Gender-related dimorphisms
in the patterns of cellular activity
and neuronal plasticity
in response to repeated stress

“Experience has shown that science frequently develops most fruitfully
once we learn to examine the things that seem the simplest,
instead of those that seem the most mysterious”

Marvin Minsky
Stress and psychopathology: is gender an issue?

Ovarian steroids produce a variety of neurochemical effects which affect multiple processes such as cognition, emotional regulation, affective style, pain sensitivity and psychopathology. Gender-related differences in the brain however, account for more than merely sex disparities in the effects of sex hormones. Reproductive as well as nonreproductive dimorphisms exist between men and women and it is intriguing to assume that non-reproductive, gender-related cognitive and emotional dissimilarities might reflect functional and/or structural differences in higher-order cortical and subcortical structures.

Gender-related differences in HPA axis regulation

Since the HPA axis represents the final effector in the modulation of the stress response, a large number of clinical and preclinical studies have attempted to define a direct link between sex-related differences in key elements of this system and the higher female susceptibility to stress and stress-related psychopathology. The HPA axis and the female reproductive system are integratively intertwined and exhibit complex relationships.

Glucocorticoids, for instance, inhibit pituitary luteinizing hormone (LH), estrogen, and progesterone secretion. Although most of the studies investigating gonadal and adrenal interactions have generally focused on stress-induced disruption of reproductive functions, the relationships between these two endocrine systems are more complex and by no means unidirectional. A partial estrogen response element has been found on in the promoter of the CRF gene. Estrogen-induced increased CRF transcription may thus represent a potential mechanism by which estradiol may enhance stress responsiveness and lead to the higher glucocorticoid levels observed in females. This finding implicates the CRF gene and, therefore, the HPA axis, as an important target of gonadal steroids and a potential element involved in the gender-related differences observed in the modulation of stress response.

Differences in the regulation of HPA axis activity may account for the differential response to stress observed between male and female rats, with the latter demonstrating a greater overall reaction, a more rapid onset of glucocorticoid secretion, and a faster elevation of plasma level of adrenal steroids. A steeper rise of circulating stress hormones seems to be necessary to elicit the faster glucocorticoid-mediated feedback inhibition needed in females. In addition, estrogen has also been shown to delay ACTH and glucocorticoid shutoff, condition that may account for the greater overall reaction to stress observed in females. Progesterone also appears to be involved in the differential modulation of stress response. It is interesting to note that progesterone shows a faster binding time than cortisol on the GR, although it binds the receptor at a different site than glucocorticoids. Female rats have a greater number of GRs in the hippocampus.
than males and this number is also modulated by progesterone. Although the majority of progesterone-induced effects on the HPA axis are mediated by GR binding, several studies have also demonstrated an affinity of this hormone for the MR, in a range similar to that of dexamethasone.

The finding of an “exaggerated” response to stress in females, even in ovariectomized animals, has very important implications for our understanding of stress responsiveness. It is now clear that a number of “stress-related” psychiatric disorders are more common in females. These include depression, post-traumatic stress disorder as well as other anxiety disorders. Ovarian hormones have been proposed as key factors in determining this higher female liability to psychopathology. If females indeed demonstrate an increased responsiveness to stress, this finding may explain, at least in part, the greater sensitivity of women to depression and anxiety. An additional aspect that should not be underestimated when considering the increased incidence of psychopathology in women is the greater resistance of female HPA axis response to glucocorticoid-mediated feedback inhibition. If we believe Munck’s hypothesis, one of the purposes of glucocorticoids is to terminate not just the HPA axis response to stress but, more in general, the entire stress “cascade.” Recent studies, documenting a role for adrenal steroids in terminating stress-induced activation of the autonomic stress system, appear to substantiate this hypothesis. In consequence of this important modulatory role of stress hormones, the higher resistance observed in women to glucocorticoid-mediated feedback inhibition would further exaggerate stress responsiveness. Therefore, whereas estrogen and progesterone exert a protective effect against the negative sequelae of hypercortisolism, they also antagonize glucocorticoid-mediated terminating action, delaying the recovery from the deleterious consequences of stress.

**Gender-related differences in cognitive processing**

Gender-related differences in structural and functional brain organization may result in sex-related dimorphisms in critical functions, such as cognitive processing and emotional regulation. The existence of sex differences on a cognitive level has become increasingly clear. Males for instance reliably outperform females on tasks that require spatial ability skills. Women, on the other hand, demonstrate superior verbal and object location memory, and rely to a greater degree on emotional content during information processing. Despite extensive research however, relatively little is known about the cellular and molecular mechanisms underlying these differences.

The fear conditioning paradigm represents a powerful model to investigate the neurobiological substrates underlying cognition and emotional regulation. Several studies have revealed that male rats exhibit greater contextual fear conditioning than females. Female rats however, acquire fear-conditioned responses much faster than
males. These differences were even more pronounced when taking into account the stage of estrous. Recent studies in humans and rodents have shown that the status of both gonadal and adrenal axes strongly influences learning and memory. In rats, for instance, the estrous cycle is approximately 5 days and is separated into four stages: proestrus, estrus, diestrus 1 and diestrus 2. The level of the primary female sex hormone, estrogen, is highest just prior to ovulation, the stage corresponding to proestrus. During this stage, female rats tend to be more active, eat and drink less, and are facilitated in learning fear-conditioned tasks. The mechanism involved in this enhanced acquisition of classically conditioned responses is unknown. Importantly, changes in synaptic efficacy are considered necessary for learning and memory, and estrogen has been shown to increase the density of dendritic spines and synapses in the hippocampus as well as the number of spine synapses formed with multiple synapse boutons. Thus, it has been recently suggested that estrogen may promote cognitive activities by controlling synaptic functions in the central nervous system and enhancing neuronal plasticity, through genomic and non-genomic actions. The latter effects seem to involve the ability of ovarian hormones to modulate the activity of ERK intracellular transduction cascade.

The influence of stress on cognitive processing

Previous exposure to acute stressful events has been shown to enhance the acquisition of several types of learning. Adrenal hormones appear to facilitate a wide variety of brain functions, an effect that is rapidly induced (within 30 minutes) and persist for at least 24 hours. Under acute physiological conditions, the elevation of glucocorticoid levels, during the post-training period, has also been proposed to determine the strength of information storage.

Animal and human studies have shown that important sex differences exist in specific cognitive abilities, particularly under stressful conditions. While adverse experiences promote associative learning and memory consolidation in males, female cognitive functions are severely impaired by exposure to the very same events. Simply re-exposing the animal to the stressful context days after stressor cessation can reinstate its deleterious influence on learning, suggesting that a psychological manipulation is sufficiently stressful to reinstate the effect of the stressor, at least in females. A growing number of studies support the view that this differential action of stress on memory formation in males and females are determined by sex-related structural differences in the brain. For instance, the induction of learning, in males, is dependent upon the N-methyl-D-aspartate (NMDA) receptor. In females, in contrast, stress-mediated effect on learning does not appear to depend on NMDA receptor activation. An additional structural difference between males and females involved the rate of neurogenesis observed in the adult brain. Neurogenesis plays a critical role in
cognitive processing and new neurons are crucially involved in hippocampal-dependent learning. Females produce more immature granule neurons in adulthood than males and some have reported that females learn hippocampal-dependent tasks better than males. Several studies have also indicated that stress inhibits the production of granule cells by suppressing the proliferation of granule cell precursors. The relationship between stress and neurogenesis is seemingly complicated when viewed in relation to learning, as exposure to stressful experiences may reduce cell proliferation in the dentate gyrus without affection and, sometimes, even enhancing hippocampal-dependent learning, at least in males. In female rats however, stress exposure immediately and persistently impairs associative learning, possibly through the inhibition of cell proliferation. These results suggest sex-related differences in the structural organization of the brain as a critical factor in both the facilitation and the impairment of cognitive processing in response to stress.

