Stress and the female brain. The effects of estradiol on the neurobiological reactions to chronic stress.
Gerrits, Marjolein

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Stress-induced sensitization of the limbic system in ovariectomized rats is partly restored by cyclic 17β-estradiol administration

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European Journal of Neuroscience (2006), 23: 1747-1756
Abstract

Chronic stress induces neurobiological alterations that have consequences for subsequent stress handling. In the current experiment ovariectomized rats were subjected daily to a stressor for 21 days. Thereafter, the rats were treated for 21 days with 17β-estradiol benzoate (10 μg/250g, 1x in 4 days) or mirtazapine (10 mg/kg, daily). In this way, we could evaluate the ability of these compounds to restore chronic stress-induced changes in the activity of the limbic system. After 21 days of recovery and treatment, the rats were re-exposed to the adverse environment of the initial stressor and perfused 2 hours later. Ovariectomized rats displayed increased numbers of c-Fos positive nuclei after re-exposure to the stressor in the paraventricular nucleus of the hypothalamus, dentate gyrus, medial prefrontal cortex and central and medial amygdala. Cyclic estradiol treatment attenuated the sensitization of the paraventricular nucleus and central amygdala. Mirtazapine increased the number of c-Fos positive nuclei in the central amygdala and dentate gyrus. Long-term transcriptional changes induced by chronic stress were determined with ΔFosB immunoreactivity. The medial prefrontal cortex showed an increased number of ΔFosB positive nuclei after chronic stress and this was not affected by estradiol or mirtazapine administration during recovery. In conclusion, cyclic estradiol administration restored chronic stress-induced sensitization induced by chronic stress in the limbic system in the paraventricular nucleus and central amygdala of female rats; output regions of the limbic system involved in fear responses. Mirtazapine did not show this restoration of stress-induced aberrations in the limbic system after 21 days of treatment.
Introduction

Stressful experiences can induce neurobiological alterations that may impede adequate handling of subsequent stressors (Kendler et al., 1995; McEwen, 1999; Kaufman et al., 2000; Trentani et al., 2002; Radley et al., 2004). Many experimental studies focusing on the pathophysiology of affective disorders have examined the effects of stress and/or antidepressants in male subjects. However, affective disorders are 2-3 times more common in women than in men (Weissman et al., 1995; Kessler, 2003) and emerge especially at times of severe changes in plasma estrogen levels, for example after giving birth or in the perimenopausal phase (Arpels, 1996; Halbreich et al., 2001; Kessler, 2003). Additionally, animal studies demonstrate sex differences in stress reactions (Galea et al., 1997; Taylor et al., 2000; Shors et al., 2001; Westenbroek et al., 2003; Renard et al., 2005), which provide important arguments to perform stress and pharmacological studies not only in male but also in female animals.

The present study was designed to evaluate the neurobiological effects of re-exposure to a stress-related environment in chronically stressed ovariectomized rats that were allowed to recover for 3 weeks. Previously, it has been demonstrated that severe stress can facilitate anxiety-like behavior for up to 21 days after the termination of the stress (Adamec et al., 1993; Ruis et al., 1999; Vyas et al., 2004). Within our experimental design, we tested the effect of the female sex hormone estradiol and the antidepressant mirtazapine for their ability to restore the stress-induced alterations in the limbic system when this treatment was started after a period of chronic stress.

Estradiol has been described as an important modulator of emotions and anxiety (Rachman et al., 1998; Marcondes et al., 2001; Bowman et al., 2002). Moreover, estrogen replacement therapy can be effective in the treatment of depressive symptoms in menopausal women (Carranza-Lira et al., 1999; Soares et al., 2001). We demonstrated previously an attenuation of chronic stress-induced aberrations by cyclic estradiol treatment in ovariectomized rats (Gerrits et al., 2005; Gerrits, submitted).

Mirtazapine is a noradrenergic and specific serotonergic antidepressant (NaSSA) which effects are mediated by blocking adrenergic \( \alpha_2 \) autoreceptors and \( \alpha_2 \) heteroreceptors, and by antagonizing 5-HT2 and 5-HT3 receptors. Besides the antidepressant actions, mirtazapine has also been described as a potent drug for treating symptoms of anxiety (Goodnick et al., 1999; Davidson et al., 2003; Asnis et al., 2004). In the forced swim test mirtazapine displayed increased swimming behavior only after chronic treatment (Reneric et al., 2002).

The approach followed in the current study is different from most other studies. Instead of comparing the stress reactions of treated versus untreated rats, a commonly used experimental design, we first disturbed normal stress processing by a chronic stress paradigm and thereafter started a chronic pharmacological intervention, with or without continuation of the stress exposure. On the last day, the rats were challenged again by exposing them to the stressful environment. This way of drug testing is more comparable to the clinical situation where patients present themselves for treatment only after considerable brain dysfunction has developed.
Materials and methods
Animals & experimental procedures

Forty-six female Wistar rats (Harlan, Horst, The Netherlands) were individually housed and kept on a reversed 12 hour light-dark cycle with lights on at 7:00 PM. Food and water was available *ad libitum*. The rats were handled daily. All experimental procedures were performed in the dark phase of the cycle. The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the protocols were approved by the Animal Experimental Committee of the University of Groningen, The Netherlands (FDC 4012).

