Effects of Long-Term Stress and Recovery on the Prefrontal Cortex and Dentate Gyrus in Male and Female Rats

Women show a higher prevalence for depression than men. However, the biological basis of gender differences in stress response and recovery still remain poorly understood. Therefore, the aim of the study was to assess the gender differences in response to acute stress, chronic stress and recovery in rats. Our results showed that stress decreased male body weight but had no effect on female rats. Open field test demonstrated behavioral changes in grooming and velocity after chronic stress and recovery. Increased activity of hypothalamic-pituitary-adrenocortical axis was reflected by adrenal hypertrophy and increase of plasma corticosterone levels except in the socially housed female rats after stress. Gender and brain region differences in response to stress and recovery were found in the expression of cAMP response element-binding protein (CREB) and phosphorylated CREB (pCREB). On the whole, expression of CREB and pCREB in male dentate gyrus (DG) and prefrontal cortex (PFC) was sensitive but in female DG and PFC it was resistant to acute and chronic stress. Interestingly, recovery restored the measured parameters to the normal level in male rats but not in female rats. In conclusion, these results suggest that male and female rats responded to stress and recovery in a different way.

Keywords: gender differences, neuroplasticity, recovery, social housing, stress response

Introduction

Depression, a common public health problem, occurs twice as frequently in women as in men (Kessler et al. 1993; Kessler 2003; Sun and Alkon 2006). Such a gender difference in depression may occur for a number of reasons, including the influence of particular sex hormones (Matheson and Anisman 2003). In rodents, females typically respond to a stress with a greater release of both adrenal corticotropic hormone and corticosterone compared with males (Rivier 1999; Tinnikov 1999). Dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis is associated with vulnerability to a number of psychiatric diseases including major depression (Howell and Muglia 2006). There are also anatomical differences (Good et al. 2001; Luders et al. 2004) and functional magnetic resonance imaging studies show different patterns of brain activity in males and females (Gron et al. 2000). Therefore, the gender differences in stress reactions and depression derived from both clinical and preclinical studies are an important argument for performing stress and pharmacological studies not only in male animals but also in female animals (Renard et al. 2005). The cellular, molecular and the psychosocial mechanisms underlying stress responses and depression may differ between males and females (Sjoberg et al. 2006).

However, many experimental studies focusing on the pathophysiology of depression have examined the effects of stress and/or antidepressants in male subjects (Palanza 2001), and the gender differences in the pathophysiology of depression remain poorly understood.

The onset of major depression is often preceded by chronic stress or stressful life events, indicating the importance of stress in the development of depression (Bale 2006). However, the cellular and molecular mechanisms underlying depression have been difficult to grasp due to the complex pathophysiology of depression. In recent years, the focus of research has been at a level beyond the serotonin and norepinephrine transporters to the intracellular signal transduction cascades that underlie the regulation of neuronal functioning (Tardito et al. 2006). Novel theories suggest that dysregulation of intracellular pathways is linked to neuroplasticity as key events in depression development (Duman et al. 1997; Manji et al. 2001). One of the action mechanisms of stress may involve the dysregulation of the cyclic adenosine monophosphate (cAMP) cascade (Duman et al. 1997). This upregulation extends to several components of the cascade: coupling of stimulatory G-protein and adenylate cyclase (Ozawa and Rasenick 1991), cAMP-dependent protein kinase (Nestler et al. 1989), transcription factor cAMP response element-binding protein (CREB) (Nibuya et al. 1996) and brain-derived neurotrophic factor (BDNF) (Nibuya et al. 1995). Among them CREB is a critical mediator of neural plasticity and has been implicated in learning, memory, and the long-term actions of opiates, psychostimulants, as well as antidepressants (Newton et al. 2002). Phosphorylation of CREB (pCREB) at serine 133 has been reported to lead to activation of gene transcription, such as BDNF (Carlezon et al. 2001). Therefore, nuclear staining of pCREB reports activation of a particular brain area in response to external or internal stimuli, as does nuclear staining immediate early genes, like fos. Despite the importance of CREB and pCREB involved in neuroplasticity, few studies were designed to elucidate the gender differences in CREB and pCREB expression induced by stress and recovery after long-term stress.

Chronic footshock exposure has been proposed as a valid animal model for affective disorders (Westenbrock, Ter Horst, et al. 2003). Chronic stress impairs the ability to anticipate reward, which resembles anhedonia and can be reversed by antidepressants (Willner 1997). At the same time, evidence shows that some patients who respond to antidepressants also respond to placebo and there is natural recovery after depression (Vallance 2007). However, gender differences in recovery after long-term footshock exposure remain unknown.