Another factor which may prove of relevance in this dual action of stress on cognitive performance is the role played by ovarian hormones. Estrogen enhances neuronal plasticity, a critical property underlying learning and memory. Importantly, the exposure to adverse events has been reported to alter estrogen and progesterone release. The crucial interaction between sex steroids and adverse experiences is supported by the observation that, contrary to cyclic females, ovariectomized animals were not impaired by stress. Furthermore, stress-induced impairment of classical eyeblink conditioning was prevented by estrogen antagonist treatment. Additional evidence to further support the involvement of estrogen in the modulation of the detrimental effects of stress comes from analysis of the consequences of adverse events in different stages of the estrous cycle. The negative impact of stress was most pronounced when females were stressed during the transition into proestrus, consistent with a rise in estrogen levels. The exact mechanisms underlying estrogen-mediated disruption of cognitive processing is unknown, although recent data suggests stress-induced supranevelation of ovarian hormone levels as a critical role in the process. Initially the latter may be perceived as inconsistent since ovarian steroids are known to stimulate neuronal plasticity and promote learning and memory. However, rising levels of estrogen, as well as impaired conditioning have been observed to be rapidly induced (within minutes) and persist for at least 24 h after stressor cessation. Since impaired learning was most evident when rats were stressed during the transition into proestrus, this suggests that a further stress-induced enhancement of estrogen release coupled with already elevated estrogen levels during proestrus and high glucocorticoid concentrations might be detrimental for the formation of new memories.
Sex-related dimorphisms during emotional processing

The functional dimorphisms between male and female brains are not limited to cognitive processing, but they also embrace other higher-order functions such as affective style and emotional regulation. The amygdala, due to its primary involvement in stimulus integration, has for many years been the main focus of clinical and preclinical research. This structure fulfills an integrative role in behavioural, vegetative, and endocrine activities of animals in relation with their environment and is involved in the modulation of mood and affective behaviors in humans. Remarkably, marked sex differences have also been observed in the activity of this limbic structure during emotional processing. Amygdala activation, for instance, differs in men and women depending upon the valence of emotion. Overall, males express more lateralized amygdala activity than females. As a result, positive emotions produce greater right than left amygdala activation for males compared to females, although both sexes showed greater left amygdala activation during fearful conditions. Gender-related differences observed during perception, experience, and expression of emotional states, may thus be related to the differential use of this structure by men and women. Notably, negative emotions, such as sadness, have been reported to activate a significantly wider portion of the limbic system in women, while men appear to rely more on cortical structures. Remarkably, depressed patients showed increased amygdala volumes compared to healthy subjects. Moreover, this enlargement in patients with first episodes of major depression is more strongly related to enhanced blood flow in the amygdala than to a particular neurodevelopmental structural predisposition. At least one study, investigating the relationship between amygdala volume and depression, found that female patients had significantly larger amygdala than males. This evidence points to differential cortical and subcortical correlates of emotional experience in males and females but also underlines the importance of the amygdala in gender-related differential processing with respect to emotions and mood. Sex-related structural and, more importantly, functional differences may thus be implicated in the mechanisms underlying the differential sensitivity to stress and psychopathology observed between males and females.
Immunohistochemical changes induced by repeated footshock stress: revelation of gender-based differences

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**Introduction**

Recent advances have been made in understanding the changes of neuronal plasticity in response to stress. Acute stressful experiences, for instance, facilitate the consolidation of new memories and promote cognitive processes. In contrast, as a growing literature has proven, adverse experiences, particularly when severe and persistent, may contribute to the development of neuronal dysfunctions and psychopathology. At the cellular level, evidence has emerged indicating dendritic atrophy and neuronal loss in response to chronic stress. At the molecular level, it has been suggested that these abnormalities, mostly detected in the hippocampus and prefrontal cortex, result from a decrease of neuronal plasticity associated with persistent exposure to elevated glucocorticoid levels. While in the short run adrenal steroids thus play a critical role in the acquisition of fear-conditioned responses, prolonged elevation of glucocorticoid levels results in functional and structural abnormalities and cognitive impairment. Remarkably however, although considerable progress has been made in elucidating the neurobiological substrates underlying the acute stress response, chronic stress-induced neurochemical changes remain poorly understood. Furthermore, although gender represents a critical aspect in both sensitivity to stress and psychopathology, most of the clinical and preclinical research concerning stress-related neuronal abnormalities have been conducted in males.

With this in mind, the cellular and molecular changes associated with short-term (2 days) and prolonged footshock stress (20 days) were investigated in male and cyclic female rats, in an attempt to gain new insights into the neuronal circuits modulating the response to acute and chronic footshock exposure as well as the mechanisms underlying the biphasic effects of stress on cognitive and emotional processes. By using identical settings (5 footshocks delivered randomly during 30-minute sessions) but extending the length of the exposure from 2 to 20 days, these footshock procedures might prove a potentially useful model for investigating the dynamic of stress-induced disruption of cognitive processing in both a gender comparative setting. Footshock-induced neurochemical changes were examined using molecular and immunohistochemical techniques, including FOS-ir, phospho-ERK1/2 expression, and gene expression microarrays, as markers of cellular activity and neuronal plasticity. This data may thus contribute to the understanding of the mechanisms underlying gender-related differences in emotional processing and their relationship with the development of stress-induced cortical-limbic dysfunctions.
Materials and Methods

Animals

The experiments were performed using adult male (n=28: 212-240 g) and cyclic female Wistar rats (n=25: 195-212 g). The animals were housed individually (cages 45 x 28 x 20 cm) with food and water available ad libitum, and maintained on a 12/12-hr light/dark cycle. All rats were weighed and handled daily for 5-8 min to minimize the non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bio-Ethics Committee of the University of Groningen (FDC: 2509).

Footshock procedure

The rodent footshock-chamber consists of a box containing an animal space placed on a grid floor connected to a shock generator and scrambler. Test-rats received one session of 30 min/day in the footshock box during which 5 inescapable footshocks were given (0.8 mA in intensity and 8 sec in duration: unconditioned stimulus; US) with different inter-shock intervals in order to make the procedure as unpredictable as possible. Each footshock was preceded by 10 sec of light (conditioned stimulus; CS). Control rats followed the same schedule in an identical setup but were exposed to CSs only, without receiving any shocks. This conditioning procedure was followed for 2 (acute challenge) or 20 days (chronic challenge). The final day of each experiment (3rd for the acute and 21st for the chronic) all rats were placed in the footshock box and exposed to 5 CSs only, without receiving any painful footshock. This allowed investigation of the pattern of protein expression (FOS-ir) and phosphorylation (phospho-ERK1/2) induced by identical and painless stimuli hereby avoiding exposure of animals to physical stress.

Acute experiment. Six male and six cyclic female rats were used in the acute experiment. Test-rats were conditioned for two consecutive days. The third and last day all rats were subjected to 5 CSs.

Chronic experiment. Eleven males and ten females were used in this experiment. Test-rats were conditioned for 20 consecutive days. On the final (21st) day all rats received 5 CSs without consequent footshocks.

Control rats. Eleven males and nine females were used as control animals. These animals were exposed to the same stimuli as the stressed rats (as they were housed in the same room and similarly exposed to the footshock chamber and CSs). They were never subjected to USs during the entire duration of the experiment.

Physiological and neuroendocrine changes following repeated footshock exposure

To define the dynamics of the response to repeated footshock stress, various physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, plasma adrenaline and corticosterone...
concentrations were measured and adrenal glands were removed and weighed. Blood samples were drawn by transcardial puncture immediately upon termination and stored at -20°C. These samples were then used to determine plasma corticosterone and adrenaline levels with HPLC. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals.

**Extraction and Chromatography**

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

**Corticosterone.** For the assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3ml 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 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/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 254nm. The detection limit of the method was 10nM.

**Histological procedure - Immunohistochemistry**

Two hours after the beginning of the final session, the rats were terminated with an overdose of halothane which preceded a transcardial perfusion 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 0.02 M, 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 40 µm were prepared on a cryostat microtome. Sections were collected in KPBS with sodiumazide and stored at 4°C.

**c-fos and phospho-ERK1/2 immunoreactivity**

The stainings were performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H2O2 for 15 min to reduce endogenous peroxidase activity, before being incubated in primary monoclonal mouse anti-phospho-ERK1/2 (New
England Biolabs, Inc., Beverly, MA, USA; www.neb.com; 1:5000 dilution in KPBS 0.02 M, pH 7.4, overnight at room temperature) or polyclonal rabbit anti-FOS antibody (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, 60-72 hours at 4°C) depending on the primary antibody host. Subsequently, sections were washed with KPBS and incubated at room temperature with biotinylated goat anti-mouse or 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. Thereafter, sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

Antibody specificity testing. To control for cross-reactivity due to aspecific binding, immunostainings were performed by incubating several sections without the presence of one of the antibodies needed for the reaction (primary, secondary or tertiary antibody). All these reactions were negative thereby confirming the specificity of all antibodies used.

Image analysis and counting procedure (semi-quantitative analysis)

FOS immunoreactive cells were quantified in 14 different brain regions or subregions with reference to the rat Swanson’s brain atlas 83 while the quantification of phospho-ERK1/2 immunoreactivity was limited to the medial prefrontal cortex (prefrontal and infralimbic areas) where an abnormal phosphorylation of these kinases was found in chronically stressed male rats 69. The quantification of FOS and phospho-ERK1/2 immunoreactivity was performed by an observer who was blind to group assignment. For counting of the immunoreactive cell nuclei, at least 4-5 sections per each brain area were analyzed. ROIs included prefrontal (prefrontal and infralimbic area; mPFC: Bregma +3.60 to +1.70) and cingulate cortices (AC: Bregma +3.20 to +0.95); hippocampal CA1 (CA1: Bregma –2.45 to –4.60) and dentate gyrus (DG: Bregma -2.00 to –3.90); central (CeA: Bregma –1.53 to –2.85), lateral (LaA: Bregma –2.00 to –3.70), basolateral (BslA: Bregma –1.78 to –3.25) and medial (MeA: Bregma –1.78 to –3.25) nuclei of the amygdala; paraventricular (PVT: Bregma –1.33 to –3.90), dorsomedial (DMT: Bregma –2.00 to –3.90) and centromedial (CMT: Bregma –1.53 to –3.90) thalamic nuclei; paraventricular (PVN: Bregma –1.08 to –2.00) and dorsomedial (DMH: Bregma –2.45 to –3.70) hypothalamic nuclei; dorsal (DR: Bregma –7.10 to –9.25) and medial (MR: Bregma –9.25 to –10.35) raphe nuclei 83. The area from structures of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany). Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). After image acquisition, FOS positive nuclei and phospho-ERK1/2-labeled dendrites were quantified. All areas were measured bilaterally (no left-right asymmetry for FOS or phospho-ERKs immunoreactivity was found) and therefore the resulting data was
reported as number of positive cells/0.1mm² (FOS-ir) or number of horizontal (H) and vertical (V) intersections (H+V contacts) between positive dendrites and an imaginary detection grid (composed by 514 horizontal x 698 vertical lines) present in the quantification field (H+V intersections/0.1mm²). Absolute regional FOS-ir (mean ± standard error (SEM)) for each region is reported in Table 1.

