Disturbed molecular and cellular adaptations play a central role in the development of stress-induced neuronal abnormalities. Stress has been proposed to impair neuroplasticity by selectively targeting expression and/or activity levels of key intracellular signaling cascade members involved in transduction of neurotrophin signals. To elucidate stress effects on expression and phosphorylation levels of specific neurotrophin cascade members, rats were exposed to prolonged footshock stress after which phospho-CREB and calcineurin immunoreactivity as well as calcineurin and CREB gene expression were investigated. The hippocampus and prefrontal cortex revealed unaltered CREB and calcineurin mRNA levels yet reduced phospho-CREB and calcineurin immunoreactivity in males, while stressed females illustrated comparatively attenuated alterations. Considering the critical role of CREB and calcineurin in regulating BDNF signaling these findings seem to confirm the deleterious action of stress on neuronal plasticity. Furthermore, this data provides new insights into the mechanisms governing distinct sex-dependent discrepancies often encountered in response to stress.

INTRODUCTION

Disturbed molecular and cellular adaptations represent common predisposing factors for the occurrence of structural and functional neuronal abnormalities. Stress, particularly when persistent and severe, can play a primary role in this pathophysiological process. Common features often observed following prolonged stress exposure for instance include hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and atrophy of apical dendrites in the medial prefrontal cortex and hippocampus. In turn these implicated prefrontal cortex and hippocampus regions, primary targets of subsequent glucocorticoid-mediated damaging effects, also modulate this stress response by actively inhibiting HPA axis activity.

Although to date very little is known about the cellular and molecular mechanisms responsible for stress-induced abnormalities, it has recently been suggested that impaired neuronal function might result from stress' selective influence on expression and/or activity levels of key members of intracellular signaling cascades underlying neuronal plasticity, particularly those involved in neurotrophin signal transduction. Recent efforts to understand the neurobiological substrates underlying neuronal plasticity have demonstrated the importance of cAMP response element binding protein (CREB). Phosphorylated (activated) CREB promotes the transcription of target genes such as brain derived neurotrophic factor (BDNF) leading to structural changes that increase synaptic efficacy and ultimately produce long-lasting alterations of cellular function. Proper maintenance of synaptic plasticity however, requires a carefully regulated balance of phosphorylation and dephosphorylation. While kinase activity represents a crucial component of a gate that regulates neuronal plasticity by phosphorylating critical elements such as CREB, activation of specific phosphatases as calcineurin (PP2B) oppose this action by dephosphorylating target proteins. The coordinated activity of protein kinases and phosphatases in response to specific stimuli thus represents a crucial feature for proper neuronal functioning, especially since abnormal regulation of these intracellular proteins has been associated with neuronal dysfunctions and in severe cases cell death.

We have recently suggested that abnormal CREB phosphorylation and calcineurin expression may account for the development of stress-induced neuronal dysfunctions in prefrontal regions of male rats. In the present study, we extend our investigation of the cellular and molecular adaptations in response to prolonged stress to include both the prefrontal cortex and the hippocampus, in order to determine whether similar mechanisms underlie stress-induced disturbances in both regions. Using this same stress paradigm, our prior findings have also corroborated marked sex differences in the activity of the hippocampo-prefrontocortical pathway as well as the HPA axis response. Since gender is known to represent a critical aspect in both stress sensitivity and psychopathology,
female rats were also included in this investigation. The results presented here may thus provide new insights into the neurobiological substrates underlying gender-related discrepancies and the occurrence of neuronal abnormalities in response to sustained stress.

MATERIALS AND METHODS

Animals

The experiments were performed on male (n°=24; 225-249 g) and female (n°=24; 200-224 g) Wistar rats. The animals were individually housed with food and water available ad libitum and maintained on a 12:12 hr light/dark cycle at 21°C. All rats were handled daily for 5-8 min to minimize the non-specific stress response. All handling was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the guidelines of the Animal Bio-ethics Committee of the University of Groningen (FDC: 2509).

Stress Procedure

The rodent test-chamber consists of a box containing an animal space positioned on a metallic grid floor connected to a shock generator and scrambler. For 3 weeks, stressed rats were subjected to one aversive session of varying duration (between 30 and 120 min/day) in the “footshock box” during which 5 uncontrollable and inescapable footshocks were applied (0.8 mA in intensity and 8 sec in duration). During these daily sessions, starting time, inter-shock interval, and total time spent in the box were randomized in order to make the procedure as unpredictable as possible.