All rats were bilaterally ovariectomized under isoflurane anesthesia. Table 1 summarizes the experimental groups and procedures. At day 10 after the ovariectomy, the stress procedure started for 41 rats. The stressor was presented daily for 21 days and consisted of 5 inescapable footshocks in combination with a long-during stay in the shock box (adverse environment as described previously (Gerrits et al., 2003; Gerrits et al., 2005)). Each day the rats underwent one session of 5 footshocks (0.8 mA, 8 sec) wherein each shock was preceded by a 5 sec during light stimulus. The stressor was presented in a randomized manner; the interval between the footshocks (3-30 min), the order in which the animals received the shocks, the start (8:30 AM-5:00 PM) and the duration of each session (15-180 min) was varied every day to prevent habituation. Five rats served as controls and were handled daily but were not exposed to the adverse environment and the footshocks. Body weights were recorded daily between 8:00 and 10:00 AM before the stress session of that day.

After 21 days of stress, the rats were divided in 4 treatment groups of 10-11 rats each. The first group was injected daily with vehicle (VEH; 0.1 ml saline with acetic acid, pH 6-6.5, i.p.). The second group received 17β-estradiol benzoate once every 4 days (E2; Sigma; 10 μg/250g in peanut oil, s.c.) and received vehicle injections on the three days between the E2 injections. As described before, this treatment paradigm led to physiological plasma E2 levels (below 50 pg/ml) with alternating high and low levels (Gerrits et al., 2005). The third group received daily injections of mirtazapine (MIR; kindly provided by Organon, Oss, The Netherlands; 10 mg/kg in saline with acetic acid, pH 6-6.5, i.p.). Other studies using this dose revealed reduction of stress-induced dopamine and noradrenaline release after chronic treatment (Dazzi et al., 2001b; Dazzi et al., 2002). The fourth group was treated with a combination of estradiol and mirtazapine in the doses as described above (E2+MIR). The 5 control rats received daily vehicle injections. All the injections were given between 9:30 and 10:30 AM and each treatment paradigm lasted for 22 days.

In this second part of the study, half of the animals of each treatment group was allowed to recover from the previous 21 days of daily stress and were kept in their home cages, like the control rats (VEH/con). The remaining 5-6 animals of each group were subjected to an additional 21 days of stress. In this continued stress protocol the rats were daily exposed to the shock box, but did not receive footshocks every day. The number of days when footshocks were presented was limited to 11 randomized days (day 22-42: SBE, FS, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS, SBE, FS; SBE=shock.
box exposure, FS=footshock).

At day 43 the rats were anesthetized with isoflurane and were transcardially perfused with 0.9 % saline, followed by 300 ml 4 % paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). Three hours prior to the perfusion the rats were injected with VEH or MIR; the last E2 treatment was 24 hours before the perfusion like in the previous experiments (Gerrits et al., 2005; Gerrits, submitted). Two hours before the perfusion the rats of all the groups were exposed to 5 light stimuli in the stress box within 30 minutes, but at this time no footshocks were presented. Using this approach only stress-induced responses related to exposure to an adverse environment were activated and pain could be avoided. For the control rats (VEH/con) this was the first exposure to the stressful environment. For the recovered rats (VEH/rec, E2/rec, MIR/rec and E2+MIR/rec) this was a re-exposure to the shock box after 21 days and for the rats subjected to the continued stress paradigm (VEH/stress, E2/stress, MIR/stress, E2+MIR/stress) this was the last exposure to the box after 43 days of stress. The brains were removed and postfixed overnight in 4 % phosphate-buffered paraformaldehyde (pH 7.4). After the perfusion the adrenals, thymus and uterus of each rat was dissected to determine the weight.

Corticosterone determination

Total corticosterone was extracted from 100 μl plasma obtained from a heart puncture prior to the perfusion and determined by high performance liquid chromatography with UV detection at 254 nm (Dawson, Jr. et al., 1984). Briefly, plasma samples were deproteinized with methanol and centrifuged. The supernatant was further cleaned by extraction on a C8 Solid Phase Extraction Column (Baker, Deventer, The Netherlands).

<table>
<thead>
<tr>
<th>Group</th>
<th>Surgery (day -9)</th>
<th>Stress (day 1-21)</th>
<th>Treatment (day 22-43)</th>
<th>Protocol (day 22-43)</th>
<th>Perfusion (day 43)</th>
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<td>Control</td>
<td>Control</td>
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<tr>
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<tr>
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<td>Stress</td>
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<td>E2/rec</td>
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<tr>
<td>MIR/rec</td>
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<td>Mirtazapine</td>
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<td>MIR/stress</td>
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<tr>
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<td>Stress</td>
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<td>Recovery</td>
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<td>6</td>
<td>OVX</td>
<td>Stress</td>
<td>E2 + Mirtazapine</td>
<td>Continued stress</td>
</tr>
</tbody>
</table>
Corticosterone was eluted with acetone and this extract was aspirated and redissolved in 25 % acetonitrile for subsequent injection onto the column (Nucleosil 100-5 C18 l=10 cm, I.D. 3 mm, particle size 5 μm; Chrompack, Middelburg, The Netherlands). Dexamethasone was used as internal standard. The minimum detection limit was 1 μg/dl.

