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Stressful experience has opposite effects on dendritic spines in the hippocampus of cycling versus masculinized females

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Abstract

Stress increases associative learning and the density of dendritic spines in the hippocampus of male rats. In contrast, exposure to the same stressor impairs associative learning and reduces spine density in females. These effects in females are most evident when they are in the proestrus phase of the estrous cycle. An injection of testosterone at the time of birth masculinizes the female brain. In adulthood, masculinized females respond like males do to stress, i.e. they learn better. Here, we hypothesized that stress would increase spine densities on pyramidal neurons in area CA1 of the hippocampus of masculinized females, because stress enhances learning ability in both males and masculinized females. To test this, we used Golgi impregnation to stain tissue from masculinized and cycling females that were exposed to the acute stressor and sacrificed one day later. There was a significant interaction between stressor exposure and testosterone treatment at birth ($p < 0.001$). In general, cycling females that were stressed tended to possess fewer spines on apical and basal dendrites in the CA1 area of the hippocampus, whereas the masculinized females possessed significantly more spines after the stressor. These findings underscore the plastic nature of dendritic spines. They suggest that their response to stress in adulthood is organized by the presence of testosterone during very early development. Such a process may represent a mechanism for altering learning abilities after an acute traumatic experience.

Keywords

Testosterone; Organizational effects of hormones; Masculinized females; Hippocampus; Spines; Stress

Introduction

It has long been thought that dendritic spines may be involved in the acquisition and retention of associative memories [13, 26]. Associative learning, as well as spatial memory tasks can increase the presence of dendritic spines in the hippocampus, especially in the area CA1 of Ammon's horn [12, 20]. In addition to learning, other experiences such as stressful encounters and environmental enrichment can alter their presence in the hippocampal

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formation [1, 14, 21, 23, 28, 39]. Moreover, changes in internal hormonal state, such as fluctuations in estrogen across the female estrous cycle, alter their presence. The densities of spines reach their highest numbers during proestrus, when estrogen levels are most elevated [28, 36, 37].

In previous studies, we observed that exposure to an acute, but intense stressful event of brief periodic tail shocks greatly increased the presence of dendritic spines in the CA1 area of the male hippocampus. In contrast, exposure to the same stressor reduced spine density in the females, especially if they were exposed to the stressor in diestrus and sacrificed in proestrus, when estrogen levels were most elevated [28, 29]. Exposure to the same stressor that alters spine density also alters associative learning during classical eyeblink conditioning. Like the effects on spines, the effect of stress on learning are expressed in opposite directions in males and females [33–35]. In male rats, acute stress increases spine density and also increases learning [27, 28, 32]. In female rats, exposure to the stressor decreases spine density and also decreases associative learning [2, 28, 30]. The effects of stress on this type of learning in both males and females depend on the hippocampus [4]. Therefore, it is clear that some type of neuronal process is occurring within the hippocampus that mediates these effects of stress on learning. It is possible that this process involves the production of dendritic spines in response to stress.

Sex differences in synaptic spines and learning are both well-documented. Such sex differences are either organized during early brain development or activated by the presence of sex hormones in adulthood. In adult females, the effects of sex hormones are evident. Numbers fluctuate across the estrous cycle and respond to exogenous application of estrogen and progesterone in ovariectomized animals [8, 19, 36, 37, 39]. In adult males, androgens exert activational effects on spine densities in the hippocampus. For example, testosterone treatment increases spine densities in gonadectomized male rats [11, 16]. The effects of stress on associative learning are also influenced by the presence of testosterone during early brain development. Females that are exposed to testosterone at birth behave like males; they learn better after stress, as do males [3, 31]. From these data, it is suggested that the presence of more dendritic spines after stress enhances learning abilities during classical eyeblink conditioning. Therefore, in this experiment, we hypothesized that stress would enhance the presence of dendritic spines in the hippocampus of the masculinized female.