Experimental Setup

To investigate the cellular and molecular changes induced by sustained footshock exposure, rats were randomly assigned to two groups:

- **STRESS** (n° = 24; 12 male and 12 female rats): stressed rats were exposed to the footshock and stressed according to the footshock procedure described above for 21 consecutive days;
- **CTR** (n° = 24; 12 male and 12 female rats): control animals followed an identical schedule in a similar setup, but did not receive any actual footshocks throughout the experiment.

On the final (21st) day of the experiment all rats were placed in the box for 15 minutes, although no footshocks were delivered. It is important to note that exposure to the box on the final day of the experiment was critical as it established a link between a harmless stimulus (environment in which footshocks were applied or “footshock box”) and aversive events (“footshocks”). This provided a way to create a stress condition without
the unwanted side effects of direct changes induced by physical or painful stimuli. The response of control and stress rats to an identical, painless stimulus could then be investigated by evaluating patterns of gene expression (CREB and calcineurin mRNA), protein synthesis (FOS and calcineurin immunoreactivity) and phosphorylation (phospho-CREB immunoreactivity). As stressed animals could learn to associate this environment with aversive footshocks, this design also allowed the opportunity to study the neuroendocrine in addition to immunohistochemical changes underlying the "psychological stress response" without exposure to actual footshock stress.

Physiological and endocrine measurements

On the final day of the experiment, rats used for immunohistochemistry were terminated two hours after the beginning of the final session, while those used for molecular biology were sacrificed 15 minutes following the end of the last stimulus. To explore their response to repeated stress, various physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis and graphs were constructed to illustrate body weight gain changes throughout the procedure. Thymus and adrenal glands were also weighed and recalculated to correct for the body weight of the animals. Blood samples drawn upon termination, were stored at -20°C and used to determine plasma corticosterone and adrenaline concentrations with HPLC. Constructed graphs serve to verify the severity of stress perceived by the animals.

Extraction and Chromatography

Catecholamines.

Noradrenaline and adrenaline were extracted from plasma using liquid/liquid extraction with 3,4-dihydroxybenzylamine as internal standard. Briefly, plasma catecholamines were bound to diphenylborate-ethanolamine at pH 8.6. Extraction was performed with n-heptane (containing 1% octanol and 25% tetraoctylammoniumbromide). Next, catecholamines were extracted from the organic phase with diluted acetic acid. Catecholamines (20 µl acetic acid extract) were analyzed using an HPLC/auto-injector (CMA, Sweden) and a Shimadzu LC-10AD pump (Kyoto, Japan) connected to a reversed phase column (Hypersil, C18, 3 µm, 150x2.0 mm), followed by an electrochemical detector (Antec Leyden, The Netherlands) working at a potential setting of 500 mV vs. Ag/AgCl reference. The mobile phase consisted of 50 mM acetate buffer, 150 mg/l octane sulfonic acid, 150 mg/ml tetramethylammonium, 15 mg/ml Na2EDTA and 3% methanol, adjusted to pH 4.1. The flow-rate was 0.35 ml/min. Temperature was 30°C. The detection limit was 0.1 nM.
Corticosterone.

For this assay, dexamethasone was used as internal standard. After addition of the internal standard, plasma was extracted with 3 ml of diethylether, vortexed for 5 min and then centrifuged for 5 min at 3000 x g. The extraction procedure was repeated twice. The organic phase was evaporated to dryness in a 50°C waterbath. The residue was reconstituted with 200 µL of mobile phase and 50 µL was injected into the HPLC system. The mobile phase (flow rate 1.0 mL/min) for the determination consisted of acetonitrile in ultrapure water (27:73 v/v). The concentration of both corticosterone and the internal standard was determined with UV detection at a wavelength of 254 nm. The detection limit was 10 nM.

