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Sex differences in synaptic plasticity in stress-responsive brain regions following chronic variable stress

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Abstract

Increased stress responsiveness is implicated in the etiology of mood and anxiety disorders, including depression and post-traumatic stress disorder. Additionally, stress-related affective disorders have a higher incidence in women than men. Chronic stress in rodents produces numerous neuromorphological changes in a variety of limbic brain regions. Here, we examined the sex-dependent differences in presynaptic innervation of the paraventricular nucleus of the hypothalamus (PVN), prefrontal cortex (PFC), bed nucleus of the stria terminalis (BST), and amygdala in response to chronic variable stress (CVS). Following 14 days of CVS, the presynaptic protein synaptophysin was assessed in male and female rats. Our results demonstrate that synaptophysin staining density was higher in females than males in all brain areas evaluated, indicating sex differences in the organization of presynaptic innervation. After CVS, the PVN, principal nucleus of the BST (BSTpr), and basolateral nucleus of the amygdala (BLA) displayed significantly reduced synaptophysin density in females but not males. Furthermore, males showed an increase in synaptophysin in the PVN after CVS, suggesting a sex difference in the modulation of presynaptic inputs to the PVN following chronic stress. Overall, these data suggest marked sex differences in PVN, BSTpr, and BLA presynaptic innervation as a consequence of chronic stress, which may be associated with differential stress responsivity and perhaps susceptibility to pathologies in males and females.

Keywords

Presynaptic; synaptophysin; amygdala; prefrontal cortex; paraventricular hypothalamus

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1. Introduction

Onset and severity of affective disorders are associated with stressful life events [1]. The lifetime prevalence of stress-related pathologies such as major depressive disorder and post-traumatic stress disorder is higher in women than men [2,3], and these sex differences in the incidence of mood disorders suggest that brain stress regulatory circuits may be differentially responsive to adverse life experiences.

Dysregulation of the hypothalamo-pituitary adrenocortical (HPA) axis is frequently associated with depression [4]. Glucocorticoid hypersecretion is linked to the symptomatology and consequences of depression, including dysphagia and cognitive deficits [5, 6]. Animal studies indicate that female rats have greater and more persistent corticosterone responses to stress [7-9]. Additionally, clinical studies report that stress induces different adrenocortical responses in men and women [10]. In particular, women have greater cortisol elevation when submitted to social rejection challenges, which may contribute to a greater vulnerability to depression [11].

Sex differences in HPA axis regulation are traced to increased central drive at the level of the paraventricular nucleus of the hypothalamus (PVN), which is the primary site for integration and regulation of corticosterone release [12] as well as sympathoadrenal activation [13, 14]. Multiple limbic forebrain structures, including the prefrontal cortex (PFC) and amygdala, regulate HPA axis activity [15, 16].

Afferents from these regions regulate HPA responses to stress via relays in multiple subcortical loci, including the bed nucleus of the stria terminalis (BST), hypothalamus and brainstem [17]. Specifically, the prelimbic region of the PFC exerts an inhibitory effect on HPA stress reactivity, whereas amygdalar nuclei appear to activate the HPA axis [For review, see 16]. These sites are also involved in the control of autonomic and behavioral stress responses [18-21], suggesting an integrative role in the whole-organism response to stress.

Chronic stress causes marked neuroplastic changes in stress-integrative circuits, including increases in excitatory innervation to the PVN [22], decreased dendritic complexity in stress-inhibitory regions such as the hippocampus [23, 24] and PFC [25, 26], and increased dendritic complexity in stress-excitatory regions such as the basolateral amygdala (BLA) [27].

Although women exhibit a higher lifetime prevalence of stress-related disorders, the effects of stress on the female brain are largely unknown. Importantly, all of the noted forebrain stress-integrative sites contain gonadal steroid hormone receptors [28, 29], and estrogens as well as androgens are known to have trophic effects on limbic neurons [30-33]. These data suggest the possibility that sex difference may dictate the efficacy of stress-inhibitory or – excitatory circuits via effects on synaptic organization. The current study tests the hypothesis that chronic stress, using a chronic variable stress design, will differentially affect stress-regulatory circuits in the female brain. To address this hypothesis, we assessed the innervation patterns of stress-responsive brain regions following CVS using synaptophysin as a marker of presynaptic nerve terminal density [34].

