Influence of gender and social environment in an animal model of affective disorders
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Chronic stress and social housing differentially affects neurogenesis in male and female rats

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Stress plays an important role in the development of affective disorders. Women show a higher prevalence for these disorders than men. The course of a depression is thought to be positively influenced by social support. We have used a chronic mild stress model in which rats received foot-shocks daily for 3 weeks. Since rats are social animals, we hypothesised that ‘social support’ might reduce the adverse effects of chronic stress. To test this hypothesis, male and female rats were housed individually or socially in unisex groups of 4 rats. The proliferation marker BrdU was injected 2 weeks before the sacrifice to investigate if stress and social housing influenced the survival of proliferating cells in the dentate gyrus. To investigate changes in proliferation, another group of rats was sacrificed the day after the last BrdU injection. Stress significantly decreased BrdU labelling in individually housed males and not significantly in socially housed males. In individually housed females, stress increased BrdU labelling, which was prevented by social housing. The increase found in females is most likely caused by differences in survival rate, since cell proliferation was not affected by stress or housing conditions. These results indicate that social support can affect neurogenesis in both female and male rats, however in a different way.
Introduction

Stress plays an important role in the onset of affective disorders. Ample evidence is accumulating from human as well as animal research that females react differently to stressors than males. While females are more susceptible to the development of affective disorders, preclinical stress research has focussed mainly on male animals and therefore little is known about the neurobiology of females coping with stress.

As stress has negative effects on mental health, social support is known to have positive effects on stress-coping and there are some indications of a gender difference in the effect of social support. Psychotherapy that could be viewed as formalised social support, improves symptoms and normalises brain activity in depressed patients, similar to antidepressant treatment. This suggests a neurobiological basis for the ameliorating effects of social support/psychotherapy.

Recent studies suggest that symptoms of depression are thought to be related to reduced synaptic plasticity in the brain, possibly resulting in the inability to respond and/or adapt to aversive stimuli. Reduced levels of neurotrophins have been found in the brains of depressed patients. One of the neuroanatomical changes found in depressed patients is a decreased hippocampal volume, which appears to be reversible after recovery from the depression. This reduction of hippocampal volume might be related to a stress-induced decrease in neurogenesis in the dentate gyrus (DG) of the hippocampus, as found after stress-exposure in rodents that can also be reversed by antidepressant treatment.

Chronic stress exposure in rodents has been proposed as a valid animal model for affective disorders. Chronically stressed rats show symptoms characteristic of depression, like anhedonia and sleep disturbances, but most studies were performed in male rats only.

Since rats are social animals, social housing during stress exposure could provide an interesting model to study the neurobiological effects of social support. We have previously shown that social housing in unisex groups ameliorates stress-coping in female rats but not males. In male rats social housing appeared to increase the stress-sensitivity, and only isolated control males showed no signs of stress, whereas isolation by itself appeared to be stressful for females. Environmental enrichment has been shown to increase neurogenesis. Although social housing does not qualify as environmental enrichment, it apparently affects the way animals cope with stress, which might be reflected in the number of new neurons born or the survival of these neurons. Rats were injected with the proliferation marker BrdU for 5 consecutive days to eliminate oestrus-cycle related variation of neurogenesis in females. The last injection was given 2 weeks before the end of the stress exposure, in order to investigate the long-term effect of chronic stress on
neurogenesis. However, the effects, evident after 3 weeks of stress exposure, could be due either to differences in cell proliferation during the time BrdU was present or to changes in survival of newly generated cells during the 2 subsequent weeks. Several studies have shown that stress decreases cell proliferation in the DG in male rats.\textsuperscript{16} So, in a follow-up experiment we investigated if females also showed this stress-induced decrease in cell proliferation.

**Material & Methods**

Male (n=24) and female (n=24) Wistar rats where either individually (males: n=10, females n=10) or socially (males: n=14, females n=14) housed in unisex groups of 4 rats. Of the individually housed rats, 5 rats were subjected to chronic stress and 5 rats to a control treatment. From each social group, two rats underwent stress exposure and two served as controls (n=7 per group). To have an equal number of 4 rats in each cage, in two cages of both genders an extra rat was added.