Methods

Experiments were approved by the Rutgers University Animal Care and Facilities Committee and are in compliance with the rules and regulations specified by the “PHS policy on Humane Care and Use of Laboratory Animals” and the “Guide for the Care and Use of Laboratory Animals”. Adult Sprague-Dawley female rats (250–350 g; 2–3 months) were housed alone with food and water ad lib. To masculinize the brain, female pups (n=13) were injected once subcutaneously, within 24 hours of birth with 0.02 ml of 6.25 mg/ml (125 µg total) of testosterone propionate (TP, Sigma- Aldrich Inc.) dissolved in sesame seed oil. Female pups (n=13) from multiple litters were injected with 0.02ml of vehicle alone (sesame oil). This treatment has been used frequently to study the organizational effects of testosterone on the brain. It effectively masculinizes the brain and many aspects of sexual behavior [6, 24, 31]. Successful masculinization of female rats was verified in adulthood by inspection of the vagina and vaginal smears [6]. Masculinized females did not undergo vaginal canalization or first estrus, which normally occurs in Sprague Dawley females during puberty around the postnatal day 35 [9]. Stages of estrus in vehicle-treated female rats were determined with daily vaginal smears, as previously described [9]. Two vehicle-treated females were excluded from the study before stress exposure, due to irregular estrous cycles.

Adult masculinized females (n=6) were stressed for 30 min by restraining them in a Plexiglas holder and exposing them to 30, 1 mA, 1 sec, shocks, delivered 1/min to two electrodes on each side of the tail [28, 29]. Vehicle-treated females (n=5) were stressed in diestrus 2 and sacrificed in proestrus, based on previous findings [28]. Groups of masculinized (n=7) and proestrus (n=6) females were not stressed and served as naïve controls. For sacrifice, rats were deeply anesthetized with an i.p. injection of 75mg/kg sodium pentobarbital and were rapidly decapitated. All animals were sacrificed in the afternoon (3:00 – 4:00 p.m.) and 24h after stress exposure. For dendritic spine analysis, we used the Golgi impregnation technique that consisted of a modified version of the FD Rapid GolgiStain™ Kit (FD NeuroTechnologies, Inc.) adapted for a vibratome [7]. Specifically, the brains were immersed in an impregnation solution containing potassium dichromate, mercuric chloride and potassium chromate (provided in the Kit) and remained undisturbed in the dark for 3 weeks. They were immersed in 30% sucrose for two days to protect them from drying. Coronal sections (100 μ m) of the entire hippocampus were cut on a vibratome and stored in 15% sucrose in the dark at 4°C. Prior to developing, all sections were mounted onto Super-frost slides (Fisher), placed in a humidity chamber in the dark, and then dried overnight at 4°C. For development, slides were incubated in 15% ammonium hydroxide for 30 min, rinsed in distilled water, incubated in undiluted Kodak photo fix for 30 min, rinsed with distilled water, taken through graded alcohol series (50–100% ethanol, 1 min each rinse) with an extra 100% ethanol rinse for 5 min, cleared in xylene for 5 min, and coverslipped with Permount.

After drying, spine density analysis was conducted blind to experimental conditions. Both apical dendrites of stratum radiatum and basal dendrites of stratum oriens were counted in fully impregnated pyramidal cells of the CA1 area of the hippocampus, as previously described in detail [12, 28, 29]. Briefly, spines were counted, with an optical microscope (Nikon Eclipse E400) using 1000x magnification, in 5 segments of apical (50 μ m away from the soma) and basal (30 μ m away from the soma) dendrites of 6 different pyramidal cells that were distinct from neighbor cells. Then spines were expressed as density (number of spines per 10 μ m of dendrite) by dividing the number of spines on a segment by the length of the segment. The densities of the spines on 5 segments of a cell were averaged for a cell mean, and the 6 cells from each animal were averaged for an animal mean.

