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SEX DIFFERENCES IN DENDRITIC ATROPHY OF CA3 PYRAMIDAL NEURONS IN RESPONSE TO CHRONIC RESTRAINT STRESS

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Abstract—The present study investigated the effects of 21 days of chronic restraint stress on neural and endocrine parameters in male and female rats. Consistent with previous results, repeated restraint stress induced apical dendritic atrophy (a decrease in the number of apical branch points and dendritic length) of the CA3c pyramidal neurons in male rats. In contrast, female rats did not show significant dendritic atrophy in the apical field in response to repeated restraint stress. Female rats did show a decrease in the number of branch points in the basal dendritic tree compared to male rats in response to repeated restraint stress. Baseline and stress levels of plasma corticosterone were higher in female rats compared to male rats. Females exhibited slightly longer increases in corticosterone levels throughout the 21 days of restraint stress than males, indicating that the male corticosterone response to stress exhibited greater habituation. Plasma corticosteroid-binding globulin levels of female rats were also higher than those of male rats throughout the experiment. There was no change in plasma corticosteroid-binding globulin levels in male rats during the restraint stress, while there was a decrease in plasma corticosteroid-binding globulin levels in female rats during the restraint stress. Plasma estradiol levels in female rats also decreased in response to the chronic stress.

In view of the qualitatively different dendritic atrophy found in males and females it appears unlikely that sex differences in the corticosteroid-binding globulin and corticosterone response can account for these morphological differences. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: sex differences, CA3 pyramidal neurons, corticosterone, repeated stress, apical dendrites, basal dendrites.

Previous experiments in this laboratory have found that 21 days of repeated restraint stress induce atrophy of the apical dendrites of hippocampal CA3 pyramidal neurons.[21,30] Both apical dendritic length and the number of apical branch points of CA3 pyramidal neurons were significantly decreased in male rats that had undergone 21 days of restraint stress compared to control male rats. This dendritic atrophy associated with chronic restraint stress was regulated by stress levels of corticosterone and *N*-methyl-D-aspartate (NMDA) receptor-mediated excitatory input.[21,30] Sex differences exist in both of these regulatory factors which suggest that a gender difference may exist in dendritic atrophy following chronic stress.

Most studies examining sex differences in the stress response have focused on the effects of acute stress[7,15,18,26,33] while the possibility that sex differences exist in effects of chronic stress has been largely ignored. Two studies that have investigated sex differences in the effects of chronic stress have found equivocal results. Mizoguchi *et al.*[24] found that 30 days of 15 min of daily cold swim stress caused a significant decrease in the number of CA3 and CA4 pyramidal cells in gonadectomized male rats, while no similar neuronal loss was observed in females. Kennett *et al.*[17] found that female rats failed to adapt (based on behavioural parameters) to five days of immobilization stress, indicating that the behavioural effects of stress were longer lasting in females than in males. To date, no studies have compared the effects of repeated stress on corticosterone or corticosteroid-binding globulin (CBG) levels in the male and female rat. This study was designed to investigate the possibility that sex differences exist in the influence of chronic restraint stress on plasma corticosterone, plasma CBG and CA3 pyramidal neurons.

EXPERIMENTAL PROCEDURES

Animals

Sixteen female (50–56 days old) and 16 male (45–49 days old) 200 g Sprague–Dawley rats were purchased from Charles River. Animals were housed in same-sex pairs in wire-mesh hanging cages. Rats were given Purina rat chow and tap water *ad libitum* and maintained under a 12 h:12 h light/dark cycle (lights on at 7.00 a.m.). All animals were

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Abbreviations: CBG, corticosteroid-binding globulin; EDTA, ethylenediaminetetra-acetate; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate.

kept under these conditions for three weeks prior to the start of the experiment. Four days prior to the start of the restraint procedure, group mean weight for female rats was 237 ± 2.4 g (range 225–255 g) while the group mean weight for male rats was 317 ± 3.85 g (range 290–345 g). All protocols were in accordance with The Rockefeller University guidelines for use of animals in research.