Immunohistochemistry

Two hours after the start of the final session, rats were deeply anaesthetized with halothane and transcardially perfused with 4% paraformaldehyde solution in 0.1M sodium phosphate buffer (pH 7.4). The brains were carefully removed and post-fixed in the same solution overnight at 4°C, before being transferred to a potassium phosphate buffer (KPBS 0.02M, pH 7.4) and stored at 4°C. Following cryoprotection of the brains by overnight immersion in a 30% glucose solution, coronal serial sections of 40µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C. All stainings were performed on free-floating sections under continuous agitation.

FOS and phospho-CREB immunohistochemistry.

The sections were preincubated in 0.3% H₂O₂ for 15 min to reduce endogenous peroxidase activity, before being incubated in primary polyclonal rabbit anti-FOS (Oncogene Research Products, brands of CN Biosciences, Inc, an affiliate of Merck KGaA, Darmstadt, Germany; 1:10000 dilution in KPBS 0.02 M, pH 7.4) or anti-phospho-CREB antibodies (Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com; 1:1000 dilution in KPBS 0.02 M, pH 7.4) for 60-72 hr at 4°C. Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 dilution) followed by ABC complex (Vector ABC kit, Vector Laboratories, Burlingame, CA, USA). After another wash, the reaction product was visualized by adding diaminobenzidine as chromogen and 1% H₂O₂ for 15 min. Then, the sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Calcineurin immunohistochemistry.

The sections were preincubated in 0.3% H₂O₂ in PBS (0.1M, pH 7.4) for 30 min to reduce endogenous peroxidase activity followed by a blocking step with 3% normal rabbit
serum for 2 hrs. Sections were subsequently incubated in a primary polyclonal goat anti-calcineurin antibody (Santa Cruz Biotechnology, Inc.; 1:250 dilution in PBS 0.1M pH 7.4, 0.25% triton, 3% normal rabbit serum) overnight at room temperature. Afterwards sections were washed with PBS, and incubated at room temperature with biotinylated rabbit anti-goat IgG (Vector Laboratories, Inc., Burlingame, CA; 1:1000 dilution in PBS 0.1M pH 7.4, 0.25% triton, 3% normal rabbit serum) followed by the ABC complex (Vector Laboratories, Inc., Burlingame, CA). After another wash, visualization of the reaction product was accomplished by adding diaminobenzidine as chromogen and 1% H$_2$O$_2$ in H$_2$O for 30 min. Finally sections were washed, mounted on slides and coverslipped with DePex.

**Antibody specificity testing.**

To control for cross-reactivity due to aspecific binding, negative controls were included by incubating several sections and performing the immunostaining without one of the antibodies needed for the reaction (primary, secondary, or tertiary). All reactions were negative thereby confirming the specificity of the antibodies.

**Image analysis and counting procedure (semi-quantitative analysis).**

FOS, phospho-CREB, and calcineurin immunoreactivity was quantified using a computerized imaging analysis system by an observer who was blind to group assignment. Selected areas from regions of interest (ROIs) included the prefrontal cortex (prelimbic (PrL) and infralimbic (InfraL); Bregma +3.60 to +1.70), the anterior cingulate (AC; Bregma +3.20 to +0.95), and the posterior cingulate cortex (postCING; Bregma +3.20 to +0.95); the hippocampal dentate gyrus (DG: Bregma –2.00 to –3.90), the hippocampal CA1 (CA1; Bregma –2.45 to –4.60) and the hippocampal CA3 (CA3; Bregma –2.45 to –4.60); the paraventricular hypothalamic nucleus (PVN Bregma –1.08 to –2.00). Coordinates are with reference to the rat Swanson's brain atlas 33. The ROIs were acquired and digitized using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany) to reduce quantification errors. After image acquisition, all measurements were performed bilaterally and no left-right asymmetry of FOS, phospho-CREB, or calcineurin immunoreactivity was found. When counting the immunoreactive cell nuclei, at least 4-5 sections per region were analyzed. Using the computer-based LEICA image analysis system (LEICA Imaging System Ltd., Cambridge, England), quantification of FOS, phospho-CREB, and calcineurin immunoreactivity was performed according to prior descriptions 17,28,34. For FOS and phospho-CREB, each digitized image was individually set at a threshold to subtract the background optical density. The numbers of positive nuclei above background were quantified and the densities were reported as the number of positive nuclei/0.1 mm$^2$. Calcineurin immunoreactivity was expressed as gray value intensity (GVI) of the selected region after correction with corpus callosum as background value and internal control for each section 17.
RNA isolation and Quantitative RT-PCR.