To address these issues above, we attempted to determine the neurobiological adaptations underlying acute stress, long-term...
stressed and recovery effects on individually housed male rats and female rats. At the same time, behaviorally, females seem to have adopted a “tend-befriend” strategy, actively seeking social contact in time of stress (Taylor et al. 2000), so we also used socially housed female rats to control for isolation stress in female rats. Particular focuses were on the prefrontal cortex (PFC) and dentate gyrus (DG), giving that studies in humans and animals have demonstrated that depression and stress-induced pathologies are implicated in DG and PFC, the key regions for HPA axis glucocorticoid feedback (Popoli et al. 2002; Sheline 2003; Radley et al. 2004; Sairanen et al. 2007). These relationships were studied by using markers of HPA axis function, behavior, and expression of CREB and pCREB. The present results may thus provide novel insight into cellular and molecular mechanisms underlying effects of stress, recovery on neuronal functions in male rats and female rats.

Materials and Methods

Animals

Male (n = 32) and female (n = 48) Wistar rats were used in the present experiment. At the start of the experiment, rats were of the same age with male rats weighing 204 ± 1.5 g and female rats weighing 186 ± 1 g. All male rats and 32 female rats were individually housed, 16 female rats were socially housed (4 rats/cage), with ad libitum access to food and water. A plastic tube (diameter 8 x 17 cm) was placed in each cage as a shelter. The light-dark cycle was reversed (lights on 19:00-7:00 h). All experimental procedures, designed to minimize the number of animals and suffering, were approved by the Animals Ethics Committee of the University of Groningen (FDC: D4145). The estrus cycle of the female rats was monitored by stroking them gently on the back, producing lordosis during estrus and weight loss was observed on the day of estrus. In the current study effects of the estrous cycle on the stress response were not specifically investigated. It was hypothesized that because female rats were exposed to the stressor during all stages of the cycle this would override sex hormone-related stress sensitivity differences (Westenbroek, Ter Horst, et al. 2003).

Individually housed male rats and female rats were randomly assigned to 4 experimental groups: 1) control group: subjected to no footshock throughout the experiment; 2) acute stress group: received 6 footshocks on day 42, and exposed to the footshock box with the light stimulus only on day 43; 3) recovery group: received footshocks daily for 3 weeks followed by a 3-week period with no footshock, and on day 43 exposure to the footshock box with only the light and no shock; 4) chronic stress group: received footshocks daily for 3 weeks followed by 3 weeks of alternating days of exposure to the footshock box with footshocks and without receiving footshock, and on day 43 exposure to the footshock box with only the light (Fig. 1). Because we have previously shown that isolation was a stressor for female rats, we had 2 extra socially housed groups of female rats: a social control group that was not exposed to the footshock box throughout the experiment, and a social acute stress group, in which rats received 6 footshocks on day 42 and were exposed only to the light on day 43. This was used to determine whether isolation for 6 weeks resulted in a changed stress response.

Stress Procedure

The “footshock chamber” consists of a box containing an animal space positioned on a metallic grid floor connected to a shock generator and scrambler. Rats in the stress group were placed in a box and received variable (2-50) inescapable footshocks with randomizing starting time (between 9:00 and 17:00 h) and intervals during a 30- to 120-min session (0.8 mA as maximum intensity and 8 s in duration) in order to make the procedure as unpredictable as possible. A light signal (10 s) preceded each footshock adding a “psychological” component to the stressor. On the last day, the stress-exposed rats were subjected to the light stimulus only, which was crucial as it provided a way to create a stress condition without the unwanted side effects of direct physical or painful stimuli. Body weight was measured daily during the whole period. Blood samples were collected through the tail vein quickly on day 20 and stored at ~20 °C to determine plasma corticosterone levels. On day 43 rats were sacrificed using isoflurane anesthesia. Three rats from each group were transecardially perfused with 50 ml of heparinized saline and 300 ml of a 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4), 2 h after the start of the last exposure to the stress box, blood samples were taken by cardiac puncture. The brains were postfixed in the same fixative overnight at 4 °C. The other 5 rats from each group were decapitated 30 min after the start of the last exposure to the stress box and the brains were removed immediately and put on dry ice and stored at ~80 °C, blood samples were also collected. The blood samples were centrifuged and plasma was removed and stored at ~20 °C. Adrenals and thymus were removed. Adrenal and thymus weight, corrected for body weight, was calculated and used as indication of the amount of stress perceived.