Behavioural and histological procedure

There were two conditions in this experiment: control and stressed, with eight males and eight females in each of the two groups. Individual rats in the stressed group were placed in wire-mesh restrainers for 6 h/day for 21 consecutive days. Control rats were not disturbed during the 21 day period except for body weight determination which occurred for all groups four days before the experiment began and on days 7, 14 and 21 of the experiment. Blood samples were obtained through the tail vein from rats in the stress condition on days 1, 3, 7, 14 and 21 in order to obtain plasma corticosterone and CBG levels. Previously, this stress paradigm has been shown to result in significant atrophy (a decrease in the number of branch points and dendritic length) in the apical dendritic tree of CA3c pyramidal neurons in male rats.[30] Twenty-four hours following the last day of restraint, rats were anaesthetized with methofane and rats were perfused in 4% paraformaldehyde in 0.1 M phosphate buffer with 1.5% picric acid. The brains were postfixed for 24 h at 4°C and processed using a modified version of the single-section Golgi impregnation technique.[30] Briefly, 100 μm coronal sections were cut using an Oscillating Tissue Slicer (Electron Microscopy Sciences) in a bath of 3.0% potassium dichromate in distilled water. Sections were then stored in this solution for 24 h, rinsed in distilled water and slide assemblies were made by mounting the sections onto plain microscope slides and coverslipped with glue at the four corners of the slide. The slide assemblies were then placed in 1.5% silver nitrate in distilled water and placed in the dark. Twenty-fours later, the slide assemblies were dismantled, the sections rinsed in distilled water, dehydrated, cleared and mounted onto slides with permount. A cell was chosen for analysis based on the following criteria: (i) the cell body and extending dendrites were completely impregnated; (ii) the cell was relatively isolated from surrounding impregnated cells; and (iii) the cell was located in the CA3c hippocampal region. Using camera lucida tracings (400×), two variables were measured for both the apical and basal dendrites: the number of branch points and the total dendritic length. Six CA3c pyramidal cells from each brain were analysed, three of the short-shaft type and three of the long-shaft type.[8] Total dendritic length was measured for each dendritic tree using a Zeiss Interactive Digitizing Analysis System. Means of each of these variables were calculated and the data were analysed using an ANOVA with sex and condition (control and stressed) as the between-subjects factors.

Radioimmunoassays

Plasma corticosterone levels were measured using a rat corticosterone Coat-a-Count kit (Diagnostic Products Corporation, Los Angeles, CA). The sensitivity of the assay was 5.7 ng/ml.

Plasma estradiol levels were measured using a kit (Coat-a-Count, Diagnostic Products Corporation) modified for low expected levels of estradiol. The sensitivity of the assay was 8 pg/ml. Due to the low volume of plasma, three time periods were analysed: (i) baseline and 30 min after stress (combined); (ii) 1 and 3 h after stress (combined); and (iii) 6 h after stress.

Measurement of corticosteroid-binding globulin

Serum CBG levels were assessed using a competitive binding assay adapted from Westphal.[32] The samples were initially diluted 1:200 in buffer consisting of 10 mM Trizma base, 1.0 mM EDTA, 10% glycerol (v/v) and 1.0 mM dithiothreitol at pH=8.0. The diluted sample was then mixed with [^{3}H]corticosterone (15 nM) or unlabelled corticosterone (10 μM) at a final dilution of 1:600 and allowed to incubate overnight at 4°C. Bound and unbound steroids were separated using activated charcoal (performed in duplicate). The bound fraction was mixed with scintillation cocktail and counted with a liquid scintillation counter (TriCarb 1600TR, Packard, Meriden, CT). Data are expressed as nmol specific [^{3}H]corticosterone binding (CBG)/litre serum.

Data analysis

The number of branch points and dendritic length were each subjected to a repeated-measures ANOVA with sex and condition (control, stress) as between-subjects factors and region (apical, basal) as the within-subjects factor. Body weight, plasma CBG and protein levels were analysed using a repeated-measures ANOVA with sex and condition as between-subjects factors and day as the within-subjects factor. A repeated-measures ANOVA with sex and condition as between-subjects factors and day and time as the within-subjects factors was conducted for the plasma corticosterone and estradiol levels. *Post hoc* tests utilized Tukey's procedure (α=0.05) unless otherwise stated.