Immunohistochemistry

The number of c-Fos and ΔFosB immunoreactive nuclei was determined. c-Fos protein is rapidly expressed after a stimulus as part of a cellular transient response for induction of AP-1 transcriptional activity (Kovacs, 1998). Full length FosB is expressed maximally 6 hours after a stimulus, after which the levels decline (McClung et al., 2004). However, the truncated splice variant ΔFosB has a very long half-life and gradually accumulates in time after successive stimuli (Chen et al., 1997; McClung et al., 2004; Perrotti et al., 2004). Induction of ΔFosB by repeated stress exposure represents long-term cellular changes. It was suggested that ΔFosB might function as a sustained molecular switch, which mediates more persistent changes of the brain in response to chronic perturbations (McClung et al., 2004). ΔFosB lacks most of the C-terminal transactivation domain of FosB but retains the dimerization and DNA-binding domains (McClung et al., 2004). We used a commercially available antibody against the N-terminus of the protein that could not discriminate FosB from ΔFosB. However, the animals were sacrificed 2 hours after the last stimulus and full length FosB is expressed maximally after 6 hours (McClung et al., 2004), therefore in our set-up we will detect predominantly ΔFosB with the N-terminus antibody. To prove this assumption we stained several adjacent sections also with an antibody against the C-terminus (kindly provided by Prof. Dr. E.J. Nestler, Dallas, TX, USA); an antibody that will only detect full length FosB. c-Fos protein expression is maximal 2 hours after a stimulus (Kovacs, 1998).

After cryoprotection of the brains in 30 % sucrose 40 μm coronal sections were cut on a cryostat microtome. Immunohistochemical procedures were performed on free-floating sections as described before (Gerrits et al., 2003). Endogenous peroxidase activity was quenched with 1 % H₂O₂. For c-Fos recognition we used rabbit polyclonal anti-c-Fos (Ab-5, Oncogene Research Products, Calbiochem-Novabiochem Int., San Diego, CA, USA; 1:10.000). For the N-terminus of FosB goat polyclonal anti-FosB (FosB (102), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:2000) and for C-terminus of FosB rabbit polyclonal anti-FosB (1:1000) was used. Biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; c-Fos, 1:1000; FosB C-terminus, 1:500) and biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA; FosB N-terminus, 1:500) were used as secondary antibodies. Immunoreactivity was visualized with a standard ABC method (Vectastain ABCkit, Vector Laboratories, Burlingame, CA, USA) followed by a 10 minute DAB-Ni reaction. Between all steps the sections were rinsed thoroughly with 0.1 M Tris buffered saline (TBS; pH 7.4). The slices were mounted on gelatin-coated slides, air-dried, dehydrated and coverslipped with DEPEX.
Recovery of stress-induced aberrations, possibilities of treatment

The c-Fos and FosB positive nuclei were quantified using a computerized image analysis system (Leica Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK).

The paraventricular nucleus of the hypothalamus (PVN; Bregma -1.08 to -2.00), the infralimbic (IL) and prelimbic (PL) area of the medial prefrontal cortex (mPFC; Bregma +3.20 to +2.15), the dentate gyrus of the hippocampus (DG; Bregma -2.45 to -3.90) and the central (CeA) and medial (MeA) nucleus of the amygdala (Bregma -2.00 to -2.85) were analyzed. The average number of positive nuclei from each rat was a calculated average of at least 3 brain slices on different levels throughout the area of interest and measured both in the left and right hemisphere. No left-right asymmetry of immunoreactivity was found. In order to make objective comparisons, counting was done in a single focus plane using the same magnification and identical gray scale settings as a correction for background staining by an observer blind for the experimental design.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analyses were done with SPSS (version 12.0.1); p<0.05 was considered significant. To describe the effects of the recovery after a 3-week stress period compared to control rats and continuously stressed rats the data of the VEH-treated rats were analyzed with a one-way ANOVA with protocol (control, recovery or stress) as between subject variable followed by an LSD pairwise comparison when applicable. To compare the effects of the different treatments the data were analyzed with a two-way ANOVA with treatment (VEH, E2, MIR or E2+MIR) and protocol (recovery or stress) as between subject variables followed by an LSD pairwise comparison when applicable.