Open field test

Animals were subjected to a voluntary open field (OF) test for a period of 8 min. The OF test was performed under red-light conditions between 9:00 and 13:00 h. during the active period of the animals, at least 16 h after the last stress session and before the stress procedure of that day. The test was repeated 3 times, on day 1, day 22, and day 40. Rats were gently placed in the tube which was connected with the OF at the start of the test. The OF consisted of a circular black arena with a diameter of 1.5 m. The behavior of rats was recorded via a videotracking system (EthoVision 3.0, Noldus information Technol- ogy, Wageningen, the Netherlands), with a sample rate of 25 samples/s. Grooming frequency in the whole arena and the ratio of the velocity in outer Q1 and outer Q4 of the arena was analyzed.

Corticosterone Radioimmunoassay

Blood samples were collected for corticosterone radioimmunoassay. The corticosterone fraction was extracted from the plasma (10 μL) with Chromosorb (Sigma-Aldrich, St. Louis) and 30% dichloromethane (Rathburn 1001). Before the extraction a trace amount of 3H labeled (200 Bq) corticosterone (TRK-406 Amersham, UK) was added to the samples to determine recovery which was 20%. A standard curve was determined by using nonlabeled corticosterone, which was treated in the same way as the extracted plasma samples. The quantification samples were mixed with 3H (500 Bq) labeled corticosterone and polyclonal antibody raised against corticosterone (rabbit nr 568 UMC). The tubes were incubated at 60 °C for 30 min and the equilibrium reaction was established in a water/ice bath for 1 h. The reaction was stopped by adding charcoal suspension and incubating for 15 min in the water/ice bath. The tubes were centrifuged for 15 min at 3000 g and 4 °C to spin down the charcoal bound with free corticosterone. The supernatant was poured into 1 mL of scintillation fluid (Ultimagold XR, Canberra Packard, Schwadorf, Austria) and samples were counted in a β-counter for 4 min or 4000 preset counts. The amount of corticosterone was calculated from the standard curve.
Western Blot Analysis
Serial 300-μm coronal sections of the cerebrum were made with a cryostat microtome (-15 °C) and kept frozen on dry ice. Tissue samples were dissected from the DG (bregma -2.45 to -2.85, Fig. 2A) and PFC (bregma +3.20 to +2.15, Fig. 2B) by using the “Palkovits Punch” technique (needle diameter 1.22 mm, Stoelting Co., IL). Two punches per animal per area were taken and homogenized in 50 μl of buffer (50 mM Tris pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1 mM NaVO₃, 10 mM NaF, 1mM ethylene glycol tetracetic acid, and complete protease inhibitors; Roche, Basel, Switzerland). Brain tissue was homogenized and then heated for 2 min at 96 °C. The samples were stored at -80 °C.

The expression of CREB, pCREB, and β-actin as a loading control was estimated by Western blot analysis. Protein samples (5 μl) were combined with sodium dodecyl sulfate (SDS)-bromophenol blue (5 μl) reducing buffer with 100 mM dithiothreitol and then heated for 5 min at 96 °C to limit the formation of high molecular weight receptor aggregates. Western blot analyses were carried out using 10% SDS polyacrylamide gels; gels were electrophoresed (Supported Nitrocellulose Membrane, BioRad, Veenendaal, The Netherlands) for 1 h using a wet electroblotting system (BioRad, Mini Trans-Blot Electrophoretic Transfer Cell), and filters were blocked for 1 h in phosphate buffered saline (PBS) buffer (pH 7.4) containing 0.5% nonfat dry milk. Blots were then incubated overnight at 4 °C with primary polyclonal antibodies of CREB, pCREB (Upstate Biotechnology, Lake Placid, NY), and β-actin (Abcam, Cambridge, UK) (1:1000 for CREB and pCREB, 1:10 000 for β-actin) diluted in 0.5% nonfat dry milk-PBS solution. Blots were washed 3 times for 10 min with PBS buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit for CREB and pCREB, peroxidase-coupled anti-mouse for β-actin, Amersham Bioscience) diluted (1:5000) with 0.5% nonfat dry milk-PBS. Immunostaining was revealed by the enhanced chemiluminescence Western blot analysis system (Syngene, Westburg, The Netherlands). The intensity of the bands was quantitated by image analysis. Membranes were washed and reprobed with the antibody to β-actin for loading control. The CREB and pCREB signals were normalized by the levels of β-actin and expressed as a percentage of the corresponding values for unstressed controls on the same blots.