RESULTS

In contrast to females, males showed dendritic atrophy in the apical tree in response to restraint stress

There were sex differences in the effects of repeated restraint stress on dendritic length, with males showing a decrease in the number and length of apical dendrites. Table 1 shows the number of branch points and dendritic length of control and stressed groups of male and female rats in both the apical and basal dendritic trees. Figure 1 represents camera lucida drawings of a representative cell for each of the four groups of rats. Specifically, there was an interaction effect of condition by sex by region on the number of branch points ($F_{1,19}$=14.24, P=0.001). *Post hoc* tests revealed that stressed males had a smaller number of apical branch points than control males (P<0.001), while there was no significant difference between stressed females and control females on apical branch points (P=0.42). For dendritic length, there was a significant main effect of condition ($F_{1,19}$=6.47, P=0.019) and a significant effect of region ($F_{1,19}$=10.05, P=0.005). *A priori*, we were interested in whether there were sex differences in the morphological response to restraint stress. The planned comparison procedure revealed that stressed males had shorter apical lengths than control males (P=0.003) while there was no significant difference between stressed females and control females (P=0.16).

Stressed females had a smaller number of basal branch points than control females

Control females had a larger number of basal branch points than control males (P=0.015) and stressed females (P=0.049). There was no reduction, however, in the total length of the basal dendrites.

Table 1. Means and S.E.Ms for the number of dendritic branch points and the total dendritic length of both apical and basal dendrites of CA3c pyramidal neurons in control and stressed male and female rats

Condition	Apical branch points	Basal branch points	Apical dendritic length (mm)	Basal dendritic length (mm)
Control male (n=6)	16.38 ± 1.19	11.97 ± 0.45	1.58 ± 0.11	1.22 ± 0.12
Stress male (n=5)	9.43 ± 2.04*	13.75 ± 0.91	1.04 ± 0.24*	1.15 ± 0.07
Control female (n=6)	14.47 ± 1.45	15.60 ± 0.76†	1.60 ± 0.09	1.20 ± 0.11
Stress female (n=6)	13.37 ± 1.04	12.75 ± 0.97‡	1.38 ± 0.15	1.03 ± 0.05

Stressed males showed significant dendritic atrophy in the apical dendrites. Stressed females showed evidence of decrease in the number of basal branch points.
*Stressed males had significantly fewer apical branch points (P<0.001) and shorter apical dendritic length (P=0.003) than control males.
†Control females had a significantly greater number of basal branch points than control males (P=0.015).
‡Stressed females had significantly fewer basal branch points than control females (P=0.049).

Stressed females had higher levels of both plasma corticosterone and corticosteroid-binding globulin levels

Plasma corticosterone levels increased in response to restraint stress in both sexes on all days of treatment, although there were sex differences in the magnitude of the response and the habituation of the response during the stress session. Figure 2 illustrates the corticosterone response to the restraint stress for males and females, respectively, across all time points on days 1, 3, 7, 14 and 21 of treatment. The results indicated that females had higher levels of corticosterone than males at all time points (main effect of sex: $F_{1,5}$=17.30, P=0.009). There were also significant main effects of day ($F_{4,20}$=3.94, P=0.016) and time ($F_{4,20}$=20.48, P<.001) and a significant interaction effect of day by time ($F_{16,80}$=2.31, P=0.008).