Results

Effects of recovery after a 3-week stress period in VEH-treated ovariectomized rats

To demonstrate neurobiological effects of re-exposure to an adverse environment after 21 days of recovery following 21 days of daily stress exposure we compared VEH/con, VEH/rec and VEH/stress rats. In the limbic system we found a significant effect of the protocol on the number of c-Fos positive cells in the PVN (F_{2,12}=21.660, p<0.001), DG (F_{2,12}=5.546, p=0.020), IL (F_{2,12}=6.009, p=0.016) and PL cortex (F_{2,12}=5.834, p=0.017), and MeA (F_{2,12}=6.836, p=0.010). The CeA displayed a similar trend (F_{2,12}=3.768, p=0.054; Figure 1A-F and 2). Specifically, daily stressed rats without recovery showed after 6 weeks a comparable c-Fos response as control animals that were exposed to the shock box for the first time in the PVN (p=0.128), IL (p=0.219) and PL cortex (p=0.061), and the DG (p=0.157) and CeA (p=0.837). In the MeA, the c-Fos response of chronically stressed rats was lower than in the control rats (p=0.005).

However, rats that were allowed to recover from the daily stress exposure and were re-ex-
posed to the shock box 3 weeks later (VEH/rec), showed a significantly higher number of c-Fos positive nuclei than rats that were stressed continuously for 6 weeks (VEH/stress) in the PVN (p<0.001), IL (p=0.005), PL (p=0.005), CeA (p=0.029, although main effect n.s.), MeA (p=0.013) and DG (p=0.006).

Besides c-Fos we have also determined the accumulation of FosB in the mPFC. Adjacent sections were stained with antibodies against the N-terminus (full length and truncated FosB) or the C-terminus (only full length FosB) of FosB and showed that most immunoreactivity achieved with the N-terminus antibody is ΔFosB (Figure 3A+B).
There was a significant effect of the protocol on the number of ΔFosB expressing nuclei in the IL cortex ($F_{2,11}=5.124$, $p=0.027$; Figure 3C) and there was a trend in the PL cortex ($F_{2,11}=3.503$, $p=0.067$; Figure 3D). Chronic stress exposure during 6 weeks increased the number of ΔFosB positive nuclei compared to control rats in the IL ($p=0.009$) and PL cortex ($p=0.023$, although main effect n.s.), whereas 21 days of recovery did not decrease the number of ΔFosB stained nuclei to control levels in the IL ($p=0.247$) and PL cortex ($p=0.229$).

Weight gain was reduced in stressed ovariectomized rats during the first 21 days ($F_{1,44}=52.634$, $p<0.001$; Figure 4A). Between days 22 and 43 of the experiment, stressed rats grew less than control rats ($p=0.040$) and rats that were allowed to recover ($p<0.001$). Rats that were exposed daily to the adverse environment for 6 weeks showed decreased thymus weight compared to control rats ($p=0.005$). The thymus weight was reversed to control size ($p=0.049$) after 3 weeks recovery. There was no main effect of the protocol on adrenal weight or corticosterone levels measured 2 hours after the last exposure to the shock box (Table 2).
The effects of E2, MIR or E2+MIR treatment on stress responses after recovery and continued stress

To reveal whether treatment with E2, MIR or E2+MIR during recovery after a 21 days period of daily stress can alleviate the stress-induced aberrations more than VEH treatment, we compared the four treatment groups. The number of c-Fos positive nuclei in the PVN was significantly affected by the protocol ($F_{1,33}=70.971$, $p<0.001$; Figure 1A and 2). Recovered rats that were re-exposed to the shock box showed an increase in the number of c-Fos positive nuclei compared to rats that were stressed for 6 weeks when treated with VEH ($p<0.001$), MIR ($p<0.001$) or E2+MIR ($p=0.011$). Remarkably, recovered rats treated with E2 during days 22-43 (E2/rec) did not display higher numbers of c-Fos positive cells following re-exposure on day 43 ($p=0.147$). There was also a significant effect of the treatment ($F_{3,33}=15.223$, $p<0.001$); after recovery E2- and E2+MIR-treated rats showed a lower number of c-Fos positive nuclei in the PVN following re-exposure than VEH- and MIR-treated rats (E2-VEH $p<0.001$, E2-MIR
Recovery of stress-induced aberrations, possibilities of treatment

Figure 4: A) Body weight gain throughout the experiment. Stress reduced weight gain in ovariectomized rats in the first 21-day stress period ($F_{1,44} = 52.634, p<0.001$) and the treatment ($F_{5,57} = 45.817, p<0.001$). B) Body weight gain over the recovery period. Body weight gain was significantly affected by the protocol ($F_{1,33} = 61.493, p<0.001$) and the treatment ($F_{3,33} = 45.817, p<0.001$). * $p<0.05$ compared to recovery, # $p<0.05$ compared to control, $\$ p<0.05$ compared to VEH.