Total RNA- and poly (A)+RNA-isolation.

Thirty minutes after the start of the final session, rats used for the molecular biology were anesthetized with halothane and decapitated. The brains were dissected to separate the prefrontal cortex and the hippocampus, which were subsequently quick-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from these brain areas by using Trizol (Life Technology, Gaithersburg, MD, USA) according to the manufacturer’s instructions. Integrity of total RNA was confirmed on a 2% agarose gel and final concentrations were assessed spectrophotometrically. Poly(A)+RNA was in turn isolated from total RNA using Micro-FastTrack™ 2.0 Kit (Invitrogen) according to the manufacturer’s instructions.

First-strand cDNA synthesis.

First-strand cDNA was synthesized from 200 ng poly(A)+RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s instructions. Before first-strand synthesis was performed, contaminating genomic DNA was removed using Deoxyribonuclease I, Amplification Grade (Invitrogen). The first-strand synthesis was primed with oligo(dT), and the RNA template was not removed from the DNA:RNA hybrid.

Primers and PCR Conditions.

All primers used were designed with Primer Express® Software v2.0 (Applied Biosystems) using default parameters (sequences are listed in table 1). PCR reactions were performed on an ABI Prism 7900HT Sequence Detection System. Amplification mixtures (25 µL divided over 3 wells, 7 µl per well) for all genes contained 2 ng cDNA from poly(A)+RNA template, 1 x SYBR Green PCR buffer, 3 mM MgCl2, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP 0.4 mM dUTP; 0.025U/µl Amplitaq Gold, 0.01 U/µl AmpErase UNG and 300 nM of each primer. The cycling conditions comprised of 2 minutes AmpErase UNG activity, 10 minutes Amplitaq Gold activation, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, and a dissociation stage: 15 seconds 95°C, 15 seconds 60°C, 15 seconds 95°C ramp rate 2%. The No Template Control contained all components except the cDNA template.
Real-time polymerase chain reaction (RT-PCR).

Real time detection was performed using an ABI PRISM 7900HT Sequence Detection System and SYBR Green PCR Core Reagents. Data analysis was performed using software provided with the instrument. The mRNAs of the different genes were relatively quantified across groups with the Comparative CT Method. The CT value, or fractional cycle number at which the defined threshold is crossed above baseline, is predictive of the input amount of target and thus useful in quantification of RNA. Here the threshold was set between 0.4 and 0.6 with a baseline range from 1 to 7. All RNA samples were measured in trip and the amount of target was normalized to an endogenous reference, the housekeeping gene, RPS29 (ΔCT). An additional reference (β-actin) was included as an extra control.

Statistical Analysis

Data are expressed as means ± standard error (SEM). Statistical significance was determined by performing one-way analysis of variance (ANOVA) and F test of variance on the corresponding parameter measures from experimental and control conditions. To compare values between male and female, and/or control and experimental conditions t tests for equal or unequal variance were performed. P<0.05 was defined as the level of significance between groups. Calculations were made using Jandel SigmaStat statistical software.
RESULTS

The validity of the aversive procedure used in the present investigation has previously been established in both genders on a behavioral and endocrinological level. The findings of the current study, however, serve to elaborate upon the latter on a molecular and cellular level. Verification of the efficacy of this chronic stress procedure is provided by various physiological correlates described below.

Physiological and Neuroendocrine Correlates

Body weight gain.

During the acclimatization period, control and stress groups of both genders displayed an identical weight gain. Immediately upon initiation of the stress procedure however, the difference in weight gain between control and stressed males increased progressively reaching a significant value on day 3 of the procedure (F=6.93; P=0.025) (fig. 1a). While weight gain in control males continued constantly as expected, a consistent reduction in body weight gain was observed in stressed males, which continued until the final day when it reached about 67% of the weight gain in controls (F=82.03; P<0.0001). In line with previous findings, body weight gain in females remained unaffected by chronic stress procedure (Fig. 1a,b).

Figure 1a.
Diagram illustrating effects on body weight gain during the chronic stress experiment. A consistent reduction in weight gain was observed in stressed males, while control males continued to grow constantly as expected. The effect on weight gain commenced immediately upon initiation of the stress procedure, reached significance on day 3 and increased progressively until the final day (a). Whereas body weight gain in stressed males was approximately 67% of controls, weight gain in female rats remained unaffected by stress (b).