We were interested *a priori* in sex differences in the corticosterone profile across 21 days of restraint stress. Thus, *post hoc* tests were analysed using sex as a factor. *Post hoc* tests (Tukey's) revealed that for both males and females there was a significant rise in corticosterone levels between baseline and 30 min after the onset of the restraint stress for all five days measured (except for males on day 14). Males had higher corticosterone levels at 30 min after the onset of stress on day 3 compared to days 14 and 21, while female rats had higher corticosterone levels on day 3 compared to day 14, indicating that females had slightly less habituation of the 30 min corticosterone response than males. The general increase in corticosterone levels at the 6 h time point for both sexes indicates the beginning of the increase in circadian corticosterone levels.[5] Figure 3 illustrates that plasma CBG levels were higher in females than males for all time points measured except for day 21 (Tukey's *post hoc* P<0.0002). *Post hoc* tests (interaction effect of condition and sex: $F_{4,40}$=4.26, P=0.006) revealed that female levels of CBG decreased across the time with all four time points showing a decrease from day 1 levels (except for day 14, P=0.09). There was no significant change over the time period for male plasma CBG levels; although there appears to be a rise in CBG levels on day 21 in males, this increase is not significantly different from day 1 (P=0.854).

Plasma estradiol levels and ovarian weight were decreased in response to chronic stress

Females had lower levels of plasma estradiol on day 21 compared to day 1 and 3 during the restraint stress paradigm (Table 2) (day by time interaction: $F_{4,28}$=2.83, P=0.04). Control females had heavier ovaries than stressed females (t_{14}=2.21, P=0.044). The mean ovarian weights for control and stressed females were 183.5 mg (± 8.7 mg S.E.M.) and 154.3 mg (± 9.9 mg S.E.M.), respectively.

Stressed rats had lower body weights than control animals

Table 3 shows the mean and S.E.M.s for all four groups of body weight across the 21 days of restraint stress. *Post hoc* tests revealed that body weight levels were higher in the control group than in the stress group (regardless of sex) during the stress paradigm, although there was no difference in body weight between the two treatment groups prior to the start of the experiment (group by day interaction: $F_{3,81}$=32.53, P<0.0001). There were also sex differences in body weight, with females weighing less than males during all four time points measured (sex by day: $F_{3,81}$=143.35, P<0.0001). Both males and females (regardless of treatment group) gained weight over the three week period.

DISCUSSION

Consistent with previous results, male rats that underwent 21 days of restraint stress showed significant apical dendritic atrophy (a decrease in number of branch points and dendritic length) compared to non-stressed controls.[21,30] Chronically restrained female rats, however, did not exhibit the severity of apical dendritic atrophy that was seen in stressed males. This result is consistent with a study in vervet monkeys, in which stressed males showed evidence of