The number of c-Fos positive nuclei was significantly affected by the protocol in the DG ($F_{1,33} = 40.116, p<0.001$), the IL ($F_{1,33} = 82.995, p<0.001$) and the PL cortex ($F_{1,33} = 71.575, p<0.001$), and in the MeA ($F_{1,33} = 43.683, p<0.001$) (Figure 1B-E). Recovered rats that were re-exposed to the shock box on day 43 showed an increased number of c-Fos positive nuclei in the aforementioned areas compared to rats that were stressed for 43 days when treated with VEH (DG $p=0.001$, IL $p<0.001$, PL $p<0.001$, MeA $p=0.006$), E2 (DG $p=0.023$, IL $p=0.001$, PL $p=0.001$, MeA $p=0.012$), MIR (DG $p=0.015$, IL $p<0.001$, PL $p=0.001$, MeA $p=0.001$) or E2+MIR (DG $p<0.001$, IL $p<0.001$, PL $p<0.001$, MeA $p=0.006$). The treatment had no effect on the number of c-Fos positive cells in the IL cortex ($F_{3,33} = 0.134, p=0.939$), PL cortex ($F_{3,33} = 0.377, p=0.770$) and MeA ($F_{3,33} = 2.410, p=0.085$). In the DG

Table 2: The effect of recovery and 6 weeks stress on the organ weights and corticosterone levels in the VEH-treated rats. The protocol had a main effect on thymus weight ($F_{2,12} = 5.891, p=0.017$), but not on uterus weight ($F_{2,12} = 0.794, p=0.475$), adrenal weight ($F_{2,12} = 1.793, p=0.207$) and corticosterone levels measured 2 hours after the stressor ($F_{2,12} = 1.359, p=0.347$). # $p<0.05$ compared to control, * $p<0.05$ compared to recovery.

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<th>Parameter</th>
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<th>recovery</th>
<th>stress</th>
</tr>
</thead>
<tbody>
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<td>100.6 ± 8.8</td>
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<td>Thymus (mg)</td>
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<td>650.6 ± 49.8</td>
<td>496.7 ± 35.0 $^{*#}$</td>
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<tr>
<td>Adrenal (mg)</td>
<td>56.0 ± 5.6</td>
<td>50.0 ± 7.0</td>
<td>69.0 ± 6.0</td>
</tr>
<tr>
<td>CORT (µg/dl)</td>
<td>39.0 ± 3.7</td>
<td>42.8 ± 4.6</td>
<td>46.3 ± 3.8</td>
</tr>
</tbody>
</table>

p<0.001, E2+MIR-VEH p<0.001, E2+MIR-MIR p<0.001). After 43 days of daily stress exposure E2+MIR-treated rats showed less c-Fos positive cells than VEH-treated rats (p=0.025).
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(F3,33=12.754, p<0.001), however, there was a treatment effect; MIR- and E2+MIR-treated rats showed more c-Fos positive nuclei than VEH- and E2-treated rats following re-exposure after recovery (VEH-E2+MIR p=0.001, E2-MIR p=0.032, E2-E2+MIR p<0.001) and after 6 weeks of daily stress exposure (VEH-MIR p=0.007, VEH-E2+MIR p=0.001, E2-MIR p=0.049, E2-E2+MIR p=0.009).

There was no effect of the protocol on the number of c-Fos positive nuclei in the CeA (F1,33=0.382, p=0.541; Figure 1F). However, the number of c-Fos positive cells in the CeA was affected by the treatment (F3,33=37.040, p<0.001); MIR- and E2+MIR-treated rats showed a significantly higher number of c-Fos positive nuclei than VEH- and E2-treated rats after recovery (MIR-VEH p<0.001, MIR-E2 p<0.001, E2+MIR-VEH p<0.001 and E2+MIR-E2 p<0.001) and after 43 days of daily stress exposure (MIR-VEH p<0.001, MIR-E2 p<0.001, E2+MIR-VEH p<0.001, E2+MIR-E2 p<0.001).

The number of ΔFosB expressing nuclei was affected by the protocol in the IL (F1,32=4.527, p=0.041) and the PL cortex (F1,32=4.950, p=0.033; Figure 3). However, posthoc tests revealed that the number of ΔFosB positive nuclei was significantly increased only in the IL cortex of MIR-treated rats that were exposed to the stress for 43 days (IL p=0.028, PL p=0.079). Treatment had no effect on the number of ΔFosB positive nuclei in the IL cortex (F3,32=0.935, p=0.435) or PL cortex (F3,32=0.712, p=0.552).

Like VEH-treated rats, MIR- (p<0.001) and E2+MIR-treated rats (p=0.030) grew less during day 22-43 when the stress was continued compared to similarly treated rats that were allowed to recover. E2- and E2+MIR-treated rats grew less than VEH- and MIR-treated rats both during recovery (E2-VEH p<0.001, E2-MIR p<0.001, E2+MIR-VEH p<0.001, E2+MIR-MIR p<0.001) and during continued stress exposure (E2-VEH p<0.001, E2-MIR p=0.020, E2+MIR-VEH p<0.001, E2+MIR-MIR p=0.004). Furthermore, rats that were allowed to recover but were treated with MIR gained less weight than rats treated with VEH (p<0.001).

