether between-group differences are solely due to the drugs administered or partially due to natural variation. Future studies should implement a crossover design to address this issue. Additionally, we did not obtain baseline measurements of subjective negative affect, and therefore group differences in PANAS-X score may also be partially attributable to natural variation. Limitations of our intervention include the fact that we measured serum levels of allopregnanolone and DHEA, and not CSF or brain levels. However, in animals, neurosteroid levels are highly correlated [Kancheva et al., 2011; Marx et al., 2006a]. Additionally, in the allopregnanolone manipulation, we administered pregnenolone, allopregnanolone's precursor. Although 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. As 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 Sripada et al., 2013b]. We also did not measure levels of additional downstream steroids. Pregnenolone administration may increase levels of pregnenolone sulfate and progesterone [Marx et al., 2009], and DHEA administration may increase estradiol and testosterone [Schmidt et al., 2005]. Thus, it is possible that increases in these downstream steroids, rather than increases in only allopregnanolone and DHEA, contributed to the reported findings. Furthermore, although the bulk of DHEA research supports an anxiolytic effect for DHEA, some studies suggest an anxiogenic role [e.g., Dmitrieva et al., 2001; van Goozen et al., 2000]. Thus, further studies should assess the behavioral impact of DHEA's reduction of amygdala resting-state functional connectivity.

Turning to methodological limitations, our results may have been impacted by the reduced average head displacement of 0.03 mm in the pregnenolone group. For instance, Van Dijk et al. [2012] have suggested that small head movements can inflate measures of local functional coupling and deflate measure of long-range functional coupling. However, we implemented scrubbing procedures as described by Power et al. [2012], which have been demonstrated to significantly reduce confounds introduced by head movement. In addition, it has been reported that DHEA and pregnenolone may potentially impact neurovascular coupling [e.g., see Liu and Dillon, 2004; Naylor et al., 2010]. PET or arterial spin labeling studies could examine this issue directly; however, we compared BOLD signal across treatment groups in control regions (i.e., visual cortex) and found no significant differences. Another potential limitation is that different methodologies were used for assaying different neurosteroids: GC/MS for pregnenolone and allopregnanolone, and enzyme immunoassay for DHEA and DHEAS. DHEA values derived from these two assay methods, however, show excellent correlation [Stanczyk, 2006], and immunoassay is much less labor-intensive and less expensive than GC/MS. In contrast, there are currently no commercially available methodologies for allopregnanolone and pregnenolone that approach the sensitivity and specificity of GC/MS, and it remains the "gold-standard" quantification approach for these steroids. It also does not appear optimal to use GC/MS for sulfated neurosteroids such as DHEAS, given recent challenges and controversies regarding their quantification [see Ebner et al., 2006; Higashi et al., 2003; Liere et al., 2004; Liu et al., 2003; Marx et al., 2009; Schumacher et al., 2008]. 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 [Li et al., 2012]. Future studies should investigate neurosteroid modulation of salience network in anxiety-disordered populations to further assess the benefits and disadvantages of these patterns.

CONCLUSION

In summary, allopregnanolone and DHEA reduced amygdala functional connectivity with other regions within amygdala/salience network and reduced amygdala connectivity 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.

FINANCIAL DISCLOSURES

Dr. Marx discloses that she is an applicant or coapplicant on pending U.S. patent applications on the use of neurosteroids and derivatives for the treatment of central nervous system disorders and for lowering cholesterol (no patents issued, no licensing in place), and she is an unpaid scientific advisor to Sage Therapeutics. The remaining authors have no potential conflicts of interest to disclose.

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