Immunohistochemistry
Following an overnight cryoprotection in a 30% sucrose solution, serial 30-μm coronal sections of the brain were made with a cryostat microtome and collected in 0.02 M potassium phosphate saline buffer. CREB and pCREB immunoreactivity (ir) in DG and PFC was performed on free-floating sections. Sections were rinsed with 0.1% Triton X-100 and then heated for 10 min to reduce endogenous peroxidase activity, thoroughly washed with 0.1 M PBS and incubated with the rabbit anti-CREB antibody (1:300, Cell Signaling, Danvers, MA) or anti-pCREB antibody (1:1000, Upstate, Danvers, MA) diluted in 0.1 M PBS with 0.1% Triton X-100 and 3% normal goat serum for 72 h at 4 °C. After thorough washing, the sections were subsequently incubated for 2 h with biotinylated goat-anti-rabbit IgG (1:1000 in 0.1 M PBS with 0.1% Triton X-100 and 3% normal goat serum) and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). After thorough washing, the peroxidase reaction was developed with a di-amino benzidine-nickel solution and 1% H₂O₂. Sections were washed for 15 min in buffer and mounted with a gelatine solution and air-dried, dehydrated in graded alcohol solutions and finally in Histoclear then coverslipped with DePeX mounting medium (BDH, Carle Place, NY). To reduce staining artifacts or intensity differences the sections from all groups were processed simultaneously.

CREB- or pCREB-positive cells in the DG (dentate gyrus granule cell layer; DG-sg) (4 slices for each rat, bregma -2.45 to -2.85) and PFC (prelimbic; PL) (8 slices for each rat, bregma +3.20 to +2.15) were blindly quantified using a computerized imaging analysis system (Westenbroek, Den Boer, et al. 2003). The selected areas were digitized by using a Sony charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at x100 magnification. Regions of interest were outlined with a light pen, measured, and the CREB- or pCREB-positive nuclei were counted using a computer-based image analysis system LEICA (LEICA Imaging System, Ltd, Cambridge, UK). Each digitized image was individually set at a threshold to subtract the background optical density. Only cell nuclei that exceeded a defined threshold were detected by the image analysis system. The resulting data was reported as number of positive cells/0.1 mm². The DG and PFC was quantified bilaterally (no left-right asymmetry of CREB or pCREB ir was found).

Statistical Analysis
Data were expressed as means ± SEM and analyzed with (SPSS Inc., Chicago, IL) (version 12.0). P < 0.05 was defined as the level of significance. Weight gain was analyzed separately for male rats and female rats with repeated measures analysis of variance (ANOVA) with days as within subject factors and treatment (control or stress) as between subject variables. Behavior was analyzed with a repeated measure ANOVA, with days as within subject variables and treatment (control or stress), gender (male or female) and housing (individual or social) as between subject variables. Adrenal weight, plasma corticosterone levels, CREB, and pCREB ir were analyzed with an ANOVA with treatment (control or stress), gender (male or female), and housing (individual or social) as between subject variables.

Results

Body Weight Gain
All rats continued to grow throughout the experiment, and there was a significant effect of day on male and female body weight gain (resp. F₁,₄₉ = 1216.449, P < 0.001; F₁,₄₂ = 150.140, P < 0.001, Fig. 3), and a significant gender difference with male rats growing faster than female rats (F₁,₄₉ = 490.839, P < 0.001, Fig. 3). Body weight gain was significantly affected by chronic stress in male rats (F₁,₂₉ = 5.694, P = 0.031, Fig. 3A) but not in female rats (Fig. 3B) and the significant effect of stress on male body weight appeared after 6 days (P = 0.046, Fig. 3A). In male rats there was a significant interaction effect between day and treatment (F₂,₄₂ = 10.096, P < 0.001, Fig. 3A). When stress was stopped, the recovery male rats restored their body weight, and the significance appeared from day 32 (P = 0.025, Fig. 3A) to
day 43 ($P = 0.047$, Fig. 3A) compared with chronically stressed male rats, whereas there was no significance between control male rats and recovery male rats ($P = 0.083$, Fig. 3A) at the end of the procedure. Social housing had no significant effect on the body weight of female rats (Fig. 3B).

**Adrenal and Thymus Weight**

Gender difference was found in relative adrenal weight, the female adrenal was significantly larger than the male adrenal ($F_{1,48} = 117.28, P < 0.001$, Fig. 4). Social housing also had no significant effect on female adrenal weight compared with individually housed control female rats. However, there was a significant increase of relative adrenal weight in recovery female rats compared with control female rats ($P = 0.029$, Fig. 4). Changes in thymus weight were not significant after acute stress, chronic stress and recovery, also no gender difference in the relative thymus weight was found (data not shown).