The uterus of ovariectomized rats was even after 5 weeks of estrogen depletion still responsive to E2 treatment. Like in the VEH-treated group, rats that were allowed to recover for 21 days had higher thymus weights compared to rats that were daily stressed for 43 days when treated with E2 (p=0.035) or MIR (p=0.002). E2 treated animals however showed lower thymus weights than VEH- and MIR-treated rats. Although reduction of stress-

<table>
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<th>E2 Recovery</th>
<th>E2 Stress</th>
<th>MIR Recovery</th>
<th>MIR Stress</th>
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<td>666.2 ± 48.57</td>
<td>706.6 ± 151.3</td>
<td>106.6 ± 7.4</td>
<td>101.5 ± 6.3</td>
<td>642.9 ± 34.25</td>
<td>551.6 ± 74.2</td>
</tr>
<tr>
<td>Thymus (mg)</td>
<td>650.6 ± 49.8</td>
<td>496.7 ± 35.0p</td>
<td>463.8 ± 30.2</td>
<td>408.5 ± 17.7</td>
<td>309.8 ± 36.4</td>
<td>407.8 ± 39.0p</td>
<td>397.3 ± 30.83</td>
<td>397.3 ± 80.5</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>50.0 ± 7.0</td>
<td>69.0 ± 6.0p</td>
<td>69.6 ± 5.1p</td>
<td>71.6 ± 5.4</td>
<td>62.1 ± 2.1</td>
<td>71.7 ± 2.7</td>
<td>73.5 ± 2.4p</td>
<td>78.2 ± 3.7</td>
</tr>
<tr>
<td>CORT (µg/dl)</td>
<td>42.8 ± 4.6</td>
<td>46.3 ± 3.8</td>
<td>53.8 ± 5.4</td>
<td>58.0 ± 5.5</td>
<td>41.3 ± 7.5</td>
<td>36.7 ± 8.9</td>
<td>51.8 ± 11.0</td>
<td>51.7 ± 11.3</td>
</tr>
</tbody>
</table>

* p<0.05 compared to recovery, ** p<0.05 compared to VEH.
induced adrenal hypertrophy was shown in recovered VEH-treated rats (p=0.022), in the other treatment groups the recovery was not associated with reduced adrenal weights. E2 treatment increased adrenal weight. Moreover, there was no effect of the treatment or the protocol on corticosterone levels measured 2 hours after exposure to the shock box on day 43 (Table 3).

Discussion

We demonstrated sensitization of the c-Fos reaction in several limbic areas in ovariectomized rats that were re-exposed to an adverse environment after 21 days recovery following 3 weeks of daily stress exposure. Cyclic E2 administration could prevent this sensitization after re-exposure in the PVN and CeA. MIR treatment increased the number of c-Fos positive cells in the DG and CeA. Transcriptional consequences of chronic stress in the mPFC, as measured by ΔFosB accumulation, were not affected by E2 or MIR treatment.

Chronic stress

Six weeks daily exposure to stress reduced growth and thymus weight and slightly induced adrenal hypertrophy in ovariectomized VEH-treated rats; known effects of chronic stress (Kioukia-Fougia et al., 2002). In the first 6 hours of the dark phase of the circadian rhythm, when rats are active, basal plasma corticosterone levels of ovariectomized rats are approximately 20 μg/dl (own unpublished findings). Exposure to the adverse environment on day 43 increased the basal plasma corticosterone concentration in all animals to at least 40 μg/dl, which indicates that this exposure is stressful for the rats. Chronically stressed rats (VEH/stress) and control rats that were exposed to the shock box for the first time (VEH/con) showed a similar c-Fos response in the PVN, DG, mPFC and CeA. The VEH/con rats did not associate the box or the light stimulus with footshocks. However, exposure to the shock box also embraces exposure to a novel environment, auditory cues of vocalization of the other rats and remaining olfactory stimuli of alarm pheromones that are present in the excretions of previously stressed rats. All these features have been described as stressful and can increase the c-Fos expression above basal level (Ishikawa et al., 1992; Handa et al., 1993; Kiyokawa et al., 2005). In a previous study we showed that non-exposed ovariectomized control rats display a negligible number of c-Fos positive nuclei in the limbic system (Gerrits et al., 2005). Based on the similarity in the c-Fos response after first time exposure and after 6 weeks daily exposure, we conclude that habituation to the currently used stress paradigm is minimal. Exposure to an environment associated with footshocks has been described to induce enhanced stress reactions compared to receiving physical footshock stress (Maier, 2001). Between days 22 and 43 in the currently used stress paradigm the rats were daily exposed to the shock box although we did not present the footshocks every day. Moreover, the rats that were exposed to the shock box could hear
the ultrasonic vocalization of other stressed rats, adding an extra psychological component (Ishikawa et al., 1992). Altogether this may explain why in the current study the rats still displayed increased numbers of c-Fos positive cells even after 43 days of daily exposure to an adverse environment, while others have reported blunted c-Fos responses after, for example, repeated restraint stress (Stamp et al., 1999).