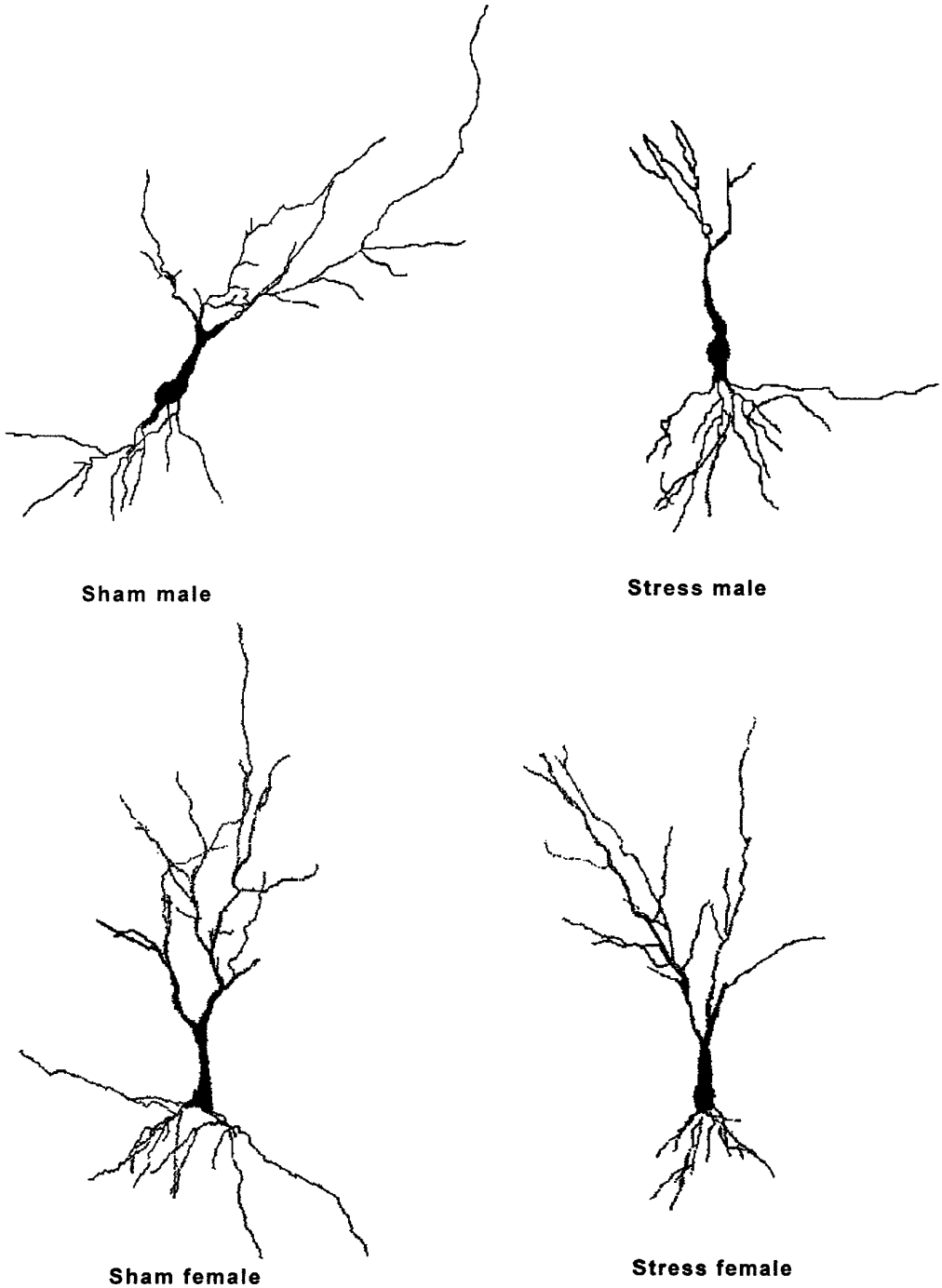

Fig. 1. Camera lucida drawings of representative CA3 pyramidal neurons from each of the four groups of animals. Male rats showed a significant atrophy in the apical dendrites after exposure to restraint stress, while females showed a decrease in the number of basal branch points after repeated restraint stress.

hippocampal pyramidal neuron loss while females did not.[28] However, in the present study, stressed female rats did show a slight decrease in the number of basal branch points compared to control females, unlike stressed males. Stressed females had higher baseline and stress levels of corticosterone than stressed males, and the profile of the female corticosterone response across the 21 days of restraint stress was more robust and longer lasting (up to 1 h compared to 30 min for males) than that of males. Plasma CBG levels were higher in the stressed female rat than in the stressed male rat at all time points measured (except day 21) during the repeated stress paradigm. Interestingly, the higher levels of CBG in stressed females decreased during the repeated stress paradigm, while there was no