**OF Test**

There was no gender difference in the grooming frequency and Q1 velocity/Q4 velocity. The effect of day was also not found in the control group. Exposure stress to male rats and female rats for 3 weeks increased the grooming frequency (resp. $F_{1,28} = 3.726, P < 0.05$; $F_{1,42} = 4.125, P < 0.05$, Fig. 5B) but decreased the Q1 velocity/Q4 velocity (resp. $F_{1,28} = 6.548, P < 0.05$; $F_{1,42} = 8.157, P < 0.05$, Fig. 5C) compared with control group. Exposure stress to male rats and female rats for 3 more weeks continued increasing the grooming frequency (resp. $F_{1,28} = 5.263, P < 0.01$; $F_{1,42} = 4.562, P < 0.05$, Fig. 5B) but decreasing Q1 velocity/Q4 velocity (resp. $F_{1,28} = 7.465, P < 0.05$; $F_{1,42} = 7.143, P < 0.05$, Fig. 5C). As shown in Figure 5, grooming behavior ($P < 0.01$, Fig. 5B) and Q1 velocity/Q4 velocity ($P < 0.05$, Fig. 5C) in recovery male rats was restored to the baseline levels compared with chronically stressed male rats on day 40. Although recovery female rats showed a slight decrease in grooming frequency on day 40, there was no significant reduction compared with chronically stressed female rats. Significant decrease in Q1 velocity/Q4 velocity was still observed in recovery female rats ($P < 0.05$, Fig. 5C).

Social housing had no significant effect on grooming behavior and Q1 velocity/Q4 velocity compared with individually housed female rats (Fig. 5B,C).

**Plasma Corticosterone Levels**

**Half Hour after Stress or Exposure to Stress Box**

Gender difference in plasma corticosterone levels was found both on day 20 and day 43 (resp. $F_{1,78} = 13.460, P < 0.001$, Fig. 6A; $F_{1,78} = 26.318, P < 0.001$, Fig. 6B), female rats showing significantly higher plasma corticosterone levels than male rats. On day 20 a significant increase in corticosterone level induced by chronic stress was observed both in male rats ($F_{1,28} = 26.826, P < 0.001$, Fig. 6A) and in female rats ($F_{1,42} = 4.629, P = 0.009$, Fig. 6A). On day 43, exposure to the conditional stimulus only significantly increased the corticosterone levels in male rats and female rats in acute stress group (resp. $P = 0.001; P = 0.002$, Fig. 6B), recovery group (resp. $P = 0.002; P < 0.001$, Fig. 6B) and the chronic stress group (resp. $P = 0.008; P = 0.008$, Fig. 6B) compared with control male and female rats, respectively. There was no significant difference among the acute, recovery and chronic male and female groups on day 43 (Fig. 6B).
housing had no significant effect on plasma corticosterone level on day 20 and day 43 compared with the individually housed female rats (Fig. 6A,B). However, exposure to the stress box induced no significant increase in the plasma corticosterone levels in social acute female rats compared with social control female rats (Fig. 6B).

**Two Hours after Exposure to Stress Box**

Gender difference in plasma corticosterone levels was also found 2 h after exposure to the stress box on day 43 ($F_{1,78} = 7.492, P = 0.012$, Fig. 6C). A significant decrease in plasma corticosterone levels was found in chronically stressed male rats ($P = 0.029$, Fig. 6C) compared with control male rats. Furthermore, in acute group there was also a significant reduction in plasma corticosterone levels in individually and socially housed female rats (resp. $P = 0.028$; $P = 0.035$, Fig. 6C).

**CREB and pCREB Expression in Male and Female PFC**

**Half Hour after Exposure to the Stress Box**

Acute and chronic stress both had no significant effect on the expression of pCREB and CREB in male and female PFC, although chronic stress induced a decreasing trend in the
expression of pCREB and CREB. Social housing also did not affect CREB and pCREB expression in female PFC (Fig. 7).

Two Hours after Exposure to the Stress Box
There was a significant decrease in the number of cells expressing pCREB ($F_{1,16} = 4.382$, $P = 0.023$, Fig. 8A), whereas no significant change in the number of CREB-positive cells in PFC of chronically stressed male rats compared with control male rats (Fig. 8A). Chronic stress also induced morphological abnormalities and irregularities in male PFC which were reflected by the “patches” (red arrow) (Fig. 8B). The size of the “patches” was different. There was no positive staining in these “patches,” and the background of these “patches” was bright, which is different from the tissue around. After recovery there was no significant change in the number of cells expressing pCREB and CREB in male PFC (Fig. 8A). In female PFC no significant change in the number of cells expressing CREB and pCREB was found (Fig. 8A), also no “patches” were examined (data not shown). Gender differences in the number of cells expressing CREB and pCREB in PFC were not found in this experiment (Fig. 8A).