Fourty-three days of daily stress exposure increased the number of cells that expressed ΔFosB in the mPFC of OVX rats compared to controls. A similar increases was reported in male rats after 10 days stress (Perrotti et al., 2004). Perrotti and colleagues revealed neuronal accumulation of ΔFosB in the mPFC after stress that was not co-localized with glial fibrillary acidic protein (GFAP) (Perrotti et al., 2004). Moreover, they reported that more than 90 % of these ΔFosB positive neurons are glutamatergic, suggesting that long-term stress a change in the activation pattern of excitatory neurons occurs.

Low expression levels of ΔFosB inhibit AP-1 transcription, whereas higher levels of ΔFosB expression increase transcription of AP-1 sites (McClung et al., 2003). An acute stressor will induce low levels of ΔFosB and high levels of c-Fos expression, while after predictable repeated stress the expression of c-Fos declines (Stamp et al., 1999) and ΔFosB increases. Transcription of AP-1 sites therefore can be induced by both acute and chronic stress but the mechanism of activation will shift from a transient activation by c-Fos towards a chronic perturbation by ΔFosB. Unfortunately, the functional consequences of accumulated ΔFosB and subsequent long-term AP-1 modulation in the mPFC are still unknown.

Sensitization

The number of c-Fos positive nuclei in the PVN, DG, mPFC, MeA and CeA following re-exposure to the stress box on day 43 was significantly higher in rats that were allowed to recover for 21 days after 21 days of daily stress exposure than rats that were exposed to this stressor continuously for 43 days. Sensitization of the c-Fos response in limbic structures by previous stress exposure was reported for male rats (Bruijnzel et al., 1999) and is now demonstrated in female rats. Moreover, sensitization of corticotrophin releasing hormone (CRH) immunoreactivity (Bruijnzel et al., 2001a), facilitation of anxiety-like behavior (Adamec et al., 1993; Ruis et al., 1999; Vyas et al., 2004), sensitization of autonomic responses including blood pressure (Bruijnzel et al., 2001b) and core body temperature (Meerlo et al., 1996) and increased release of dopamine, noradrenaline (Irwin et al., 1986; Gresch et al., 1994) and serotonin (Adell et al., 1988) have been described after re-exposure. Besides it has been demonstrated that stressful events during adulthood, including postnatal stress have significant effects on stress reactivity later in life (Meaney et al., 1996; Pryce et al., 2005; Matsumoto et al., 2005). These data indicate that previous stress exposure can modulate the reactions to subsequent events and cause excessive activity to seemingly mild stressors. Indeed, in subjects that were diagnosed for post-traumatic stress disorder (PTSD) exposure to a stressor induced exaggerated responses in most (Butler et al., 1990; Orr et al., 1995; Metzger et al., 1999; Heim et al., 2000; Bremner et al., 2003),
but not all studies (Klumpers et al., 2004). Interestingly, levels of accumulated ΔFosB in the mPFC were not returned to control levels following 21 days of recovery in the present study, which shows that the preceding stress period still can have transcriptional consequences in this limbic area.

Effects of E2 treatment

One of the aims of the present study was to test whether E2 treatment of ovariectomized rats, depleted of estrogen for 5 weeks, could reverse stress-induced changes in limbic system reactivity. Because treatment with gonadal hormones has considerable side effects in humans (Rossouw et al., 2002), we examined the effects of a low dose of E2 that resembles physiological conditions. We used a once every 4 days cyclic treatment paradigm to induce alternating high and low plasma E2 levels that mimics the natural E2 cycle instead of often used pellets or daily injections that may induce estrogen receptor down-regulation (Gerrits et al., 2005).

E2-treated rats do not display increased sensitization of the c-Fos immunoreactivity in the PVN after 21 days recovery. Moreover, the number of c-Fos positive nuclei in the PVN of E2+MIR-treated rats was lower than in VEH- or MIR-treated rats, indicating a strong effect of E2 administration on PVN activation. Previously we have reported an attenuation of the stress-induced expression of c-Fos positive nuclei in the PVN after cyclic E2 administration when the stress exposure and E2 treatment were combined for 21 days (Gerrits et al., 2005). In the present study, however, the cyclic E2 treatment was started after a daily chronic stress exposure of 21 days. We proposed previously that reduction of the c-Fos response by E2 during stress was mediated by increased dendritic release of oxytocin. Twenty-one days of daily stress increased the number of ERβ positive cells in the PVN; the main location of oxytocin containing cells (Gerrits et al., 2005). E2/ERβ complexes can bind to estrogen responsive elements located on the oxytocin gene and induce transcription of the gene (Mohr et al., 1991; Loven et al., 2001; Nomura et al., 2002). Windle and colleagues have reported that oxytocin can repress the stress-induced c-Fos expression in the PVN (Windle et al., 2004). Except for the PVN, cyclic E2 treatment also attenuated stress sensitization in the CeA, an area that also expresses oxytocin receptors and is a known target for oxytocin-induced modulation of the autonomic fear response (Huber et al., 2005). Cyclic E2 administration did not have an effect on the excessive c-Fos response that was found after re-exposure in the mPFC, DG and MeA.