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<th>Area (ARS)</th>
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<th>Acute Males</th>
<th>Chronic Males</th>
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<th>Acute Females</th>
<th>Chronic Females</th>
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<td>34±5**</td>
<td>29±6°</td>
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Table 1. Absolute FOS-ir following acute and chronic stress in male and female rats

* = p<0.05; ** = p<0.01; *** = p<0.001; non-stressed vs. stressed-rats;
° = p<0.05; † = p<0.01; ‡ = p<0.001 non-stressed males vs. non-stressed females.

Statistics

One-Way-Anova and F test of variance were run on numbers of FOS and phospho-CREB immunoreactive cell nuclei from individual brain ROIs from experimental and control conditions. To compare cell counts from individual ROIs, t tests for equal or unequal variance were performed. P<0.05 was defined as the level of significance between groups.
Histological procedure - Molecular biology

Tissue and RNA Preparation

Thirty minutes after the beginning of the final session, rats used for the molecular biology were anesthetized with halothane and decapitated. The prefrontal cortex was dissected, quick-frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the prefrontal cortex of each animal by using Trizol (Life Technology, Gaithersburg, MD, USA) according to the manufacturer's instructions. Integrity of total RNA was confirmed on an agarose gel and final concentrations were assessed spectrophotometrically.

cDNA microarray

RNA was extracted from the prefrontal cortex of 4 or 5 rats within the groups participating in the chronic experiment and their controls. RNA (2-5µg/rat), subsequently converted into a 32P-labeled first-strand cDNA, was used to hybridize cDNA microarrays (rat atlas cDNA 1.2; Clontech, Palo Alto, CA, USA). Use of a broad coverage array instead of a stress array was intentionally chosen because of our interest in the role of transcription factors and second messengers in stress-induced neuronal dysfunction, which could involve the expression of numerous candidate genes. In this microarray, plasmid and bacteriophage DNAs are included as negative controls, along with several housekeeping cDNAs as positive controls. A complete list of the genes and controls spotted on the array, as well as array coordinates and GenBank accession numbers, is available at Clontech’s web site, (http://www.clontech.com). In order to suppress non-specific background each membrane was prehybridized for 30 min at 68°C in 5ml of hybridization solution (ExpressHyb, Clontech) with continuous agitation. Hybridization was subsequently carried out by the addition of the denatured, labeled cDNA to the prehybridization solution at 68°C for overnight incubation to reach a final probe concentration of 2-5 x 10^6 cpm/ml. Membranes were stringently washed with continuous agitation at 68°C in 2 x SSC, 1% SDS (4x30 min) and then in 0.1 SSC, 0.5% SDS (30 min) After a final rinse in 0.1 x SSC (5 min), membranes were mounted on Whatman paper, plastic-wrapped, exposed to x-ray film overnight at -80°C followed by exposure to a phosphoimager screen for 3 days.

PhosphoImaging analysis

Membranes were scanned using a Molecular Dynamics STORM PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA, USA), and images were analyzed by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA, USA). According to the Array manufacturer, Clontech, the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. An admonition of this quantitative analysis however, is that the accuracy for the extremely low abundant genes may not be reliable due to the detection limitation of this technique. The expression level for each gene was measured by the phosphoimager in arbitrary signal intensity units. This original raw output was subsequently used to perform the statistical analysis.
Statistics
To perform the following analysis, use was made of Delphi 5.0 and SPSS 11.0 software. Data from the 19 hybridizations was analyzed by log transforming the data points and converting to sequential format. Using analysis of variance the data was checked for systemic position effects. A significant effect was found in the y coordinate of the genes, but since this only explained 1% of the variance, a correction step for this effect would have minimal influence on data quality, and was thus omitted. After transformation to a parallel format, principal component analysis was performed to assess the first principal component. This common component was then removed to yield a better representation of true differences and outliers in a means plot using analysis of variance. To further analyze the data, a reference set of genes was chosen with which to test the hypothesis against a smaller, analysis set. Genes selected for inclusion in the analysis set were those which displayed the greatest variation compared to other genes. To verify the significance of the results, the criterion for inclusion of genes was expression over a range of standard deviations (>1.5, >1.8, >1.9 >2.5). In order to identify the strongest alterations of individual genes a plot was made of the regression factor scores. On the horizontal axis de common variation or expression of the genes was set out against the inter-array differences or variation (second principal component).
Results

Physiological and neuroendocrine changes following repeated footshock exposure

To define the dynamics of the response to prolonged footshock stress, various physiological and neuroendocrine parameters were measured, including body weight gain throughout the experiment, plasma adrenaline and corticosterone concentrations as well as adrenal weights upon termination.

Body weight gain

Body weights were measured daily during the acclimatization period and the chronic stress procedure in control and stressed rats (fig. 1a). During the acclimatization period both groups showed an identical weight gain. Immediately after initiation of stress exposure however, a consistent reduction in body weight gain was observed in stressed males, while weight gain in non-stressed animals continued constantly as expected. The difference in weight gain between non-stressed and stressed males continued to increase progressively until the final day (F=18.09, p<0.0019). No differences were found between non-stressed and chronically stressed females. This finding was in accordance with previous preclinical data showing that stress exposure, in female rats, does not affect body weight gain as much as it does in males.

Plasma corticosterone levels

Corticosterone and adrenaline concentrations were measured by HPLC. Although chronically stressed rats, on the final day, were only exposed to psychological (CSs and exposure to the box) and not to physical stress (USs), they showed significantly elevated plasma corticosterone concentrations (F=8.14, p<0.021, non-stressed vs. chronically stressed males; F=9.81, p<0.014, non-stressed vs. chronically stressed females). Adrenaline
concentrations, although higher in chronically stressed rats, did not reach a statistical difference compared to non-stressed animals (F=2.87, p<0.12, males; F=1.75, p<0.22, females) (fig. 1b). This evidence seems to suggest the lack of habituation in the neuroendocrine response to repeated footshock stress, both in male and female rats. It is interesting to note that females, both under stressed and non-stress conditions, reported higher plasma corticosterone levels than males (F=3.86, p<0.085, non-stressed males vs. females; F=6.26, p<0.037, chronically stressed males vs. females) (fig. 1b).

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Adrenal weights
Both male and female rats repeatedly subjected to footshock stress showed a significant adrenal hypertrophy (F_{males}=24.20, p_{males}<0.001: F_{females}=6.05, p_{females}<0.039) that appears to suggest a prolonged HPA axis activation (fig. 1c). Additionally, female rats showed higher
adrenal weights compared to males, both under non-stressed and stressed conditions (fig. 1c).

**Immunohistochemistry**

In the present study, two experiments were performed: a short-term training, consisting of two daily sessions of footshock stress during which 5 electric shocks were delivered, and a chronic experiment, consisting of 20 consecutive daily sessions of footshock stress.

![Figure 3. Phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites.](image)

After each experiment, FOS-ir (fig. 2) and phospho-ERK1/2 expression (fig. 3) were quantified throughout several cortical and limbic regions involved in the modulation of emotional and stress responses, including the frontal cortex, the hippocampus, the amygdala, the thalamus, the hypothalamus, and the midbrain.

**Acute footshock challenge.** Gender-related differences in the patterns of cortical-limbic activity were found in response to acute challenge. Male rats showed a significantly increased FOS-ir in the AC (F=14.35, p<0.0043) (fig. 4a), the CA1 (F=5.39, p<0.045) (fig. 4b), the CeA (F=13.45, p<0.0063), the LaA (F=16.39, p<0.0037), the BslA (F=8.93, p<0.017), the MeA (F=6.12, p<0.038) (fig. 4c), the CMT (F=9.54, p<0.013), the PVT (F=11.42, p<0.008) (fig. 4d), the PVN (F=6.45, p<0.032) (fig. 4e), the MR (F=6.24, p<0.034), and the DR (F=8.46, p<0.02) (fig. 4f). In contrast, female rats, acutely exposed to footshock stress, showed a reduction of FOS-ir in the mPFC (F=88.35, p<<0.001), the AC (F=5.81, p<0.039) (fig. 4a), the CA1 (F=94.88, p<<0.001), the DG (F=20.96, p<0.0013) (fig. 4b), the LaA (F=30.52, p<<0.001), the BslA (F=9.49, p<0.013), the MeA (F=11.43, p<0.008) (fig. 4c), the CMT (F=28.15, p<0.001), the DMT (F=26.15, p<0.001), the PVT (F=28.89, p<0.001) (fig. 4d), the DMH (F=5.24, p<0.048) (fig. 4e), and the MR (F=7.18, p<0.028) (fig. 4f). An opposite effect was found in the PVN, where a significantly increased FOS-ir was observed (F=6.61, p<0.033) (fig. 4e).
**Figure 4.** Effect of acute and chronic stress on absolute FOS-ir in: a) medial prefrontal cortex; b) hippocampus; c) amygdala; d) thalamus; e) hypothalamus; f) raphe nuclei. The symbol * expresses the comparison of absolute FOS-ir between stressed rats, both acutely or chronically, and non-stressed animals (*=p<0.05; **=p<0.01; ***=p<0.001).
Unexpectedly, acute footshock exposure caused a significant decrease of phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites in male rats while no changes were found in cyclic females (fig. 5).