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Adrenal glands.

Repeated exposure to stressful conditions caused a significant increase in adrenal weight in both males ($F=26.41; P<0.0001$) and females ($F=8.32; P=0.014$). A gender difference was also evident as females had heavier adrenal glands than males under both basal ($F=82.71; P<0.0001$) and stress conditions ($F=218.14; P<0.0001$) (Fig. 2a).

Thymus.

Chronic footshock stress resulted in a significant loss of thymus weight in female rats ($F=7.12; P=0.020$). Although thymus weight tended to decrease in males, this trend did not reach statistical significance. Females also demonstrated larger basal thymus weights compared to males ($F=10.20; P=0.009$) (Fig. 2a).

Plasma adrenaline concentrations.

Following chronic stress exposure, plasma adrenaline levels were significantly elevated in males ($F=7.34; P=0.024$). Although peripheral adrenaline concentrations tended to be higher in chronically stressed females, this trend did not reach statistical significance. No gender differences were observed in the basal level of this catecholamine (Fig. 2b).

Plasma noradrenaline concentrations.

While noradrenaline levels, in males, remained unchanged, a significant increase of peripheral noradrenaline concentration was detected in chronically stressed females ($F=6.02; P=0.032$). A basal gender difference was evident under control conditions, as non-stressed females illustrated significantly lower noradrenaline levels than males ($F=7.22; P=0.023$) (Fig. 2b).
Plasma corticosterone levels.

In line with corresponding total adrenal and adrenal cortex findings, chronic footshock stress resulted in significantly enhanced plasma corticosterone release in both sexes. Chronic stress exposure resulted in a 45% increase peripheral glucocorticoid levels in males ($F=6.68; P=0.032$) and a 77% rise in females ($F=12.30; P=0.005$). Plasma corticosterone concentrations were also higher in females and this gender-related difference was found in both control ($F=9.40; P=0.012$) and stressed animals ($F=31.12; P<0.0001$) (Fig. 2b).
Immunohistochemistry

In order to evaluate the chronic stress-induced neurochemical changes in male and female rats, FOS, phospho-CREB, and calcineurin immunoreactivity was quantified in prefrontocortical (PFC) and hippocampal (HIP) regions. Interest in these two structures is attributable to their primary role in the regulation of HPA axis activity as well as their sensitivity to the deleterious effects of prolonged stressful conditions.

c-fos immunoreactivity.

Repeated footshock stress was associated with significantly increased FOS-ir the PVN in both male (F=6.58, p=0.028) and female rats (F=15.78, p=0.0026). Increased FOS expression in this region on the final day is indicative of a lack of habituation of this hypothalamic nucleus in response to repeated stress exposure. No baseline differences were evident between the genders (Fig. 3).

Figure 3.

Photomicrograph (a) and diagram (b) illustrating stress-induced c-fos immunoreactivity in the PVN. After chronic stress both genders illustrated increased PVN activity, confirming continual stress discernment without habituation in male and female rats. Values are shown as mean ± SEM. * represents a significant stress effect (p<0.05); # represents a significant sex effect (p<0.05).
Phospho-CREB immunoreactivity.

The analysis of the pattern of CREB phosphorylation revealed significant gender-dependent stress effects in both prefrontocortical and hippocampal regions. In males, prolonged stress induced a significant reduction in the level of phospho-CREB immunoreactivity in the infralimbic, ($F=38.1, p<0.0001$) the prelimbic ($F=16.6, p=0.002$), the anterior cingulate ($F=10.6, p=0.008$), and the posterior cingulate cortices ($F=6.84, p=0.026$) (fig. 4a), as well as the hippocampal dentate gyrus ($F=11.9, p=0.006$) (fig 4b). On the contrary, only slight changes of CREB phosphorylation were detected in chronically stressed females. Although females showed a trend towards a decreased phospho-CREB immunoreactivity in the dentate gyrus, this trend did not reach statistical significance ($F=4.4, p=0.081$). A significant gender effect was evident however under stress conditions in all regions, with females showing elevated phospho-CREB levels compared to males in the infralimbic and ($F=60.6, p<0.0001$) prelimbic cortex ($F=48.9, p<0.0001$), the anterior cingulate ($F=23.2, p=0.001$), the posterior cingulate cortex ($F=20.7, p=0.002$), and the hippocampal dentate gyrus ($F=5.6, p=0.045$). Although investigated, phospho-CREB immunoreactivity in the hippocampal CA1 and CA3 was almost absent. Due to the low number of phospho-CREB-positive nuclei and the lack of differences between experimental groups, we excluded these areas from further analysis.
Figure 4b.