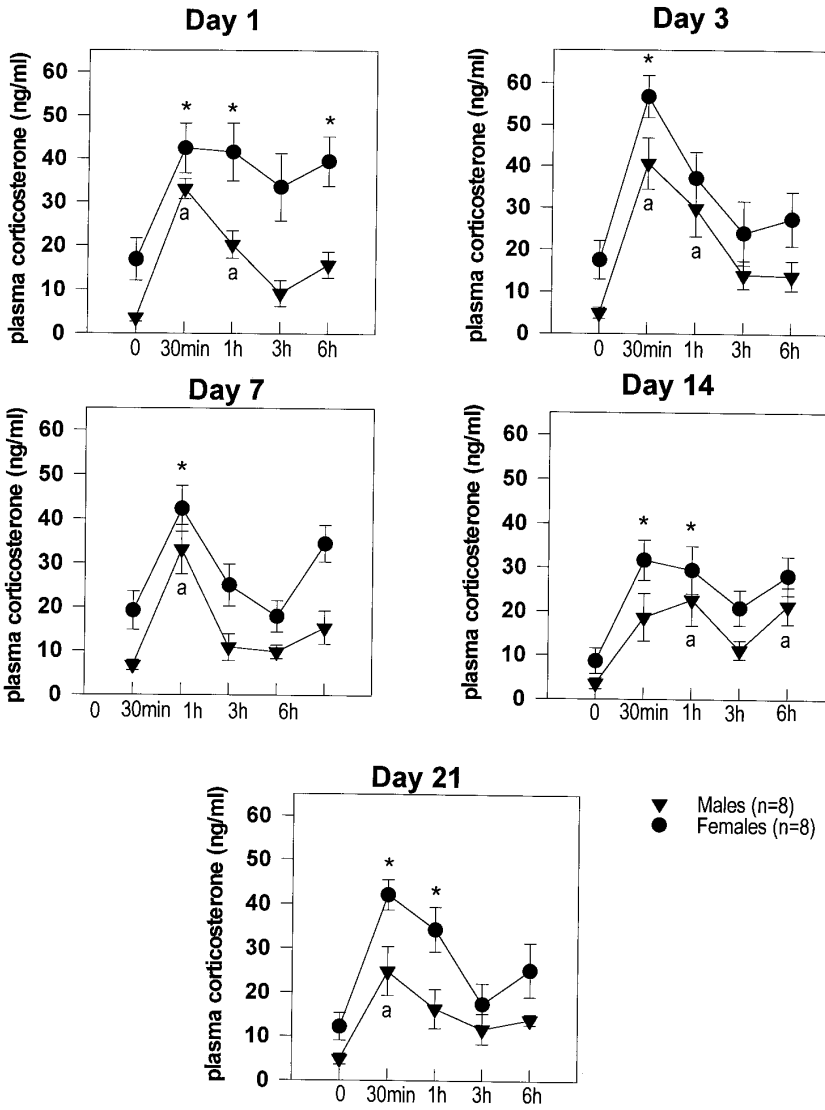

Fig. 2. Group mean (±S.E.M.) levels of total plasma corticosterone at baseline, 30 min, 1 h, 3 h and 6 h during restraint stress for days 1, 3, 7, 14 and 21 in male and female rats. The asterisks signify significant difference from baseline levels for female rats; "a" denotes significance from baseline levels for male rats (Tukey's procedure). $n=8$/group.

significant change in male CBG levels across the stress paradigm.

Possible role of corticosterone, corticosteroid-binding globulin levels and N-*methyl*-D-*aspartate receptors in the sex differences in response to stress*

The stress-induced rise in corticosterone levels is one factor that mediates the dendritic atrophy in repeatedly stressed males.[21] Thus, it is perhaps unexpected that despite the higher stress levels of corticosterone compared to males, stressed females did not show significant atrophy of the apical dendrites. Higher stress levels of corticosterone observed during repeated stress in the female is consistent with studies examining the effects of acute stress.[7,15,18,26,33] This suggests that there may be a protective effect in female rats that prevents the dendritic atrophy in response to repeated stress. Consistent with past literature, female rats in the present study also had higher levels of CBG, which reduces the level of free corticosterone.[23] Higher CBG levels have been shown to decrease glucocorticoid receptor activation among rat strains differing in stress responsitivity.[6] The higher levels of CBG in females seen in the present may be a factor in the "protective" effect seen in females as the free levels of corticosterone may be similar to that of males due to the higher levels of plasma CBG. However, exposure to repeated restraint stress decreased plasma CBG levels in females (presumably resulting in an increase in relative corticosterone levels) while no change in plasma CBG levels was observed in males. Thus, plasma levels of corticosterone and CBG may not completely account