**Chronic footshock challenge.** Gender specific patterns of neuronal activity were also observed in response to repeated footshock exposure. Male rats showed an increased cortical-limbic FOS-ir in 12 of the 15 regions (80%) (table 1): the increase was significant in 8 of the 15 areas (53%), including the mPFC ($F=36.90$, $p<0.0003$) (fig. 4a), the CeA ($F=6.52$, $p<0.034$), the BslA ($F=14.72$, $p<0.005$), the MeA ($F=12.25$, $p<0.0081$) (fig. 4c), the DMH ($F=10.05$, $p<0.013$), the PVN ($F=14.72$, $p<0.005$) (fig. 4e), the MR ($F=9.95$, $p<0.016$), and the DR ($F=28.65$, $p<0.0011$) (fig. 4f). Only DG ($F=6.63$, $p<0.033$) and CMT showed an opposite effect such as a decreased FOS-ir after prolonged footshock stress (fig. 4b). Chronically stressed females, in contrast, showed a widespread reduction of cortical-limbic FOS-ir (11 out of 15 regions analyzed). The effect was significant in the mPFC ($F=8.62$, $p<0.026$) (fig. 4a), the CA1 ($F=11.14$, $p<0.016$), the DG ($F=8.96$, $p<0.024$) (fig. 4b), the LaA ($F=45.60$, $p<0.001$) (fig. 4c), the CMT ($F=6.08$, $p<0.049$), and the DMT ($F=44.20$, $p<0.001$) (fig. 4d). An opposite effect was found in the PVN where a significantly increased FOS-ir was observed after chronic stress exposure ($F=8.04$, $p<0.03$) (fig. 4e). In addition, chronic footshock stress caused a selective and prolonged ERK1/2 hyperactivation in dendrites of the higher medial prefrontocortical layers (II and III) in males but not in cyclic female rats (fig. 5).
Basal level of protein expression and phosphorylation. A significant gender-related dimorphism in the level of basal FOS immunoreactivity (FOS-ir quantified under non-stressed conditions) was found in several cortical and subcortical areas (fig. 4). Non-stressed females showed in fact a significantly higher FOS-ir than male rats in the mPFC ($F=131.45$, $p<0.001$), the AC ($F=55.54$, $p<0.001$) (fig. 4a), the CA1 ($F=65.96$, $p<0.001$), the DG ($F=28.23$, $p<0.0011$) (fig. 4b), the CeA ($F=9.69$, $p<0.017$), the LaA ($F=77.98$, $p<0.001$), the BslA ($F=30.81$, $p<0.001$), the MeA ($F=31.82$, $p<0.001$) (fig. 4c), the CMT ($F=97.25$, $p<0.001$), the DMT ($F=68.07$, $p<0.001$), the PVT ($F=51.23$, $p<0.001$) (fig. 4d), the PVN ($F=64.79$, $p<0.001$) (fig. 4e), the MR ($F=8.09$, $p<0.025$), and the DR ($F=11.10$, $p<0.013$) (fig. 4f). No differences however were observed in the pattern of medial prefrontocortical phospho-ERK1/2 between male and cyclic female rats (fig. 5).

Molecular biology - Gene expression patterns
Since functional and morphological changes have been reported in medial prefrontocortical regions following chronic stress exposure, nineteen animals where assigned randomly to 4 groups and used for the analysis of gene expression patterns in this cortical region in response to prolonged footshock stimulation. The animals were assigned as follows:

- CTR-males (n=5) and CTR-females (n=5): these rats were exposed to the footshock box and CSs but did not receive any footshocks.
- STR-males (n=4) and STR-females (n=5): These animals were exposed daily to the footshock procedure for 20 consecutive days. On the final day of the experiment they only received CSs without consequent footshocks.

The results illustrate a significant gender difference with regard to gene expression following repeated footshock exposure. The males responded with stronger changes following stress and display an opposite change compared to stressed females. Whereas females illustrated a reduced mRNA transcription (-0.0080 to -0.0252), males demonstrated a strong increase in prefrontocortical gene expression following chronic footshock stress (-0.284 to 0.0594). This interaction effect is quite significant ($p=0.006$) although it only explains a minimal amount of variation (~0.1 %). If the genes to be compared relative to the others are selected for higher variance, the number of analyzed genes decreases and noise becomes more important. The significance of the results depends on the amount of data included, yet despite the chosen threshold, the trend remains the same. The strongest effect, illustrated below, was obtained with a standard deviation threshold of 1.8 (table 2).
These opposing findings in gene expression seem to confirm FOS-ir data concerning a sex-related dimorphism in the pattern of protein expression and, possibly, neuronal activity in response to repeated footshock exposure. In support of recent studies that documented atrophy of prefrontocortical dendrites in chronically stressed male rats, the present investigation reports an abnormal pattern of prefrontocortical ERK1/2 phosphorylation in chronically stressed males (fig. 5). Due to the pivotal role played by ERK1 and 2 in this neuronal function, the expression arrays were further analyzed to identify changes in genes that have been reported to modulate synaptic plasticity in the medial prefrontal areas and might be affected by prolonged footshock exposure. When regression factor score 1 was plotted against factor 2 a skewed distribution was evident (table 3).

Table 2

In line with the gender effects, the majority of outliers were located on the negative part of the X axis, coinciding with the greatest variation between highly expressive genes in males and low expressive genes in females. It is of interest to note that the expression of several genes involved in the modulation of neuronal plasticity, such as synapsin II, SNAP25, calmodulin, and ERK2, was differentially affected by repeated footshock stimulation in male compared to female rats (table 4).
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Table 4. Differential gene expression in prefrontal cortex of male and female rats exposed to prolonged footshock stress evaluated by cDNA array.
Discussion

Upon analysis of neuroendocrine and immunohistochemical changes induced by acute and prolonged footshock exposure, important gender-related differences emerged in the patterns of cortical-limbic FOS-ir and prefrontocortical phospho-ERK1/2 expression. The choice of investigating the level of expression and phosphorylation of these specific proteins was made upon reviewing their specific cellular functions. Changes of FOS-ir have been widely used as molecular marker of neuronal activity [75-77]. The analysis of FOS-ir has thus become a molecular tool to investigate complex processes, such as learning [85-91] and memory [92-95] as well as the neurocircuits activated by stress [96-105]. The extracellular signal-regulated kinase (ERK) is a member of a family of serine/threonine protein kinases implicated in the transduction of neurotrophic signals from the cell surface to the nucleus [78]. The ERK cascade plays a central role during neurodevelopment in the regulation of cell growth, proliferation, and differentiation but, interestingly, several family members, including ERK1 and ERK2, are also widely expressed by post-mitotic neurons in the mammalian nervous system [106]. This evidence has suggested that ERKs might contribute to the regulation of important functions in the adult brain, including neuronal plasticity, learning, and memory [107,108]. A critical step in the regulation of ERK-mediated activities is the dual phosphorylation of these kinases that leads to their transient activation and translocation from the cytoplasm to the nucleus [109]. Only phosphorylated ERKs (phospho-ERKs) are able to interact with and activate cytoplasmic and nuclear targets, and modulate such critical neuronal functions [78]. Changes in the levels of ERK1/2 phosphorylation may thus provide important indications concerning the ability of stress to influence neuronal plasticity.

Immunohistochemical changes in response to acute footshock challenge

Acute footshock exposure activated, in male rats, cortical and subcortical structures, including the cingulate cortex (fig. 4a), the hippocampal CA1 (fig. 4b), the central, lateral, basolateral, and medial nucleus of the amygdala (fig. 4c), the centromedial and paraventricular nucleus of the thalamus (fig. 4d), the paraventricular hypothalamic nucleus (fig. 4e), the median and dorsal raphe nucleus (fig. 4f). In contrast, acute footshock stress was associated, in female rats, with a significant reduction of FOS-ir in most of the above-mentioned cortical-limbic regions (fig. 4), with the only exception of the PVN where, similarly to males, a marked increase of neuronal activation was observed (fig. 4e).