CREB and pCREB Expression in Male and Female DG

Half Hour after Exposure to the Stress Box
In male rats, acute and chronic stress slightly decreased the expression of pCREB in DG, but the effect was not significant. Stress also had no significant effect on the expression of CREB in male DG. In female DG stress and social housing had no significant effect on the expression of CREB and pCREB (Fig. 9).

Two Hours after Exposure to the Stress Box
In male rats, a significant effect of stress on the number of cells expressing pCREB was found in DG ($F_{1,16} = 8.673$, $P = 0.007$, Fig. 10A). The number of pCREB-positive cells was significantly decreased in acute ($P = 0.002$, Fig. 10A) and chronically stressed male rats ($P = 0.008$, Fig. 10A). Morphological abnormalities and irregularities were demonstrated by many “patches” (red arrow) (Fig. 10B) especially around granule cell layer (stratum granulosum [SG]) and dentate gyrus medial blade molecular layer (DGmb-mo) in male DG. Similar to the “patches” observed in male PFC, in male SG there was also no positive staining in these “patches,” and the background of these “patches” was bright, which is different from the tissue around. In male DGmb-mo “patches” were also found, although positive staining was not observed in DGmb-mo. However, in recovery male rats there was no significant change in the number of pCREB-positive cells compared with male control rats on day 43 (Fig. 10A). Stress had no significant effect on the number of CREB-positive cells in male DG (Fig. 10A). In female DG there was no significant change in the number of CREB and pCREB-positive cells after acute stress, chronic stress, recovery, and social housing compared with individually housed control female rats (Fig. 10A). Acute and chronic stress also didn’t
induce “patches” in female DG (data not shown). Gender differences in the number of CREB- and pCREB-positive cells in DG were not found in this experiment (Fig. 10A).

Discussion
Despite the striking gender differences in the prevalence of depression, attempts to identify corresponding gender differences in stress response in animal models have met with limited success (Altemus 2006). In the present study gender differences following exposure to stress (acute and chronic) and recovery after long-term stress were further investigated using different markers like physical measure, behavioral tests, HPA axis markers and also focusing on detecting changes and differences in CREB and pCREB in both male rats and female rats in the DG and the PFC, regions that are involved in depression and stress-induced pathologies. In addition to gender differences in stress reactivity, the beneficial effects of social housing have been examined in female rats.

Effects of Acute Stress on Individually Housed Rats
Gender differences in the expression of CREB and pCREB in PFC and DG after acute stress were observed in this study. On the whole acute stress had no significant effect on the expression of CREB and pCREB in female PFC and DG. Acute stress impacted the expression of pCREB time dependently and caused a significant reduction in pCREB ir, whereas not CREB ir, in male DG 2 h after exposure to the stress box. These results suggest that DG and PFC responded to acute stress in a different way.

The gender difference in the expression of pCREB in DG after acute stress may be related to the changes in corticosterone level. Acute stress induced a significant increase in plasma corticosterone levels both in individually housed male rats and female rats. However, 2 h after stress individually housed female rats, whereas not male rats, showed a significant reduction in the plasma corticosterone levels which may be due to increased negative feedback (Gunderson et al. 2003), suggesting that in female rats a protective mechanism may have become activated. Rises in corticosteroid levels after acute stress impair synaptic plasticity in the rat hippocampus when compared with the situation where levels are basal under rest (Alfárez et al. 2003), for hippocampus is a prime target for glucocorticoid action (De Kloet 1995). The changes of pCREB subsequently may result in the alterations in neurotrophin
expression, for CREB and pCREB plays a central role in mediating neurotrophin responses in neurons (Finkbeiner et al. 1997; Finkbeiner 2000). For example, acute stress reduces neurotrophin expression in the hippocampus of male mice (Pizarro et al. 2004).

Activation of the infralimbic and PL area of the PFC is necessary for stressor controllability (Amat et al. 2005). In a previous study acute stress was found to increase the expression of pCREB in PFC (Pardon et al. 2005), suggesting that protective effects of stressor controllability are mediated by the PFC. However, in the present study, although an increasing trend in the expression of pCREB in male PFC was observed, no significance was found. The differences from the previous study may result from the different breed of animal, stressor, methods used, etc.