Both the PVN and the CeA are output regions of the limbic system that mediate autonomic and endocrine stress responses (Luiten et al., 1985; Roozendaal et al., 1991) and play a critical role in anxiety behavior. E2-induced suppression of the exaggerated stress response of the PVN and CeA after re-exposure may prevent excessive hypothalamus-pituitary-adrenal-axis and cardiovascular activity, providing a neurobiological basis for the anxiolytic effects of E2 (Rachman et al., 1998; Marcondes et al., 2001; Bowman et al., 2002). Moreover, these results may explain the conflicting data regarding the stress responses of PTSD.
patients. Possibly, some of these studies included more women in the late follicular phase, when E2 levels are high.

Surprisingly, when we continued the exposure to the adverse environment during the E2 treatment between days 22 and 43, E2 had no effect on the c-Fos response. Probably other, not yet identified, factors overrule the positive effects of E2 when the stress was continued for 43 days.

Cyclic E2 treatment could not prevent or diminish ΔFosB accumulation in the mPFC, and therefore does not attenuate the long-term transcriptional alterations induced by stress.

Effects of MIR treatment

The sensitization in the PVN, DG, mPFC and MeA induced by previous stress exposure as shown with c-Fos expression after re-exposure was not affected by MIR treatment during 21 days of recovery, which suggests that chronic MIR administration cannot restore the deleterious effects of stress in these structures.

MIR increased the number of c-Fos positive nuclei in the DG and CeA compared to VEH; an effect that was observed in MIR and E2+MIR-treated rats. Other studies also have reported increased c-Fos expression in the CeA after antidepressant treatment, including MIR (Sumner et al., 2004; Slattery et al., 2005). However, these results were obtained following acute treatment in male rats. So, 21 days MIR treatment of ovariectomized or cyclic E2-treated rats following chronic stress gave similar results in the CeA as in non-stressed male rats that were subjected to a single, and lower, dose of MIR. Since the CeA is involved in autonomic stress reactions, the increased neuronal activity observed in the present study after antidepressant treatment may indicate increased autonomic responses. No sensitization was found in the CeA of MIR- and E2+MIR-treated rats after 21 days of recovery; it is conceivable that MIR treatment somehow activates a maximal number of CeA neurons, which prevent recruitment of additional CeA cells during re-exposure. This, in turn, may have positive effects on perception of the anxiety-inducing stimuli.

ΔFosB accumulation in the mPFC was not prevented or reduced by MIR treatment, so the long-term transcriptional alterations induced by stress were not attenuated.

Although, chronic MIR administration has been described to prevent noradrenaline, dopamine and acetylcholine release elicited by acute footshock stress in male rats in the mPFC (Dazzi et al., 2001a; Dazzi et al., 2001b; Dazzi et al., 2001c; Dazzi et al., 2002), MIR administration for 21 days following 21 days of daily adverse environment exposure did not induce different effects on c-Fos expression than VEH except in the CeA. However, here the antidepressant treatment was started after development of stress-induced aberrations. MIR has, as far as we know, never been tested before in a paradigm that was setup to investigate the ability of MIR to restore limbic system functions after chronic stress exposure. It is possible that a longer treatment period is necessary to restore the abnormalities induced by long-term stress. From patient studies it is known that antidepressants have a delayed onset of therapeutic effects which can amount to several weeks (Gelenberg et al., 2000;
Gumnick et al., 2000).

**Effects of the combination E2+MIR**

Cyclic E2 administration did not augment the actions of MIR or vice versa. MIR did not cancel out the effects of E2 in the PVN and the effects of MIR administration in the DG and CeA were not counteracted by E2.

**Concluding remarks**

Altogether, we demonstrated that 21 days of daily stress exposure in an adverse environment induces neurobiological changes in the limbic system of female rats that has consequences for subsequent stress processing. After 21 days of recovery the c-Fos response to re-exposure was sensitized. It would be very interesting to see if these changes persist for a longer time in this paradigm or that a time-dependent washout occurs. Cyclic E2 administration could prevent the sensitization of the PVN and CeA, which may underlie anxiolytic effects. Effects of MIR treatment during recovery of chronic stress were not different from VEH treatment in the currently used paradigm, except for a prevention of sensitization of the CeA. The stress-induced long-term ΔFosB accumulation in the mPFC was not affected by either of these treatments. When the exposure to the adverse environment was continued during days 22-43, we did not find an advantage of E2 or MIR treatment compared to VEH. This finding may have consequences for understanding the confounding response to antidepressant drugs in patients that are still exposed to the stressful conditions that trigger their disease. Most studies interested in the efficacy of antidepressants have tested stress reactions after chronic administration, while in the current study we have evaluated the capacity of antidepressants to restore stress-induced aberrations in the limbic system, which is in our view more relevant for understanding the neurobiological basis of stress-induced affective disorders and the potential positive effects of antidepressants.

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