Acute emotional experiences have been reported to promote learning and memory [37,110,111]. A growing body of evidence has pinpointed in particular the amygdala and the hippocampus as core components of the brain’s fear system [112-118]. Thus, the
increased FOS-ir reported by males in the amygdala (CeA, LaA, BslA, and MeA) (fig. 4c) and hippocampus (CA1) (fig. 4b) may support the participation of these limbic structures in the modulation of acute fear-related responses. Surprisingly, a different pattern of cortical-limbic FOS-ir was observed in female rats following short-term aversive challenge. In contrast to males, acutely stressed females reported a significant reduction of FOS expression in both amygdala (LaA, BslA and MeA) and hippocampus (CA1) compared to non-stressed animals (fig. 4b, c). This differential pattern of neuronal activation was not limited to these two limbic structures but also involved the anterior cingulate cortex (fig. 4a), the thalamus (centromedial and paraventricular nuclei) (fig. 4d), and the midbrain (median raphe nucleus) (fig. 4f). Interestingly, a decreased phospho-ERK1/2 immunoreactivity was observed in medial prefrontocortical dendrites of acutely stressed males while no changes in the level of kinase phosphorylation were found in cyclic females (fig. 5). A critical step in ERK-mediated facilitation of neuronal plasticity involves their dual phosphorylation followed by translocation from the cytoplasm to the nucleus. Reduced phospho-ERK1/2 immunoreactivity in medial prefrontocortical dendrites of male rats, might thus illustrate the translocation of these enzymes from the periphery to the nucleus (fig. 3a, b, 5) and support the molecular changes underlying the consolidation of fear-related memories.

In recent years, a cumulative body of evidence concerning the existence of morphological and functional differences between the male and female brain has emerged. Cognitive processes, such as learning and memory, as well as behavioral responses to stress are influenced by sex. The gender-related patterns of neuronal activity observed in the present study following acute challenge might represent sex-specific coping strategies under aversive conditions. The notion that males and females may differ in their coping strategies has been proposed by Taylor and colleagues. Taylor stated that the male response to stress in humans, along with some animal species, is characterized by a “fight-or-flight” response whereas the female response is more typically characterized by a pattern termed “tend-and befriend.” These gender-related behavioral responses may reflect the involvement of different neural pathways and our results might offer indirect immunohistochemical evidence linking such gender-related coping styles with differential patterns of neuronal activity. A closer look at our data reveals that the divergent response to acute footshock challenge appears to be strongly related to the different level of basal FOS-ir (FOS expression under non-stress conditions) (fig. 4). Non-stressed females in fact, illustrated overall higher neuronal activation than males (up to 5-7 times higher), especially in frontocortical areas (fig. 4a). In line with these findings, neuroimaging investigations have also found gender-related diversities in brain activity patterns in humans, as women illustrated higher values than men. Furthermore, Esposito and colleagues reported substantial gender-related differences in the frontal lobe rCBF during
performance of a variety of cognitive tasks with women showing a significantly higher activation. Although functional brain imaging studies have illustrated sex differences in global as well as in regional brain activity, reports of differential activation in the frontal lobes have been particularly prevalent. The results presented here may thus provide new insights into gender-related differences in the neuronal circuitry engaged in the acute stress response and the molecular mechanisms underlying its modulation.

**Gender-related dimorphism following repeated footshock exposure**

Brief elevations of glucocorticoid levels play a critical role in the modulation of fear-related responses, promoting learning acquisition and memory consolidation. This beneficial effect of adrenal steroids, however, is only temporary as prolonged exposure to high glucocorticoid concentrations has been shown to impair cognitive processes, possibly through the deleterious effects of stress hormones on neuronal plasticity. It is intriguing to speculate that gender-related differences in FOS-ir and ERK1/2 phosphorylation, detected following repeated footshock stress, may illustrate the deleterious effects of prolonged exposure to hostile conditions on functional and structural integrity of the brain. The latter is supported by physiological and neuroendocrine evidence, such as the reduction of body weight gain, the significant elevation of corticosterone levels, the hyperactivity of the PVN, and the adrenal hypertrophy, observed in both chronically stressed male and female rats. Footshocks have been reported to strongly activate the PVN, elevating plasma corticosteroid concentrations. Given the pivotal role of this hypothalamic nucleus in the regulation of the HPA axis, these neuroendocrine changes seem to substantiate a lack of habituation and, possibly, an abnormal HPA axis activation in response to repeated stress. An intriguing possibility is that prolonged footshock exposure promotes functional and morphological impairments by persistently elevating corticosteroid concentrations in the brain. Glucocorticoids have been known to exert a deleterious influence on neuronal plasticity and cause functional and morphological abnormalities in vulnerable regions, such as the hippocampus and the prefrontal cortex. Functional cortical-limbic alterations included, in chronically stressed males, a reduced neuronal activation in the DG and an increased FOS-ir in the mPFC, amygdala, hypothalamus, and raphe. The abnormal ERK1/2 phosphorylation in medial prefrontocortical dendrites may, instead, document a stress-related structural impairment. In female rats, on the contrary, prolonged aversive stimulation was associated with a general reduction of FOS-ir in most of the cortical and subcortical regions, including the mPFC, the hippocampus, the LaA, and the thalamus (CMT and DMT). No changes in the level of phospho-
ERK1/2 immunoreactivity were observed (fig. 5). These gender-related patterns of neuronal activity were also confirmed at the molecular level, as gene expression analysis illustrates a general up-regulation of gene transcription in the frontal lobe of chronically stressed males, while a differential response was observed in females. It is interesting to note that, in addition to the gender-related dimorphism observed in the level of FOS and phospho-ERK1/2 immunoreactivity following repeated footshock exposure, differential gene expression patterns were also detected between male and female rats, including various key genes underlying neuronal plasticity (table 4).
Amygdala-prefrontocortical involvement
in response to chronic footshock stress:
a gender comparative view*

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*Adapted from the manuscript in preparation for “Neuroscience”
Introduction

Exposure to stressful events represents a predisposing factor in the development of depression 153, chronic psychiatric illness characterized by complex cortical and subcortical defects 154,155 and marked gender-related prevalence 156. These abnormalities include functional and structural deficits, such as neuronal pathology 157, reduced prefrontocortical function 158,159 as well as abnormal amygdala 160,161 and HPA axis activity 162-165. A troubling aspect of depression involves the prolonged processing of negative emotions and the preferential memory for adverse emotional events 166,167. Recent clinical findings have confirmed the existence of malfunctions in the coordinated interplay between amygdala and prefrontal cortex as a critical element in the development and maintenance of depressive symptoms, such as recollection of intrusive traumatic memories and persistent low mood 158,168,169.

In the present study, we investigated the cortical-limbic response to prolonged footshock stress in male and female rats, using c-fos (FOS-ir) and phospho-CREB expression as immunohistochemical correlates of cellular activity 170-172 and neuronal plasticity 172-174. Reliability of these molecular markers to study complex neurocircuits underlying cognitive and emotional regulation 170,175-185 as well as neuronal plasticity changes 186-188 has been established. Analysis of FOS-ir and phospho-CREB expression focused primarily on cortical and limbic regions involved in the modulation of stress, cognitive, and emotional responses such as the prefrontal and anterior cingulate cortices, the hippocampus, amygdala, thalamus, and hypothalamus. This data may contribute to the understanding of the mechanisms underlying gender-related vulnerability to stress and provide new insights into the cortical-limbic circuits involved in the modulation of the response to chronic aversive stimulation.
Materials and Methods

Animals

Adult male (n=12: 212-240 g) and cyclic female (n = 12: 195-212 g) Wistar rats were used in the present investigation. The animals were individually housed (cages 45 x 28 x 20 cm) with food and water available ad libitum and maintained on a 12/12-hr light/dark cycle. They were weighed (09:00) and handled daily for 5-8 min to minimize non-specific stress response. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and with the guidelines of the Animal Bioethics Committee of the University of Groningen (FDC: 2509).

Chronic footshock paradigm

The rodent footshock-chamber consists of a box containing an animal space placed on a grid floor connected to a shock generator and scrambler. Test rats received one session of 30-60 min/day in the footshock box during which 5 inescapable footshocks were given (0.8 mA in intensity and 8 sec in duration) with different inter-shock intervals in order to make the procedure as unpredictable as possible. This procedure was followed for 20 days. The final day of the experiments (21st day) all rats were placed in the footshock box without being exposed to any painful electric shocks.

Non-stressed rats. Twelve rats (6 males and 6 cyclic females) were used as control animals. These animals were exposed to the same environmental stimuli as stressed rats. They were housed in the same room as stressed rats and regularly exposed to the footshock chamber, although they were never subjected to footshocks or other psychological stressors (such as vocalizations produced by stressed rats) during the entire duration of the experiment.

Physiological and neuroendocrine changes in response to chronic footshock stress

To define the dynamics of the response to chronic footshock stress, changes in physiological and neuroendocrine parameters were measured. Weight gain was monitored on a daily basis throughout the experiment, and upon termination, adrenal glands and thymus were removed and weighed. Graphs were constructed to serve as a reference to verify the severity of stress perceived by the animals during the experiment. In addition, blood samples were taken after the final session (no footshocks were given) and stored at -80°C, until determination of plasma corticosterone, noradrenaline, and adrenaline concentrations by HPLC.