Effects of Chronic Stress on Individually Housed Rats

Stress generated an inhibitive effect on male weight gain, whereas no such effect was found in female rats. These results were in accordance with previous preclinical data showing that stress exposure did not affect body weight gain in female rats as much as it did in male rats (Duncko et al. 2001). Chronic stress induced behavioral changes such as increased grooming frequency and decreased Q1 velocity/Q4 velocity both in male rats and female rats, whereas no gender difference was found. Stress for 21 days induced adrenal hypertrophy both in male and female rats (Westenbroek, Den Boer, et al. 2003), whereas no significance was found after 43 days’ stress in this study due to an unknown mechanism (Kioukia-Fougia et al. 2002; Kuipers et al. 2006).

As expected (De Kloet et al. 1998), acute and chronic stress induced classical features of prolonged elevations of circulating corticosterone levels from the adrenal gland 0.5 h after stress or exposure to stress box. However, female rats demonstrated higher plasma corticosterone levels than male rats not only under normal condition but also during stress (Rivier 1999; Tinnikov 1999). Interestingly, 2 h after exposure to the stress box plasma corticosterone levels were significantly decreased through an unknown mechanism (maybe negative feedback, breakdown etc) in chronically stressed male rats. In our experiment chronic stress significantly reduced pCREB expression in male DG but had no obvious effect on female DG. CREB expression remained unaffected by chronic stress both in male and female DG, suggesting that CREB activity in the hippocampus was insensitive to chronic stress (Miller et al. 2006). Consistent with our results, chronic mild stress (CMS) was also found to result in a significant decrease in the expression of pCREB in male DG, whereas the expression of total CREB was unaffected by CMS (Gronli et al. 2006). In view

Figure 8. Number of cells expressing pCREB and CREB in PFC (PL) measured by immunohistochemistry in male rats and female rats 2 h after exposure to the footshock box with the light only on day 43. (A) Number of cells expressing pCREB and CREB 2 h after exposure to the stress box on day 43 in male and female PL, *P < 0.05 versus control group. (B) Representative photomicrographs of pCREB ir in the PL of control male rats (×100). (C) Representative photomicrographs of pCREB ir in the PL of male rats exposed to chronic stress (×100). "patches" were clearly observed (red arrow) in chronically stressed male rats. The filled arrows → pointed to cells that were counted as labelled (positive), and open arrows ← pointed to cells that were counted as unlabeled (negative).
of the importance of CREB and pCREB in neuroplasticity, our results were also consistent with other studies showing that chronically restrained female rats did not exhibit the severity of apical dendritic atrophy that was seen in stressed males (Galea et al. 1997), and that stressed male vervet monkeys showed evidence of hippocampal pyramidal neuron loss, whereas females did not (Uno et al. 1989).

Besides the hippocampus, the male PFC also showed neurochemical changes in response to stress. As revealed in the present experiment, pCREB expression was reduced in male PFC. pCREB expression was also reported to be reduced by chronic stress in PFC in male rats (Laifenfeld et al. 2005). Significantly reduced pCREB expression in both cortical regions suggested reduced synaptic plasticity in male rats. Recent animal experiments showed that chronic stress resulted in altered dendritic morphology, and reduced spine density in the PFC (Cook and Wellman 2004; Radley et al. 2004, 2006). Through downregulating pCREB expression, chronic stress may thus compromise PPC plasticity required for proper response and/or adaptation to (stressful) stimuli (Kuipers et al. 2003). “Patches” in male PFC and DG after chronic stress were observed, which may be due to the dysfunction of HPA axis and prolonged elevation of corticosterone, for treatment with the synthetic glucocorticoid dexamethasone resulted in neuronal loss and atrophy in the mPFC as well as in the granule cell layer of the DG (Cerqueira et al. 2005). Pathological changes associated with stress and depression in limbic regions such as hippocampus, PFC and amygdala were exhibited not only in animals but also in depressed patients (McEwen 2000; Manji et al. 2001). The “patches” observed in the present study suggest a rate of the neuronal loss or an appearance of pycnotic cell, which may due to the activation of necrotic and/or apoptotic cellular death pathways that underlie the structural impairments (Sapolsky 2000). However, the demonstration that neurogenesis occurs in the human brain into senescence (Eriksson et al. 1998), raises the possibility that ongoing impairment of neurogenesis may also play a role (Henn and Vollmayr 2004). Furthermore, there is evidence that the cytoskeletal microtubular system was involved in the neuronal plasticity, stress, and antidepressant effects (Bianchi et al. 2003), suggesting that the pathological changes in our experiment may also due to the disturbance of cytoskeletal
microtubular system. Nevertheless, more studies are needed to clarify this interesting and important phenomenon in the future. Interestingly, we did not find obvious changes in morphology ("patches") and the expression of pCREB and CREB in female PFC and DG. Based on the results from the expression of CREB and pCREB and structural changes ("patches") in PFC and DG, it may be concluded that female PFC and DG were resistant, whereas male PFC and DG were susceptible to the aversive effects of chronic stress and acute stress.