2. Materials and Methods

2.1 Subjects

Male and female Sprague-Dawley rats (Harlan, Indianapolis) weighing 260-295 g (age matched 10-12 weeks) at the beginning of the study were housed two per cage in conventional shoebox rat cages with food and water available *ad libitum* in a temperature and humidity controlled vivarium with a 12/12-hr light /dark cycle (Lights on at 0600 hr). All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2 Chronic Variable Stress

The 14-day chronic variable stress (CVS) paradigm consisted of twice-daily (morning and afternoon) exposures to randomly assigned stressors, with occasional overnight stressors. Stressors included 20 min hypoxia (8% oxygen, 92% nitrogen), 20 min warm swim (26-30°C), 10 min cold swim (17-18°C), 1 hour in cold room (4°C) and 1 hour on platform shaker (100 rpm). The overnight stressor (social crowding, six rats per cage) began immediately after the cessation of the afternoon stressor and ended with the initiation of the next morning stressor.

2.3 Tissue Collection

The morning following the last afternoon stressor, rats received an overdose of sodium pentobarbital and were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde and then transferred to 30% sucrose in KPBS at 4°C. Following 48 hours, brains were sectioned at 25 μm on a sliding microtome and stored in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at -20°C until processing for immunohistochemistry.

2.4 Immunohistochemical Procedures

Sections were transferred from cryoprotectant to 50 mM potassium phosphate buffered saline (KPBS; 40 mM potassium phosphate dibasic, 10 mM potassium phosphate monobasic, and 0.9% sodium chloride) at room temperature (RT). Sections were rinsed (5 x 5 min) in KPBS then transferred to blocking solution (50 mM KPBS, 0.1% bovine serum albumin (BSA), and 0.2% Triton X-100) for 1 hour at RT. Sections were incubated overnight at 4°C in primary synaptophysin antibody diluted in blocking solution (Zymed Laboratories - San Francisco, CA, rabbit antisera, dilution 1:300). The following morning sections were rinsed in KPBS (5 x 5 min) and incubated in fluorescent Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA), diluted 1:500 in KPBS + 0.1% BSA for 1 hour at RT. Sections were rinsed five times in KPBS at RT following the final antibody incubation, mounted onto Superfrost Plus slides and coverslipped with Gelvatol.

2.5 Quantification of synaptophysin immunoreactivity

The images were taken on a Zeiss fluorescent microscope with Apotome attachment (AxioImager.Z1 microscope). The images from PFC, amygdala and BSTpr were collected using a 40x oil immersion lens while those from PVN were collected using a 63x oil immersion lens. These images were analyzed based on the Atlas of Swanson [35] and quantified by individuals blind to the sex and treatment (control or CVS). Using Apotome deconvolution software, twenty five bilateral z-stacks from each animal were collected for each site investigated .

All z-stack image collecting was organized using Zeiss LSM Image Browser Software version 3.5.0.376. Five consecutive z-stack images were compressed and a single projection for each combination of z-stack was created. Three projections from the middle of each z-stack were created and subsequently used to quantify the percentage of the field occupied by synaptophysin immunoreactivity.

The field percent area occupied by the labeled synaptophysin was obtained using Apotome deconvolution and Axiovision 4.6. software. The threshold for pixel inclusion was obtained by analysis of several random projection images and was held constant for all images analyzed. The field density occupied by synaptophysin immunoreactivity was determined by averaging across the projections taken from each animal. Finally, the field percent area was averaged across animals by sex and treatment group.

2.6 Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Adjusted thymus and adrenal weights were calculated as organ weight divided by final body weight $\times 100$. Body weights, organ weights and the area occupied by synaptophysin-labeled terminals were analyzed by two-way factorial analysis of variance (ANOVA) with experimental condition (control or CVS) and sex as factors. When significant main effects for sex, treatment, or interactions were obtained, data were further analyzed by post hoc Duncan tests. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Body and Organ Weight

Body and organ weights were assessed to confirm the efficacy of CVS. Both male and females exposed to CVS decreased body weight gain ($F(1,37) = 40.23$, $p < 0.05$) and increased adrenal weight ($F(1,37) = 8.87$, $p < 0.05$) relative to respective controls (Table 1). However, there was no main effect of CVS on thymus weight ($F(1,37) = 0.06$, $p > 0.05$) in either females or males.