Extraction and Chromatography

Adrenaline and Norepinephrine. Catecholamines were extracted from plasma using liquid/liquid extraction with 3,4-dihydroxybenzylamine as internal standard. Briefly, plasma adrenaline and norepinephrine were bound to diphenylborate-ethanolamine at pH 8.6. The extraction was performed with n-heptane (containing 1% octanol and 25%
tetraoctylammoniumbromide). Finally, catecholamines were extracted from the organic phase with diluted acetic acid. Adrenaline and noradrenaline (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, 3µm, 150x2.0mm), followed by an electrochemical detector (Antec Leyden, The Netherlands) working at a potential setting of 500mV vs. Ag/AgCl reference. The mobile phase consisted of 50mM acetate buffer, 150mg/l octane sulphonic acid, 150mg/l tetramethylammonium, 15mg/ml Na2EDTA and 3% methanol, adjusted to pH 4.1. The flow-rate was 0.35ml/min. Temperature was 30°C. The detection limit of the method was 0.1nM.

**Corticosterone.** For this assay, dexamethason was used as internal standard. After addition of the internal standard, plasma was extracted with 3ml 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 200µL of mobile phase and 50µL was injected into the HPLC system. The mobile phase (flow rate 1.0mL/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 254nm. The detection limit of the method was 10nM.

**Immunohistochemistry**

Two hours following the beginning of the final session, rats were sacrificed with an overdose of halotane which preceded blood sampling and transcardial perfusion 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 0.02 M, 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.

**FOS and phospho-CREB immunohistochemistry**

The stainings was performed on free-floating sections under continuous agitation. The sections were preincubated in 0.3% H2O2 for 15 min to reduce endogenous peroxidase activity, before being incubated in a primary polyclonal rabbit anti-FOS antibody (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 (Upstate Biotechnology, Charlottesville, VA, USA: www.upstatebiotech.com; 1:1000 dilution in KPBS 0.02 M, pH 7.4). 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 in KPBS 0.02 M, pH 7.4) 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% H$_2$O$_2$ for 15 min. Finally, sections were washed, mounted on slides, dehydrated and coverslipped with DePex.

**Antibody specificity testing.** To control for cross-reactivity due to aspecific binding, immunostainings were performed by incubating several sections without the presence of one of the antibodies needed for the reaction (primary, secondary or tertiary antibody), thereby confirming the specificity of all antibodies used. All these reactions were negative confirming the specificity of the antibodies.

**Quantification and data analysis**

FOS and phospho-CREB positive nuclei were quantified using a computerized imaging analysis system. The quantification was performed by an observer who was blind to group assignment. For counting of the immunoreactive cell nuclei, at least 4-5 sections per each brain area were analyzed. The selected area from regions of interest (ROI) were digitized by using a Sony (SONY Corporation, Tokyo, Japan) charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (LEICA, Wetzlar, Germany) at x100 magnification. ROIs included the medial orbitofrontal cortex (mORB: Bregma +4.85 to +3.60), the prefrontal cortex (prelimbic (PrL) and infralimbic (InfraL) area; mPFC: Bregma +3.60 to +1.70), the anterior (AC: Bregma +3.20 to +0.95) and posterior cingulate cortex (postCING: Bregma +1.70 to -1.08); the dentate gyrus (DG: Bregma -2.00 to -3.90); the central (CeA: Bregma -1.53 to -2.85), the lateral (LaA: Bregma -2.00 to -3.70), and the basolateral nucleus of the amygdala (BslA: Bregma -1.78 to -3.25); the paraventricular thalamic (PVT: Bregma -1.33 to -3.90) and hypothalamic nucleus (PVN: Bregma -1.08 to -2.00), the median raphe nucleus (MR: Bregma -9.25 to -10.35) 191. ROIs were outlined with a digital pen and their areas were measured. Each digitized image was individually set at a threshold to subtract the background optical density, and the numbers of cell nuclei above the background were counted by using the computer-based image analysis system LEICA (LEICA Imaging System Ltd., Cambridge, England). FOS and phospho-CREB positive immunoreactivity was reported as number of positive cells/0.1mm$^2$. Although all areas were measured bilaterally, no left-right asymmetry in FOS or phospho-CREB expression was found.

**Relative regional cortical-limbic FOS-ir**

This analysis allows one to consider individual cortical-limbic structures as parts of a larger, more complex system. In order to perform this calculation, we determined the average regional surface (ARS) of all the regions of interest. The c-fos positive cell densities of each cortical-limbic region was then multiplied by the average regional surface for all animals (regional cell density $n$ * ARS). This was done in order to correct for eventual differences in quantified areas between different rats, thereby providing c-fos positive cell numbers across a similar cortical-limbic quantified surface area in all rats, suitable for comparison. For a detailed description of relative FOS-ir analysis, the reader is referred to Chapter 1, Materials and Methods section 192.
Statistics

One-Way-Anova and $F$ test of variance were run on numbers of FOS and phospho-CREB immunoreactive cell nuclei from individual brain ROIs from experimental and control conditions. To compare cell counts from individual ROIs, $t$ tests for equal or unequal variance were performed. $P<0.05$ was defined as the level of significance between groups.
Results

Physiological and neuroendocrine changes in response to prolonged stress

To define the dynamic of the response to prolonged footshock exposure, physiological and neuroendocrine changes, including body weight gain, adrenal and thymus weight, plasma adrenaline, noradrenaline, and corticosterone levels, were analyzed.

Body weight gain

Body weights were measured daily throughout the experiment in non-stressed and stressed rats (fig. 1). A consistent reduction in body weight gain was observed in chronically stressed males (F=9.21, p<0.013), while weight gain in control animals continued constantly as expected. No differences were detected between control and stressed females.

![Figure 1. Body weight gain following prolonged stress exposure.](image)

Plasma corticosterone, adrenaline and noradrenaline concentrations

Blood samples were collected by transcardial injection upon termination and corticosterone, adrenaline, and noradrenaline concentrations were subsequently measured by HPLC. Although stressed rats were not exposed to footshocks during the final session, they showed significantly higher plasma catecholamines and corticosterone levels (fig. 2a). Plasma adrenaline (F=7.34, p<0.024) and corticosterone (F=5.96, p<0.036) concentrations were significantly higher in chronically stressed males, while increased noradrenaline (F=6.32, p<0.036) and corticosterone levels (F=8.1, p<0.022) were observed in stressed females. Chronic stress also induced thymus hypotrophy in females (F=5.1, p<0.048) and adrenal hypertrophy in both male (F=26.41; p<<0.001) and female rats (F=5.94, p<0.035) (fig. 2b). These results suggest the lack of habituation in the response of the HPA axis to repeated
stress but also support the view concerning a persistent hyperactivity of this stress response system following prolonged footshock exposure.

**Figure 2.** Plasma catecholamine and corticosterone concentrations in chronically stressed male and female rats (a). Prolonged stress exposure also caused a significant adrenal hypertrophy in both genders (b).

**Immunohistochemistry**

**Absolute FOS-ir**

In the present study, FOS-ir was quantified throughout various forebrain structures, including the frontal cortex, the hippocampus, the amygdala, the thalamus, and the hypothalamus (fig. 3).

**Figure 3.** Effect of chronic stress on absolute FOS-ir in: a) medial prefrontal cortex; b) amygdala; c) hippocampal, thalamic and hypothalamic areas.
Chronic footshock stress resulted, in male rats, in a significantly decreased absolute FOS-ir in the mPFC ($F=5.17, p<0.046$), the mORB ($F=5.36, p<0.043$), the AC ($F=12.92, p<0.0049$) (fig. 3a), and the DG ($F=5.17, p<0.046$) (fig. 3c). Only the PVN showed an opposite tendency, showing a significant induction of FOS-ir ($F=6.58, p<0.028$) (fig. 3c). Chronically stressed females, in contrast, reported a selective increased FOS-ir in the CeA ($F=7.1, p<0.024$), the LaA ($F=8.25, p<0.017$), and the BslA ($F=6.2, p<0.032$) (fig. 3b). No changes were instead detected in any prefrontocortical region examined (fig. 3a). Likewise males, chronic footshock exposure resulted, in females, in a significantly increased FOS-ir in PVN ($F=15.78, p<0.0026$) (fig. 3c).

**Figure 4.** Effect of prolonged stress exposure on relative FOS-ir in: a) medial prefrontal cortex; b) amygdala; c) hippocampal, thalamic and hypothalamic areas; d) prefrontal vs amygdala relative activity.

**Relative FOS-ir**

Chronic footshock stress was associated, in males, with increased relative FOS-ir in the BslA ($F=5.36, p<0.046$) (fig. 4b) and PVN ($F=16.38, p<0.002$) (fig. 4c), while a decreased relative regional activity was detected in the AC ($F=25.82, p<0.001$) (fig. 4a). Chronically stressed females illustrated a selective increase of relative FOS-ir in the LaA ($F=6.92, p<0.025$) (fig. 4b) and PVN ($F=8.25, p<0.017$) (fig. 4c). In contrast, a significantly decreased relative regional FOS-ir was found in the mPFC ($F=5.23, p<0.045$) (fig. 4a).
Phospho-CREB immunohistochemistry

In male rats, repeated footshock exposure caused a general reduction of phospho-CREB immunoreactivity in both cortical and subcortical regions (fig. 5). Decreased phospho-CREB immunoreactivity was detected in the mORB (F=36.06, p<<0.001), the PrL (F=16.59, p<0.002), the InfraL (F=38.18, p<<0.001) (fig. 5a), the AC (F=10.59, p<0.009), the postCING (F=6.84, p<0.026) (fig. 5b), the hippocampal DG (F=11.99, p<0.006), the LaA (F=24.68, p<<0.001), and the BslA (F=42.32, p<<0.001) (fig. 5c). Surprisingly, no changes in CREB phosphorylation were detected in cyclic female rats with the only exception of the hippocampal dentate gyrus where a marked, although not significant, reduction was observed (F=4.38, p<0.081) (fig. 5c).