Figure 4.
Diagrams illustrating phospho-CREB immunoreactivity patterns in the prefrontal cortex (a) and hippocampus (b) of chronically stressed rats. Whereas stress in males induced a significant reduction of phospho-CREB expression in all regions observed, phospho-CREB expression in females remained unaffected by stress exposure. In turn, a significant gender effect was revealed however in all regions under stress conditions, with females showing higher phosphorylated CREB levels than males. Values are shown as mean ± SEM. * represents a significant stress effect (p<0.05); # represents a significant sex effect (p<0.05).

Figure 5.
Photomicrographs illustrating phospho-CREB immunoreactivity patterns in the hippocampal dentate gyrus (a,c,e,g) and prefrontal cortex (b,d,f,h) of control (a,b) and stress males (c,d) and control (e,f) and stressed females (g,h).
Calcineurin immunoreactivity.

Like phospho-CREB immunoreactivity, analysis of calcineurin expression also revealed sex and region-specific stress effects. Following chronic stress, females illustrated a decreased calcineurin immunoreactivity in the prelimbic cortex ($F=7.37$, $p=0.01$), while chronically stressed males showed a reduced expression in the posterior cingulate cortex ($F=8.22$, $p=0.01$). Gender-related differences in the expression of this phosphatase were also evident in the posterior cingulate cortex as female rats showed higher calcineurin immunoreactivity compared to males following chronic stressful conditions (fig. 6a). In the hippocampus, while calcineurin expression in females remained unaffected, significantly reduced immunoreactivity was observed in the dentate gyrus ($F=6.05$, $p=0.033$) and CA3 area of chronically stressed males ($F=5.67$, $p=0.038$) (fig. 6b).

Figure 6a.

Figure 6.

Diagrams illustrating calcineurin immunoreactivity patterns in the prefrontal cortex (a) and hippocampus (b) of chronically stressed rats. Chronic stress induced gender and region specific calcineurin immunoreactivity patterns in the prefrontal cortex. Whereas stressed males showed decreased expression in the posterior cingulate cortex, stressed females illustrated reduced expression in the prelimbic cortex. A gender effect was also seen in the posterior cingulate cortex as females expressed higher levels under stress conditions. Stress effects in the hippocampus were only observed in males however as calcineurin immunoreactivity was significantly reduced in the hippocampal CA3 and dentate gyrus. Values are shown as mean ± SEM. * represents a significant stress effect ($p<0.05$); # represents a significant sex effect ($p<0.05$).
**Molecular Biology - Gene expression patterns**

**CREB mRNA levels.**

Neither males nor females demonstrated stress-induced alterations of CREB mRNA levels in either the prefrontal cortex or hippocampus (fig. 7a).

**Calcineurin mRNA levels.**

Like CREB expression, prefrontal cortex and hippocampal calcineurin mRNA levels also remained unaltered by stress in both genders (fig. 7b).

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**Figure 7.**

CREB (a) and calcineurin (b) mRNA levels in the prefrontal cortex and hippocampus of male and female rats following repeated footshock stress. Despite altered protein expression, stress failed to alter CREB and calcineurin mRNA levels in the prefrontal cortex or hippocampus of both sexes. mRNA levels on the y axis are depicted by the inverse fractional cycle number at which the defined threshold is passed (1/\(\Delta ACT\)). Values are shown as mean ± SEM.
DISCUSSION