3.2 Prefrontal Cortex

The effect of chronic stress on pre-synaptic terminal density in stress-related brain regions was quantified by synaptophysin immunoreactivity. There was no significant effect of CVS or sex $\times$ stress interaction on synaptophysin staining density in infralimbic (Fig. 1B) or prelimbic prefrontal cortices (Fig. 1C). However, our statistical analysis revealed a

significant main effect of sex. Synaptophysin immunoreactivity was significantly higher in females than males in both prelimbic ($F(1,34) = 31.10$, $p < 0.05$) and infralimbic ($F(1,34) = 49.06$, $p < 0.05$; Fig 1A-C) cortices.

3.3 Amygdala and Bed Nucleus of the Stria Terminalis

Statistical analysis revealed a significant sex $\times$ stress interaction on synaptophysin staining density in the BLA ($F(1,31) = 5.60$, $p < 0.05$) and in the principal subnucleus of the BST (BSTpr) ($F(1,28) = 4.82$, $p < 0.05$). Post-hoc analysis indicated a significant reduction in the area occupied by synaptophysin-labeled terminals within the BLA (Fig. 2C) and BSTpr (Fig. 2D) of females, but not males. In addition, significant effects of sex on synaptophysin densities were observed in the CeA ($F(1,32) = 10.95$, $p < 0.05$), MeA ($F(1,31) = 24.57$, $p < 0.05$), BLA ($F(1,31) = 20.93$, $p < 0.05$), and BSTpr ($F(1,28) = 51.98$, $p < 0.05$), with females exhibiting higher staining density than males (Fig. 2A-D).

3.4 Paraventricular Hypothalamus

In the PVN, there was a significant interaction of CVS and sex ($F(1,33) = 33.16$, $p < 0.05$). Synaptophysin staining was attenuated in females, but increased in males (Fig. 3A and B). Furthermore, there was a significant main effect of sex ($F(1,34) = 7.95$, $p < 0.05$), with females having overall greater synaptophysin staining than males.

4. Discussion

The results of this study support the hypothesis that chronic stress produces significant neuromorphological changes in stress-regulatory brain regions. Exposure to chronic stress (14 days of CVS) altered presynaptic innervation in both female and male rats. Synaptophysin staining suggests that chronic stress causes retraction of synaptic inputs to key stress regulatory regions, including the BLA, BSTpr and PVN, in females but not males. Indeed, synaptophysin staining was increased rather than decreased in the PVN of males, consistent with a previous study from our group [22]. The data are consistent with a differential effect of stress on limbic synaptic plasticity in females.

Synaptophysin, an integral Ca^{2+} -binding synaptic vesicle membrane glycoprotein [36], is found in presynaptic neurons of diverse vertebrate species [37]. Synaptophysin is required for vesicle fusion and neurotransmitter release [38] and is used as a marker of pre-synaptic nerve terminal density [39]. Reduction in the level of synaptophysin is consistent with a decrease in synaptic density [40]. For instance, synaptophysin mRNA and protein in the hippocampus are down-regulated by stress [41-43] and up-regulated by antidepressant drugs [44]. Furthermore, electron microscopy confirms that repeated stress decreases the total synaptic density in CA3 hippocampal subfield [45-46]. Together, these findings confirm that chronic stress decreases synaptic density in brain areas important for negative feedback control of the HPA axis, which may be involved in impaired HPA axis inhibition.

Repeated stress exposure leads to changes in the function of the HPA axis in rodents, including hypersecretion of corticosterone during the circadian trough [47] and facilitated HPA responses to novel stressors [48]. This increase in HPA axis responsiveness after

chronic stress is associated with increased excitatory and/or decreased inhibitory innervation of the parvocellular PVN [22, 49, 50].

In males, chronic stress increases glutamatergic and noradrenergic innervation of CRH neurons [22], suggesting an enhanced excitatory drive. However, presynaptic innervation of the parvocellular PVN decreases, rather than increases in females. In combination with the demonstrated efficacy of the CVS protocol (decreased body weight, adrenal hypertrophy) and known excitatory effects of CVS on CRH mRNA expression [47], the data raise the possibility that plasticity in the female may take the form of reduced inhibitory innervation (e.g., reduced GABAergic tone). Additional experiments are required to establish the phenotype of PVN synaptic changes in the female.