**Figure 5.** Effect of chronic stress on phospho-CREB immunoreactivity in: a) medial prefrontal cortex; b) cingulate cortex; c) hippocampus and amygdala.
**Discussion**

Chronic stress has been associated with functional and structural neuronal dysfunctions\textsuperscript{139,142,193-195}. In this study, we explored at a cellular level, the alterations of cortical-limbic activity and neuronal plasticity in response to prolonged footshock stress in male and female rats. A significant reduction in body weight gain (fig. 1), elevation of plasma adrenaline and corticosterone levels (fig. 2a), adrenal hypertrophy (fig. 2b), and increased absolute and relative FOS-ir in the PVN (fig. 3c, 4c) were detected in chronically stressed males. Similarly, chronically stressed females reported higher plasma noradrenaline and corticosterone concentrations (fig. 2a), significantly reduced thymus weight, adrenal hypertrophy (fig. 2b), and enhanced absolute and relative PVN FOS-ir (fig. 3c, 4c). Since the PVN plays a key role in HPA axis regulation, the increased activity observed in this nucleus, combined with the increased corticosterone concentrations and adrenal hypertrophy, suggests a prolonged HPA axis hyperactivity in both sexes. It is interesting to note that both stressed male and female rats exhibit similar neuroendocrine responses (fig. 2a,b). However, despite these similarities in the response to stress, differential, gender-related patterns of absolute FOS-ir and phospho-CREB expression were detected (fig. 3, 4, 5). Chronic footshock exposure selectively affected prefrontocortical and hippocampal regions in male rats, causing a significant reduction of absolute regional FOS-ir in the medial orbitofrontal cortex, the medial prefrontal cortex, the anterior cingulate cortex (fig. 3a), and the dentate gyrus (fig. 3c). Interestingly absolute FOS-ir in the amygdala remained unaffected (fig. 3b). A marked reduction of CREB phosphorylation in various cortical and subcortical structures was also observed (fig. 5). In contrast to males, chronically stressed females showed significantly increased absolute FOS-ir in the central, lateral, and basolateral nuclei of the amygdala (fig. 3b). No relevant changes in the level of neuronal activity were observed in cortical regions (fig. 3a) and, surprisingly, only a slightly decreased phospho-CREB immunoreactivity was detected (fig. 5).

The circuitry underlying neuroendocrine regulation has been characterized in both humans and rodents and involves coordinated interactions amongst frontocortical areas (orbital, medial prefrontal and anterior cingulate cortices), the hippocampus, the amygdala, the hypothalamus, and several brainstem nuclei \textsuperscript{196,197}. The prefrontal cortex and the hippocampus, in particular, modulate the activity of the HPA axis by maintaining this system under functional inhibition \textsuperscript{198,199}. Lesions in prefrontocortical and hippocampal areas result, at least in male rats, in pronounced activation of both HPA axis and sympathetic nervous system \textsuperscript{200}. On the contrary, the amygdala stimulates HPA axis activity, either directly through its connections to the PVN and/or indirectly by modulating the activity of various noradrenergic brainstem nuclei \textsuperscript{199}. The amygdala also participates in the modulation of acute stress and fear conditioned...
Thus, while acute fear is characterized by increased amygdala activity and reduced prefrontal activation \(^{203}\), the termination of this very same response has been associated with increased frontocortical activity and reduced amygdala activation \(^{204}\). Exposure to acute threatening stimuli strongly stimulates the amygdala \(^{203}\) promoting its activation that prevails over the functional inhibition mediated by cortical and limbic structures, such as the medial prefrontal and the anterior cingulate cortex. As a result, activation of the amygdala may ultimately lead to functional inhibition of prefrontocortical areas \(^{203}\). The response profile of the amygdala however, habituates from early to late stages of the aversive response \(^{201}\). This structure has been found most consistently activated in the early phases of acute stress or conditioned-fear acquisition and this activation was then found to progressively decrease \(^{205}\). It is intriguing to hypothesize that the gradual reduction of amygdala activity may “free” the frontal cortex from the functional inhibition mediated by this limbic structure. In addition, during the final phase of the aversive response, the level of prefrontocortical activation slowly increases, perhaps overcoming amygdala-mediated inhibition and leading in turn to the extinction of the aversive response \(^{204}\). In the absence of new adverse stimuli, the prefrontocortical activation remains elevated thereby maintaining the amygdala under inhibition and avoiding the onset of a new “stress” response until the animals are faced with a novel potential threat. Frontocortical deficits have also been shown to release the amygdala from this inhibition \(^{206}\), resulting possibly in a slower extinction of stress response. It is possible that the reduced frontocortical and hippocampal activation seen in males (reduced absolute FOS-ir) (fig. 3a,b), combined with a general reduction of neuronal plasticity (reduced phospho-CREB immunoreactivity) (fig. 5), may compromise the coordination between frontal and/or subcortical structures, thereby impairing the appropriate regulation of the neuroendocrine response to stress. The probability of an abnormal response to adverse stimuli (slower termination) seems also supported by the prolonged HPA axis hyperactivity, as confirmed by both neuroendocrine (fig. 2) and immunohistochemical findings (fig. 3c, 4c).

This hypothetical framework however, does not explain why the lack of change in the number of FOS-labeled neurons observed in the male amygdala (fig. 3b), since a significant activation would be expected in this limbic structure following stress. However, absolute FOS-ir provides only a general indication of the regional response to specific stimuli since it does not consider, for instance, the individual differences in basal expression of this immediate early gene amongst animals. Therefore, while absolute FOS-ir analysis suggested that long-term footshock stress in males did not engage the amygdala (fig. 3b), relative FOS-ir analysis displayed a different scenario (fig. 4b). Relative analysis revealed a significant chronic stress induced activation of both the BslA and the PVN (fig. 4b,c). In accordance with absolute FOS-ir analysis, a significant reduction of relative FOS-ir was also observed in the mPFC and DG (fig. 4a,c). An
interesting possibility, supported by clinical and preclinical evidence, is the ability of chronic stress to permanently elevate glucocorticoids levels by impairing stress response regulation 162,207-210. The BslA has been involved in many aspects of stress and conditioned-fear responses 211-214, and a permanent hyperactivity of this nucleus may facilitate, directly and indirectly, HPA axis activation by maintaining the prefrontal cortex under enduring inhibition. Ultimately, amygdala hyperactivity and prefrontocortical hypofunction may act synergistically to promote HPA axis hyperactivity and further elevate glucocorticoid levels. Corticosteroids, in turn, promote stress-induced hippocampal structural impairments 215,216, possibly through inhibitory effects on CREB phosphorylation 143,217. The combination of these effects may explain the reduced hippocampal absolute FOS-ir and phospho-CREB expression observed in the DG and CA3 (fig. 3c, 5c), important regions for feedback inhibition to the HPA axis209,218,219. This complex series of events thus provides a hypothetical pathway by which chronic footshock exposure may lead to HPA axis hyperactivity, elevated glucocorticoid levels, and selective impairment in the circuitry underlying stress response regulation, promoting further glucocorticoid secretion and leading, eventually, to neuronal defects.