**Effects of Recovery on Individually Housed Rats**

Opposing effects of recovery were found in male rats and female rats justified by adrenal hypertrophy, behavior, and...
neurochemical changes. The effects of stress on male rats were not permanent and once the stress was removed, different parameters applied in this study restored to the baseline level. McEwen also noted that the remodeling of the hippocampus in response to stress was largely reversible if chronic stress was terminated at the end of the 3rd week (McEwen 2004). Surprisingly, recovery female rats showed even more adrenal hypertrophy and abnormal behavior, indicating female rats were still suffering from the consequences of stress and thus apparently exhibited a resistance to recovery. In a previous study of our research group, levels of accumulated ΔFosB in the mPFC of female rats had not returned to control levels following 21 days of recovery, which showed that the preceding stress period still had transcriptional consequences in this limbic area (Gerrits et al. 2006). All these data suggest that recovery for 21 days was not enough for female rats to recover.

In light of the effects of chronic stress and recovery, it seems contradictory that on one hand, female rats were resistant to stress-induced neurochemical changes in DG and PFC, and on the other hand, female rats still showed stressed after recovery. Therefore, other proteins related to neuropaoplasticity or more brain regions besides DG and PFC in female brain should be investigated, especially in brain regions containing estrogen receptors, such as amygdala and hypothalamus where estrogen receptor (ER)-α dominates and entorhinal cortex and thalamus where ER-β dominates, for compelling evidence now exists for estrogen’s involvement in depression and schizophrenia (Ostlund et al. 2003). Estrogen and progesterone administration to ovariectomized rats resulted in elevated levels of pCREB and BDNF in hippocampus (Franklin and Perrot-Sinal 2006; Sharma et al. 2007). Furthermore, depression in females emerges especially in time of severe changes in plasma estrogen levels, like after pregnancy, prior to menses and during and shortly after menopause (Arpels 1996; Halbreich and Kahn 2001; Kessler 2003), implying that gonadal hormone may be a complex factor in females during stress.

**Effects of Social Housing on Female Rats**

In this study social housing prevented significant increases of plasma corticosterone levels after acute stress, suggesting that social housing maintained the HPA axis at lower activity. Social housing was also found to help female rats to cope with chronic stress (Westenbroek, Den Boer, et al. 2003; Westenbroek, Ter Horst, et al. 2003), and had positive effects on neuronal survival (Westenbroek et al. 2004). All these results indicate that social housing has positive effects on female rats, whereas contradictory effects of group housing on males were reported (Karolewicz and Paul 2001; Garey et al. 2002; Chourbaji et al. 2005). Oxytocin could be a possible mediator of the positive effects of social housing because social contact in rats was shown to increase oxytocin release (Uvnas-Moberg 1997). In humans social contact was also associated with increases in plasma oxytocin levels and found to reduce plasma cortisol levels (Carter 1998; Turner et al. 1999).

In conclusion, female rats appeared to be quite different from male rats. The parameters used in this study suggest that the female rats were impacted less and showed more resistance to stress effects, at least with the footshock stress used here. Therefore, further studies are needed to argue logically on one hand, women are more susceptible to depression, and on the other hand, female rats are relatively resistant to the effects of stress.

**Funding**

Bernoilli grant from the University of Groningen.

**Notes**

We would like to thank Rob Visser and Britta Kuest in the Graduate School Behavioral and Cognitive Neurosciences of the University of Groningen for administrative support. Conflict of Interest: None declared.

Address correspondence to Prof Gert J. Ter Horst, PhD, Department of Psychiatry, University Medical Center Groningen, University of Groningen, Graduate School of Behavioral and Cognitive Neurosciences, Hanzeplein 1, P.O. Box 30.001, 9700 RB Groningen, The Netherlands. Email: g.j.ter.horst@med.umcg.nl.

**References**


Vallance AK. 2007. A systematic review comparing the functional neuroanatomy of patients with depression who respond to placebo to those who recover spontaneously: is there a biological basis for the placebo effect in depression? J Affect Disord. 98:177-185.