The phosphatase calcineurin and the transcription factor CREB have been established as essential components of the intracellular transduction apparatus involved in the regulation of neuronal plasticity. Stress however, particularly when persistent and severe, has been associated with impairment of neuroplasticity, as illustrated by reduced neurogenesis in the dentate gyrus and atrophy of apical dendrites in selective prefrontocortical and hippocampal areas. Although our understanding of the exact mechanisms underlying the deleterious effects of stress on neuronal integrity is limited, it is intriguing to speculate that altered expression and/or phosphorylation of key proteins could account for stress-induced disruption of the coordinated regulation of synaptic plasticity thereby promoting structural and functional abnormalities. Interestingly, despite numerous reports of hippocampal atrophy following prolonged stress exposure in males, ample evidence also supports a gender discrepancy with females being more sensitive to stress than males. In the present study, neurochemical changes in response to repeated stress were thus investigated in male and female rats, on a cellular and molecular level with immunohistochemistry and quantitative RT-PCR, respectively. This data in turn may ultimately help to elucidate two fundamental questions: Is gender a critical determinant for the impact of chronic stress on neuronal plasticity? If so, how do male and female rat reactivity to chronic stress compare at the cellular and molecular level?

Various physiological and neuroendocrine parameters served to verify the severity of the stress procedure in rats of both genders. These included reduced body weight gain in males and prolonged HPA axis hyperactivity in both sexes following chronic stress. The latter was in turn substantiated by several adrenocortical parameters including significantly elevated plasma corticosterone and catecholamine levels (adrenaline in males and noradrenaline in females), reduced thymus weights (in females), enlarged adrenal glands, and increased FOS-ir in the paraventricular hypothalamic nucleus.

On a molecular level, sustained stress and subsequently elevated glucocorticoid levels have been associated with reduced expression of BDNF. Since CREB is known to play a crucial role in modulating BDNF transcription, a possible mechanism by which stress could limit availability of this fundamental neurotrophin is through decreased CREB transcription. Alternatively, chronic stress can also impair neuronal integrity by affecting transcription of other specific genes involved in the modulation of synaptic plasticity such as calcineurin. Despite the marked neuroendocrine effects observed here following stress however, quantitative RT-PCR analysis revealed unaltered CREB and calcineurin mRNA levels in the frontal cortex and hippocampus of both genders. This data therefore appears to refute a direct effect of chronic stress on transcript levels of these two genes as a possible mechanism by which stress impairs neuronal plasticity.

Analysis of phospho-CREB and calcineurin immunoreactivity however proved more revealing. Whereas males illustrated stress-induced reduction of calcineurin immunoreac-
tivity and CREB phosphorylation in the prefrontal cortex and hippocampus (particularly the dentate gyrus and the cingulate cortex) female rats exposed to the same stressor, displayed comparatively attenuated changes (fig. 4,6). The CREB protein family is critical for induction of gene expression in all eukaryotic cells 24,25 and the crucial step underlying its ability to initiate the transcription of several target genes lies in its phosphorylation at serine 133 25. CREB phosphorylation is considered a molecular switch to transform short-lasting into long-lasting changes of synaptic plasticity 48, especially in the hippocampus, enabling long-term memory storage in mammals 25. Previous data from a similar stress experiment support a facilitatory role for CREB in the modulation of neuronal plasticity following acute stress in prefrontocortical regions 17. However, while acute stress has been reported to promote learning acquisition and memory formation 49,50, sustained stress has been associated with cognitive impairment and emotional dysregulation 51-53. Accordingly chronic stress caused significantly reduced phospho-CREB immunoreactivity in the same cortical regions 17,28. In line with these prior findings, the current results in males confirm a stress-induced reduction of CREB phosphorylation in several subregions of the prefrontal cortex including the infralimbic, prelimbic, anterior and posterior cingulate cortex (fig. 4a). Moreover this decrease was not only limited to cortical areas as this study revealed similar findings in the hippocampal dentate gyrus (fig. 4b), suggesting that the negative impact of chronic stress on CREB phosphorylation extends beyond the prefrontal cortex.

Neurotrophins regulate various neuronal functions, including survival, differentiation, and plasticity. Although phosphorylated CREB mediates the transduction of neurotrophin signals and BDNF transcription 47, exposure of neurons to BDNF in turn also stimulates CREB phosphorylation 22. This can occur via at least two signaling pathways: a Ras-MAPK-dependent pathway 26 and a calcium/calmodulin-dependent kinase-regulated pathway, activated by the release of intracellular calcium. 22 Calcium influx also regulates protein phosphatase 2B (calcineurin) activity, another key component of the calcium/calmodulin-dependent kinase-regulated pathway 54.