Contrary to males, different chronic stress-induced patterns of absolute cortical-limbic FOS-ir (fig. 3) and phospho-CREB expression (fig. 5) were observed in female rats. Stressed females showed a selective increase of absolute FOS-ir in the amygdala (fig. 3b) and PVN (fig. 3c), and display only a slight reduction phospho-CREB immunoreactivity (fig. 5). In the past few years, a growing amount of literature has reported both functional 220-225 and morphological differences 226-232 between the male and the female brain. These gender-related differences might offer important insights into the understanding of the dimorphic patterns of frontocortical-amygdalar FOS-ir and phospho-CREB expression. Sustained stress has been associated with cortical-limbic abnormalities and emotional dysregulation 233-235. The amygdala, in particular, plays a key role in the modulation of stress response and the pathophysiology of affective disorders 161,169,236. Whereas such disorders are often associated with marked gender differences 154,155, clinical studies have also revealed sex-related dimorphisms in the involvement of the amygdala during emotional processing 221,223,224,237. A significant enlargement of this subcortical structure has also been reported in depressed subjects 236. It is intriguing to speculate that chronic aversive conditions selectively affect the amygdala in females, leading to its abnormal activation (fig. 3b), which in turn may alter the coordinated interactions between cortical and subcortical structures. It is also possible that chronic footshock stress, in addition to the amygdala, targets prefrontocortical regions in females as well, without however causing the state of functional deactivation observed in males, but disrupting the orchestrated coordination between cortical and subcortical regions. Relative FOS-ir analysis seems to facilitate the
interpretation of these results (fig 4). Although marked gender-related differences were seen in the distribution of absolute FOS-ir in response to prolonged stress, the patterns of relative FOS-ir were surprisingly similar in both sexes (fig. 4). Chronic stress resulted in amygdala hyperactivity (BslAmales vs. LaA females) (fig. 4b) and frontocortical hypoactivity (ACmales vs. mPFC females) (fig. 4a) in both males and females. Chronic stress might thus impair the ability of frontocortical regions to exert an adequate inhibition on the amygdala, resulting in its functional hyperactivity that causes malfunctions in the modulation of the stress response as well as the impairment of cognitive and emotional regulation. A troubling aspect of depression is represented by the prolonged processing of negative emotions 169. Intriguingly, the amygdala has been shown to play a central integrative role in the elaboration of emotional stimuli and the retrieval of emotional memories 211,238-240. One however, may question if the hyperactivity observed in the amygdala is related to the persistent recollection of intrusive emotional memories. An important indication to answer this question may be provided by the analysis of phospho-CREB immunoreactivity. CREB phosphorylation has been reported to be crucial in the modulation of neuronal plasticity and the consolidation of new fear-related memories 181,241. The phosphorylation of this transcription factor appears to be fundamental for the stability of new and reactivated fear memories 241. Interestingly, chronically stressed females did not show the general and significant decrease of phospho-CREB expression observed in male rats (fig. 5). We can thus speculate that the availability of phosphorylated CREB in the amygdala may support the prolonged processing of negative emotions leading, in females, to a condition of functional hyperactivity, as documented by the significantly increased absolute and relative FOS-ir (fig. 4b).

The molecular mechanisms underlying this differential effect of chronic footshock stress on phospho-CREB expression remain obscure although an intriguing candidate to explain these differences might be represented by ovarian hormones. As illustrated in chapter 1, chronic footshock stress may cause cortical-limbic abnormalities by impairing the activity of intracellular elements involved in the transduction of neurotrophic signals. By influencing CREB expression and/or phosphorylation, prolonged stress/glucocorticoid exposure may affect neurotrophin availability, reducing neuronal plasticity and increasing the vulnerability of neurons to subsequent insults. Similarly to the previous study, chronically stressed males showed, a marked reduction of phospho-CREB immunoreactivity (fig. 5). Following chronic footshock stress however, no changes of CREB phosphorylation were observed in females (fig. 5). Estrogen and neurotrophins activate similar signaling transduction pathways that culminate with CREB phosphorylation 242,243. Ovarian hormones also protect neurons from the effects of oxidative stress 244. It is thus intriguing to hypothesize that ovarian hormones may protect the female brain from the deleterious influence of glucocorticoids on neuronal
plasticity by preventing stress-induced reduction of phospho-CREB immunoreactivity. On the contrary however, ovarian hormone-induced trophic actions may also provide critical substrates (such as phospho-CREB and phospho-ERK1/2) that promote the formation, consolidation and ultimately the recollection of intrusive traumatic memories.
Conclusions

A wide variety of sexual dimorphisms, both structural and functional, between sexes have been described in the brains of many vertebrate species, including humans. In the first part of this chapter, we explored the neurochemical changes induced by prolonged footshock stress in male and cyclic female rats. Remarkably, marked sex differences emerged in the patterns of FOS-ir and ERK1/2 phosphorylation in response to repeated stress. Sex hormones may account for some of the gender discrepancies since cyclic changes in levels of circulating estrogen and progesterone have been established to play a central role in the differences observed in stress-sensitivity and psychopathology between men and women. The neurocircuits underlying cognitive and emotional processes are also prime targets for ovarian hormone action and stress. By influencing the hormonal state of the animals, stress may play a central role in determining gender-related dimorphisms. In a parallel study specifically performed to assess the overall effects of ovarian steroids in central stress integration, immunohistochemical changes associated with sustained stress exposure were analyzed in both cyclic and ovariectomized female rats\textsuperscript{245}. Surprisingly, no differences absolute FOS-ir patterns were found between cyclic and ovariectomized females following long-term aversive stimulation in any of the cortical and subcortical regions examined. Therefore, although important, the presence or cyclic fluctuations of sex hormones does not account for all the differences observed between male and female rats.

It is likely however, that the action of ovarian hormones during the early developmental phases along with other factors might also be critical in influencing the structure of the CNS. Morphological sex differences in brain areas underlie sex differences in function, and it has become increasingly clear that male and female brains are two different and separate entities both from a functional and a morphological perspective. Remarkably, while substantial and compelling evidence exists for gender-related differences in brain structure and function, our understanding of the molecular and cellular mechanisms that give rise to these dimorphisms remain poorly explored. Ovarian steroids may prevent the abnormal ERK1/2 phosphorylation that was detected in chronically stressed males. Estrogen and neurotrophins activate similar signaling transduction pathways, including the ERK1/2 cascade\textsuperscript{242,243}, and protect neurons from the effects of stress by modulating the activity of this pathway\textsuperscript{244}. It is thus intriguing to speculate that estrogen and/or progesterone may protect the female brain against some of the consequences of chronic stress by preventing stress-induced alterations of ERK1/2 activity. Sex steroids may substitute the function of neurotrophins reduced by chronic stress, in sustaining the plastic changes needed by the brain when faced with prolonged aversive conditions. The immunohistochemical changes presented here in male and female rats might represent neurochemical evidence for a differential role of stress on
cognitive processing. More importantly, the functional dimorphism within the cortical-limbic network observed following long-term aversive stimulation might illustrate gender-related differences in the sensitivity to the deleterious effects of stress on these neurocircuits. It seems plausible that the reduction of cortical-limbic absolute FOS-ir following acute stress as well as the presence of ovarian hormones may protect female rats from the harmful consequences of chronic footshock stress, thereby preventing both structural (phospho-ERK1/2 hyperphosphorylation) and functional (neuronal hyperactivity) prefrontocortical abnormalities. This view however is in contrast with a growing body of literature, both clinical and preclinical, that supports the notion of higher stress sensitivity in female under both acute and chronic conditions.

In the second part, we have presented additional evidence in support of the destabilizing effects of chronic stress on the coordinated regulation of cortical-limbic activity and neuronal plasticity. Chronic footshock exposure resulted in a significant reduction of absolute prefrontocortical FOS-ir in males while promoting amygdala hyperactivity in females. Moreover, a general reduction of cortical-limbic phospho-CREB immunoreactivity was observed in chronically stressed male rats, while no changes were found in females. The prefrontal cortex and the amygdala may thus represent primary targets of the dimorphic and detrimental influences of stress. It is possible that chronic footshock stress may induce differential gender-related immunohistochemical changes, due to the divergent and sex-dependent role that cortical and subcortical structures plays during cognitive and emotional assessment. Consequently, the negative impact of prolonged stress exposure may differ depending on the gender of the organism. However, due to the profound interconnections amongst cortical and limbic structures necessary to guarantee a coordinate brain functioning and assure the proper regulation of stress responses, the destabilizing action of stress may lead to similar abnormalities, independently with where the defect primarily appears. In male rats, stress-induced prefrontocortical impairment may release the amygdala and the HPA axis from their functional inhibition, generating an auto-sustaining positive feedback loop in which glucocorticoids stimulate their own release and lead to hippocampal and frontocortical dysfunctions. Female rats, in contrast, possibly due to the protective action of estrogen, did not exhibit the same reduction of neuronal plasticity seen in males. Thus, our data does not support the traditional view of a higher female susceptibility to the effects of stress. In fact, although female rats displayed a higher sensitivity to acute stress, they demonstrated a lower susceptibility to the detrimental influence of prolonged footshock exposure on neuronal plasticity. Nevertheless, we cannot draw definite conclusions with regard to these findings, as it cannot be excluded that the data presented here is the result of the specific markers that were used in this investigation. It remains plausible however, that chronic stress exposure may affect female brain integrity through different molecular and cellular pathways than those found in males.
It is of interest to note that although prolonged stress exposure caused similar neuroendocrine alterations in male and female rats, different changes in FOS-ir were detected in the two investigations presented in this chapter. These discrepancies were particular evident in frontocortical regions of chronically stressed males. Thus, while in the first section ("Immunohistochemical changes induced by repeated footshock stress: revelation of gender-based differences") an increased absolute FOS-ir was observed in prefrontocortical regions in animals exposed to chronic stress, a reduced FOS-ir was instead detected in the second investigation ("Amygdala-prefrontocortical involvement in response to chronic footshock stress: a gender comparative view"). The reason for this discrepancy is unknown, although the two studies markedly differ with regard to the duration of daily aversive sessions, making the footshock procedure in the second investigation (daily sessions ranging from 30 to 60 minutes) more stressful than the one used in the first part of the chapter (daily sessions ranging from 15 to 30 minutes). Besides the previously mentioned differences in absolute FOS-ir patterns, chronically stressed males in both investigations illustrated similar neuroendocrine alterations (adrenal hypertrophy and PVN hyperactivity) which might indicate abnormal HPA axis activity and reduced neuronal plasticity.
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