Reduced PVN input in the female may be related to changes in upstream regions also affected by CVS exposure. For example, the posterior BST, which encompasses the BSTpr, receives limbic input and provides GABAergic inhibition of the PVN [16, 51]. In addition, the BLA, a stress excitatory region, is innervated by GABAergic synapses from multiple types of inhibitory interneurons [52, 53]. Given knowledge of an anatomical pathway interconnecting the BLA, posterior BST and PVN, the data support a multi-synaptic model of chronic stress-induced decreases in presynaptic GABAergic terminals that can lead to disinhibition of HPA responsiveness.

Regardless of experimental condition, synaptophysin staining density was significantly higher in females than males in all stress-responsive brain areas investigated. This sex dependent effect on presynaptic innervation patterns may be determined by trophic influences of ovarian hormones. For example, ovarian hormones (i.e., estradiol) modulate neurotrophic factor systems [31, 54-56], suggesting a connection between estrogens and growth-related processes supporting synaptic connectivity and efficacy.

In addition, estrogens play a critical role in the organization of synapses in the developing brain (for review, see [32, 57]), suggesting that similar effects may be in play during the process of synaptic reorganization. In fact, the appearance of dendritic spines in some brain regions is dependent on variations in estradiol during the estrous cycle [58-61]. Thus, the observed increases in synaptophysin staining density may be due to organizational and/or activational effects of gonadal steroids. Future studies will be required to determine the necessity of estrogens and/or androgens for limbic synaptic density in the two sexes.

Previous studies support sexual dimorphisms in synaptic innervation, as females have a greater density of dendritic branching and spines in the arcuate nucleus of the hypothalamus [62] and more dendritic spines and axospinous synapses in the hippocampal subiculum [63]. Increased availability of post-synaptic structures support our findings that females may have increased pre-synaptic innervation in stress-related brain regions, and further implicate differences in brain organization in the sexual dimorphism of behaviors.

5. Conclusion

Overall, the current data present a general sex difference in the synaptic plasticity of stress-regulatory neural circuitry that may be associated with differential acute stress responsivity

in males and females. In addition, the pronounced sex differences in limbic presynaptic reorganization after stress may be linked to male-female differences in the susceptibility and/or severity of stress-related diseases in humans.

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Figure 1.

Effects of sex on synaptic terminal density in PFC. Images (A) were taken from the infralimbic cortex of female and male control rats, and represent the projections of five optical section $0.5\mu\text{m}$ apart using 40x objective. Graphs (B and C) depict the effect of chronic stress exposure on synaptophysin staining density in infralimbic (B) and prelimbic (C) regions. Data are expressed as mean percentage of occupied area $\pm$ SEM; $n = 8-10$. # Male is significantly different from female at $p < 0.05$ by Duncan *post hoc* test. Scale bar = $100\mu\text{m}$.

Figure 2.

Effect of chronic stress exposure on synaptophysin staining density in the central (A), medial (B), and basolateral nuclei (C) of the amygdala and principal nucleus of the BST (D). Data are expressed as mean percentage of occupied area $\pm$ SEM; $n = 8-10$. *CVS is significantly different from control at $p < 0.05$ by Duncan *post hoc* test. # Male is significantly different from female at $p < 0.05$.

Figure 3.

Effects of CVS on the density of synaptic terminals in the PVN. Images (A) were taken from the PVN of control females and CVS females, and represent the projections of five optical section $0.5\mu\text{m}$ apart using 63x objective. Panel B represents the effect of chronic stress exposure on the synaptophysin staining density in the PVN of male and female rats. Data are expressed as means percentage of occupied area $\pm$ SEM; $n = 8-10$. *CVS is significantly different from control at $p < 0.05$ by Duncan *post hoc* test. # Male is significantly different from female at $p < 0.05$. Scale bar = $100\mu\text{m}$.

Table 1

Body and organ weight.

	Experimental Groups			
	Female control (n = 10)	Female CVS (n = 12)	Male control (n = 10)	Male CVS (n = 9)
Body weight change (% from original weight)	2.94 ± 1.75	-1.22 ± 0.89 *	11.50 ± 1.61	-1.15 ± 0.78 *
Thymus weight (% body weight ×100)	7.66 ± 0.61	8.33 ± 0.35	11.69 ± 0.46	11.24 ± 0.64
Adrenal weight (%body weight ×100)	2.37 ± 0.09	2.64 ± 0.10 *	1.18 ± 0.07	1.45 ± 0.08 *

Data are expressed as mean ± SEM.

* p < 0.05 compared to respective control group by Duncan test.