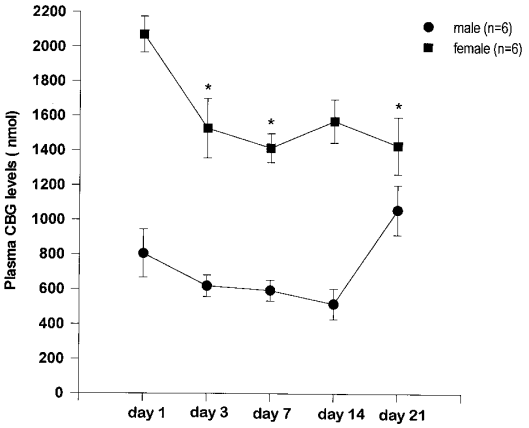

Fig. 3. Group mean (±S.E.M.) levels of total plasma CBG levels for males and females during days 1, 3, 7, 14 and 21 during the restraint stress paradigm. The asterisks signify significant difference from the levels on day 1 (Tukey's procedure). Female CBG levels on day 3, 7, and 21 were significantly decreased from day 1 ($P<0.05$). $n=6$/group.

for the sex differences seen in dendritic atrophy. This indicates that central mechanisms may be involved in the regulation of these sex differences. Some of the potential central factors that may be involved are excitatory amino acids and sex differences in innervation of the CA3 region.

Previous research has shown that both the stress levels of corticosterone and the degree of NMDA receptor activation regulate the dendritic atrophy seen in males after exposure to repeated stress.[21] Thus, sex differences in central NMDA receptor function may contribute to the sex differences in dendritic atrophy. A previous study has shown that there was a stronger NMDA receptor activation in male rats relative to females in the dentate gyrus after high-frequency stimulation of the perforant path.[22] In contrast, female rats showed a greater behavioural response to a systemic injection of the non-competitive NMDA receptor antagonist dizocilpine maleate (MK-801) than did males.[12] The results from these studies suggest that there may be regional differences in NMDA receptor function. Indeed, Weiland[31] has shown that estradiol regulates NMDA receptor agonist binding in the CA1 region of the hippocampus and NMDA receptor antagonist binding in the dentate gyrus in female rats. In addition, after exposure to acute swim stress there are sex differences in the binding of MK-801 and $GABA_A$ in the mouse forebrain, with males showing a greater change in the former and females showing a greater change in the latter.[1,2] Thus the effects of NMDA receptor and corticosterone antagonists on the sex differences in dendritic atrophy should also be explored.

Sex differences in hippocampal morphology

In the present study, repeatedly stressed females showed no significant atrophy of the apical dendrites but did show a decrease in the number of basal branch points compared to control females. Thus, the "protective" effect in females appears to be restricted in one area of the hippocampus and indicates that there may be sex differences in hippocampal neuroanatomy. The main afferent to the CA3 region is from the dentate gyrus and there are sex differences in the dentate gyrus, with male rats having a larger dentate gyrus than female rats.[20,27] However, there are no significant sex differences in the number of synapses from the mossy fibres to the CA3 pyramidal neurons synapses[20] despite significant sex differences favouring males in the number of granule neurons and the volume of the dentate gyrus in some strains of rodents.[20,34] Mossy fibres from the dentate gyrus project to both the apical and basal dendrites of the CA3 neurons. However, the basal dendrites receive input mainly from the infrapyramidal blade while the apical dendrites receive input from all parts of the dentate gyrus.[35] In the present study, sex differences were found in the effects of repeated restraint stress, with apical dendrites affected in males and basal dendrites affected in females. These sex differences in affected region may reflect as yet unreported sex differences in the innervation of these two dendritic fields. However, the fact that we found that females have more basal branch points than males is consistent with this possibility.

Juraska has found sex differences in different regions of the hippocampus, usually with males showing more dendritic intersections (intersection between concentric rings and dendritic field developed by Sholl) in granule cells located in the dentate gyrus than females, although these sex differences depend on the type of housing environment.[13,14] Contrary to previous results, the present study found a sex difference favouring control females in the number of basal branch points in CA3 pyramidal neurons. Juraska *et al.*[14] did not find any sex difference in the number of branch points in the basilar dendritic tree. However, in their study, pyramidal neurons were taken predominately from the CA3b region as opposed to the present study in which pyramidal neurons were taken exclusively from the CA3c region, raising the possibility that there are regional differences. Furthermore, different species of laboratory rats (Juraska *et al.*, Long–Evans hooded rats; present study, Sprague–Dawley rats) were used in the two studies which may also account for the discrepancy.