Results

Females in proestrus possessed ~14–15 spines per 10 μ m on both the apical and basal dendrites of the pyramidal neurons in area CA1 of the hippocampus. One day after exposure to the stressor, the numbers decreased by ~10%. Masculinized females possessed slightly fewer spines than intact females. After exposure to the stressor, however, the numbers of spines increased by ~20%. This relatively dramatic response to stress was apparent on both the apical and basal dendrites of the CA1 area of the hippocampus (Figure 1a and b). The data were analyzed with analysis of variance using testosterone treatment (testosterone versus vehicle) and stressor exposure (stress versus no stress) as the independent measures and the density of spines as the dependent measure. Two-way ANOVA revealed an interaction between treatment at birth (testosterone versus vehicle) and condition (stress versus no stress) on the density of dendritic spines in the CA1 area of the hippocampus [basal dendrites: $F_{(1,20)} = 21.3$; $p < 0.001$, apical dendrites: $F_{(1,20)} = 22.3$; $p < 0.001$]. *Post-hoc* analysis with Tukey tests revealed that stress increased spine density in the apical and basal dendrites of masculinized females (testosterone-treated), in comparison to vehicle-treated controls ($p < 0.01$) (Figures 1 and 2). Conversely, exposure to the stressor tended to decrease spine density on apical and basal dendrites of vehicle-treated females, although the effect was not significant ($p = 0.24$ and 0.14 , respectively) (Figures 1 and 2). Therefore, the

density of dendritic spines on apical and basal dendrites of stressed masculinized females was higher than in the stressed vehicle-treated females ($p < 0.01$) (Figure 1).

Discussion

The results presented here indicate that exposure to one acute stressful event has long-lasting repercussions on the numbers of dendritic spines in the female hippocampus. Moreover, the effect of stress and the “direction” of that effect seem to depend on the presence of gonadal hormones during very early development. Specifically, we found that exposure to one episode of brief intermittent tail shocks (1 sec shock, 1 per minute over 30 minutes) enhances the density of dendritic spines in the hippocampus of masculinized female rats. The effects of stress on spine density were evident on the apical and basal dendrites of the area CA1 pyramidal neuron. The effects are very similar to those that have been reported for adult males and the opposite of those reported for intact females [28, 29].

Exactly how testosterone exposure in development alters the female response to stress in adulthood is unclear. It may be that testosterone preconditions the animal to respond with an increase in available synapses after an acute stressful experience. This process may represent an anatomical substrate for preparing an animal to learn well and make rapid associations between events in the future [13], one that is typically suppressed in females by stress. Because exposure to this stressor enhances associative learning in males and masculinized females, but impairs learning in intact females, the data support our initial hypothesis that the presence of dendritic spines in response to stress predicts the effects of that stressor on associative learning. The present analysis provides yet another link between the presence of dendritic spines and associative learning of the classically conditioned eyeblink response [31]. It is noted that we have also recently found that the effects of stress on learning in both sexes are dependent on the hippocampal formation [4]. Thus, changes in spines within the hippocampus are a viable mean for altering rates of learning or minimally, performance of classically conditioned fine motor responses.

During development, testosterone, through its conversion to estradiol in the brain, organizes male sexual behavior, as well as the morphology and function of sexually dimorphic brain regions, such as the hypothalamus [17, 18]. However, recent findings suggest that testosterone can also exert developmental effects on the hippocampus, as well as on aspects of learning and memory [10, 18, 24, 31]. The present data similarly suggest that stress effects on the hippocampus can also be organized during the perinatal critical period of steroid exposure, although the exact mechanism is unknown. Upon puberty and into adulthood, these organizational effects are combined with activational effects of hormones to produce sex differences in learning and the opposite effects of stress on learning in males versus females [9, 30].

In the present study, stressor exposure tended to decrease spine density in the unstressed females, as before [28, 29], but the effect on its own was not significant. The lack of a greater effect may be attributed either to the stress of the injection on the day of birth or perhaps to hormone concentrations at the time of sacrifice. Estrogen and progesterone levels are critical for the effects of stress on females and a decrease in dendritic spines after stress is only apparent when females are sacrificed in proestrus, when estrogen levels and density of spines are high [28, 36]. Essentially then, exposure to the stressor prevents the increase in spine density that would normally occur during proestrus. In the present study, all vehicle-treated females expressed a normal estrous cycle. However, it was not feasible to sacrifice all animals at the exact same time during proestrus. Therefore, there is likely some difference in the concentrations of estrogen and progesterone among cycling females, at the time of sacrifice. It is also noted that masculinized females do not cycle and have very low