Interestingly, functional interactions between calcium/calmodulin kinase and the MAPK cascade have been reported and both calcineurin and CREB appear to represent common links between these two intracellular pathways 55-57. More specifically, calcineurin plays a pivotal role in modulating neuronal plasticity by actively regulating the phosphorylation state of several kinases and transcription factors such as Elk1 and CREB 58,59. In line with the decreased CREB phosphorylation, a significant reduction of calcineurin immunoreactivity was also observed in the frontal cortex (posterior cingulate) (fig. 6a) and hippocampus (DG and CA3) (fig. 6b) of stressed males. Given the fundamental role of this phosphatase in the maintenance of neuronal plasticity, persistent stress-induced reduction of calcineurin levels, particularly in the hippocampus (fig 6b), may lead to abnormal activation of different intracellular transduction members, ultimately disrupting CREB phosphorylation.

As noted however, an interesting finding emerged with regard to the gender-dependent nature of these results, as significant gender divergent regulation of calcineurin immunoo-
Impaired regulation of HPA axis activity for instance, common to both genders may constitute the mechanism by which chronic stress leads to persistently elevated glucocorticoid levels and PVN activation, although the data shows gender-dependent means of attainment. Impaired prefrontocortical and/or hippocampal control over the HPA axis seem to represent the mechanism by which repeated foot shocks lead to abnormal stress response regulation in male rats, whereas imbalanced GR/MR expression, disturbed autonomic function, and abnormal amygdala activity could instead illustrate the events involved in the development of abnormal HPA axis responses in female rats (figure 1,2).

On a more cellular/molecular level, stress-induced effects of neuronal plasticity and synaptic integrity seem to represent a possible mechanism by which this effect is realized in males given the changes observed in their general gene expression, specific second messenger signalling cascade members and synaptic vesicle associated proteins. Considering the potential role of these proteins and functional neurotransmission in BDNF activity, the data may represent indirect indications for impaired neurotrophic plasticity in males. In view of the lesser effects observed on these parameters in females, it raises the interesting possibility that the differential impact of stress on neuronal plasticity may depend on gender-related mechanisms that either potentiate or suppress the deleterious influences of adverse events. Dependent upon the interpretation, in the case of the latter, ovarian hormones and their established neurotrophic effects, may protect the female brain against the insults of chronic stress by stimulating specific intracellular cascades underlying neuronal plasticity and preventing the reduced neuronal plasticity (CREB phosphorylation) observed in chronically stressed males. On the other hand, these neurotrophic effects might have a paradoxically damaging effect if these “blocked” neuronal/synaptic changes are required in order to attain an adaptive response. After all, neurogenesis/proliferation was predominantly affected in these females. Whatever the pathway however, both dysregulations seen in the genders are known to contribute to final volumetric changes often reported in stress and psychopathology.

The final question regarding the sensitivity of the sexes remains and unfortunately it is not as unambiguous as one might desire. In view of the data presented here and illustrated in figures 1 and 2, the answer to this seems to depend on the parameter of interest, since both sexes appear to be affected albeit through different mechanisms. In view of the future of stress research, it would be of interest from a clinical perspective to further pursue the neurobiological substrates and neurochemical profiles underlying effects of stress in both genders. As illustrated by the antidepressant data, each gender should be evaluated independently to determine the stress and/or treatment response and thus new avenues of therapeutic approach. The data presented here offer ample indications to argue for further investigations of the role of steroid hormones in multi-level research. With this in mind the paramount message is that findings associated with one gender cannot be automatically extrapolated to the other; and that which may appear “negative” in one could represent a physiological condition in the other and vice versa.
that the deleterious action of prolonged footshock exposure involve primarily post-transcriptional changes, at least in the male prefrontal cortex and hippocampus. Specifically chronic stress appeared to alter CREB phosphorylation and calcineurin levels without affecting coincident transcription of the respective genes. In turn this lack of altered gene expression may ultimately deprive cortical and hippocampal neurons of what is most essential, namely a continuous supply of newly synthesized CREB and calcineurin to antagonize the negative impact of prolonged stress exposure. Although one can only speculate the latter, current endeavors are being pursued to further explore this notion.
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