The possible role of gonadal steroids in sex differences in response to stress

The present finding of sex differences in the response to repeated restraint stress implies that gonadal hormones may be involved. There are known interactions between gonadal steroid levels and the stress response.[11] Plasma corticosterone levels are higher in response to acute restraint stress during the high estrogen phase of the estrous cycle or in estrogen-treated female rats.[3,29] The estrogen state

Table 2. Group mean (±S.E.M.) plasma estradiol levels for stressed females during days 1, 3 and 21 of the stress paradigm for the three time periods during the restraint stress (n=8)

	Baseline and 30 min	1 and 3 h	6 h
Day 1	39.5±6.0	38.9±6.9	31.5±2.2
Day 3	38.7±3.5	35.1±3.6	43.8±0.8
Day 21	21.8±2.6*	23.7±2.1*	21.5±1.5*

Day 21 estradiol levels were significantly different from all time points on days 1 and 3 (P<0.05).

Table 3. Group mean (±S.E.M.) body weights for all four groups for four days prior to the experiment and on days 7, 14 and 21 of the stress paradigm

	Body weight (g)			
	day −4	day 7	day 14	day 21
Control male (n=8)	321±5.2	390±10.8	421±13.9	456±16.7
Stress male (n=8)	313±5.7	342±7.0	367±8.7	398±10.7
Control female (n=7)	234±3.6	246±3.7	256±5.7	265±7.1
Stress female (n=8)	240±3.2	228±4.1	230±4.2	238±4.9

Control rats weighed more than stressed rats during the stress paradigm (P<0.001). Male rats weighed more than female rats (P<0.001).

of a female mouse has profound effects on the neurochemical expression of swim stress-induced analgesia.[16,25] In the adult male rat, gonadectomy appears to affect the amount of neuronal death seen in the CA3 field of the hippocampus as a result of repeated stress.[24] In female rats, estradiol levels mediate structural changes in the CA1 region of the hippocampus and regulate NMDA receptor antagonist binding in the dentate gyrus, the main afferent to the CA3 pyramidal cells.[31] Thus, estradiol level may be an important factor in observing structural changes in the CA3 region after exposure to stress. In the present study, we did not control for differences in estrous cycle and it is possible that there may be differences in the morphology of the CA3c pyramidal neurons depending on the reproductive status of the female rat. Ovarian weight and plasma estradiol levels were, however, significantly decreased in the female rats that had undergone chronic restraint stress, indicating a "shut-down" of gonadal function in the stressed females compared to control females. Experiments should be conducted in order to determine whether estrogen or testosterone level alters dendritic morphology after chronic restraint stress.

Possible functional relevance of CA3 dendritic atrophy in response to repeated stress

After 21 days of restraint stress, male rats perform worse on spatial tasks.[4,19] Female rats that have undergone repeated stress may also show a similar deficit in spatial learning that is related to the reduced basal dendritic branching. A possible confound, however, is that stressed females have lower levels of estradiol than control females, and lower estradiol levels have been linked to better spatial performance.[9,10] It is important to note that the dendritic atrophy seen in males is not permanent and once the stress is removed, the apical dendrites return to baseline levels within seven to 10 days (Magarinos and McEwen, unpublished observations). Interestingly, the behavioural impairment seen in the radial arm maze in chronically stressed male rats is also transient.[19]

CONCLUSIONS

In addition to presenting the first comparison of morphological changes in the male and female rat hippocampus after repeated stress, this study also presents the first comparison of plasma corticosterone and CBG levels in male and female rats exposed to repeated stress. Females had higher basal and stress levels of both plasma corticosterone and CBG than males. They also had a more prolonged increase in plasma corticosterone levels in response to stress than males. Moreover, whereas plasma CBG levels remained unchanged in male rats during exposure to restraint stress, CBG levels were decreased by stress in female rats. In spite of these endocrine sex differences indicating an increase in plasma corticosterone levels overall, females did not show significant apical dendritic atrophy of CA3c pyramidal neurons, unlike male rats. Female rats did, however, manifest an unexpected decrease in basal dendritic branching that points to another structural and functional sex difference in the rat brain.

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