levels of estrogen relative to cycling females [31]. This might explain, at least in part, the increase in spine density observed after stress in the masculinized female hippocampus. Interestingly, we observed a similar increase, but of a smaller magnitude in females that were stressed in estrus and sacrificed in diestrus, when estrogen levels are low. These results suggest that the increase in spine density after stress may relate to very low levels of estrogen in the bloodstream, perhaps like those observed in males [28]. However, more experiments would be necessary in order to evaluate this idea. It would be informative to administer exogenous estrogen to masculinized females. In this case, one might predict that these masculinized females would produce fewer spines after stressor exposure, as cycling females do. Recently, it was reported that the local synthesis of estrogen in the hippocampus itself mediates the changes in spine density across the estrous cycle [22, 23]. However, it was also reported that cyclic changes in the gonadotrophin-releasing hormone during the estrous cycle modulate the local production of estrogen in the hippocampus [22]. These findings may relate to the present ones because the production of the gonadotrophin-releasing hormone is highly sexually dimorphic and its pattern of secretion is organized during perinatal development [18].

From the present findings, it seems that testosterone's high circulating levels in the bloodstream of adult males are not necessary for the enhancement in dendritic spines after stress exposure, because masculinized females, which have relatively low circulating testosterone concentrations [31], also produced a greater number of dendritic spines after stress. Nonetheless, we expected that unstressed masculinized females would possess fewer spines than intact females, resembling adult males of previous studies [28]. We did not observe such an effect in the present study. However, it is important to note that masculinized females differ from intact males in two aspects. First, they have not undergone through puberty and the surge in testosterone and secondly, as adults they have low testosterone circulating levels, both of which affect spine densities in males [5, 15]. It would probably be useful to compare the effects of stress on spine density in castrated males versus masculinized females.

Stress effects on dendritic spines in the present study cannot be attributed to changes in circulating corticosterone levels for several reasons. First, corticosterone levels are elevated in the plasma of both males and females in response to acute stress, but the density of spines can be either increased or decreased, depending on sex [28]. Second, spine density was assessed 24 hours after stressor exposure when circulating levels of corticosterone have returned to basal levels [34]. That said, it is certainly possible that other components of the HPA axis contribute to these distinctive effects of stress on spine density in the masculinized brain [25].

The enhancement of spine density during proestrus, as well as the opposite effects of stress on dendritic spines in the hippocampus of males and females, depend on the activation of NMDA receptors [29, 38]. Thus, the effects of stress in the present study are also likely mediated by changes in the density, sensitivity, or activity of the NMDA-receptors in the hippocampus [18]. Whatever the mechanism, these results underscore the powerful organizing effects of gonadal hormones in the hippocampus and the consequences for the stress response in adulthood.

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Figure 1. Stress increases density of dendritic spines in the apical and basal dendrites of masculinized females

(a) Graph presents the density of spines (number of spines/ 10µm) in the apical dendrites of pyramidal cells in the CA1 area of the hippocampus (stratum radiatum) in unstressed and stressed females treated with vehicle (oil) or testosterone at birth (masculinized females). (b) Graph presents the density of spines (number of spines/10µm) in the basal dendrites of pyramidal cells in the CA1 area of the hippocampus (stratum oriens) in unstressed and stressed females treated with vehicle (oil) or testosterone at birth (masculinized females). Dendritic spines were assessed 24h after exposure to an acute stressful experience. Vehicle-treated females were in diestrus 2 during stress exposure and in proestrus during sacrifice. Means $\pm$ standard errors are presented in the graph. Asterisks depict statistical significant *post-hoc* differences ($p < 0.05$).

Figure 2.

(a) Representative picture of the apical dendrites of a pyramidal cell in the CA1 area of the hippocampus (stratum radiatum) of a vehicle-treated unstressed female. (b) Schematic of a Golgi-impregnated CA1 pyramidal cell illustrating the apical and basal dendrites. (c) Representative pictures of dendritic spines in the apical dendrites of pyramidal cells in the CA1 area of the hippocampus of the four groups: no stress/oil-treated females, stress/oil-treated females, no stress/ testosterone- treated females (T) and stress/ testosterone- treated females (T).

Golgi impregnation was used to stain complete neurons and dendritic processes. All pictures were taken with an optical light microscope at magnification 1000x. Scale bar is 1μm.