At the start of the experiment rats were of the same age with males weighing 298 ± 3 g and females weighing 214 ± 1 g. The light-dark cycle was reversed (lights on 19.00-7.00 hr) and water and food was provided ad lib. All experimental procedures were approved by the Animals Ethics Committee of the University of Groningen (FDC: 2509).

Rats were subjected to a chronic stress protocol for 3 weeks. During the dark/active period of the rats, daily at random times, rats in the stress group were transferred to a footshock box and received 5 inescapable footshocks at random intervals during a 30-120 minute session (0.8 mA in intensity and 8 sec in duration). A light signal (10 sec) preceded each footshock adding a ‘psychological’ component to the noxious event. Control rats were placed in similar, non-electrified, cages. To study the effects of chronic stress on neurogenesis rats were treated with the thymidine analog bromodeoxyuridine (BrdU) (i.p. 100 mg/kg) for 5 consecutive days to eliminate oestrous-cycle related variation of neurogenesis in females.\textsuperscript{32} The last injection was given 2 weeks before the end of the stress protocol (Figure 1B).

In the follow-up experiment female rats were either individually or socially housed (n=6 per group), and subjected to the same stress exposure protocol but now they were sacrificed after the last BrdU injection on the eighth day of the protocol (Figure 1A). At the end of the experiments, rats were deeply anaesthetised on day 22 with sodium pentobarbital (1 ml, 6%) and transcardially perfused with 50 ml heparinised saline and 300 ml of a 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4). Adrenal and thymus weights, corrected for body weight, were calculated and used as indication of the amount of stress perceived.

BrdU immunohistochemistry was carried out on 40 μm free floating sections as described previously.\textsuperscript{19} For the DAB-BrdU staining the following antibodies were used: rat-anti-BrdU (1:1000), Oxford biotechnology, (www.immunologicalsdirect.com), 2nd antibody, biotinylated goat anti-
rat IgG and avidin-biotin-peroxidase complex (1:900). Fluorescent triple staining were applied in TBS with 3% normal donkey serum with 0.1% Triton-X-100. The primary antibodies used were: mouse anti-NeuN (1:200, Chemicon), rabbit anti-Cow GFAP (1:500, DAKO), and rat anti-BrdU (1:300, Oxford biotechnology). The corresponding fluorescent antibodies used were: Donkey-anti-Mouse rhodamine Red-X-conjugated (1:200, Jackson), Donkey-anti-Rabbit Cy5-conjugated (1:200, Jackson), Donkey-anti-Rat biotin-SP-conjugated, (1:200, Jackson) together with Fluorescein (DTAF)-conjugated streptavidin (1:200, Jackson).

Sections were digitised by using a Sony charge-coupled device digital camera mounted on a LEICA Leitz DMRB microscope (Leica, Wetzlar, Germany) at 100x magnification. The number of DAB stained BrdU-positive cells in the granule cell layer (GLC) of the DG per 0.1 mm$^2$ were quantified and group differences were expressed as percentage change with the isolated controls (per sex) at 100%. Immunofluorescent labelling was visualised under 40x magnification with a confocal laser microscope (Zeiss LSM510 META-NLO). Percentages of double labelled BrdU-positive cells in the GCL were quantified. None of the BrdU labelled cells was labelled with both GFAP and NeuN.

Statistical analyses were done with SPSS (version 10.0), and p $<$ 0.05 was considered significant. Weight gain for each gender was analysed with a repeated measures ANOVA with days as within subject factors and treatment (control or stress) and housing (individual or social) as between subject variables. BrdU data were analysed with an univariate ANOVA with gender, housing and treatment as between subject factors. Sphericity assumed modelling, with Greenhouse-Geisser and Huynh-Feldt adjustments, was applied.29

Results

Weight gain was significantly affected by chronic stress in male rats ($F_{1,20} = 39.37$, p $<$ 0.001), reducing the growth rate in both individually and socially housed males (resp. $F_{1,20} = 27.63$, p $<$ 0.001 and $F_{1,20} = 12.17$, p = 0.002). However no significant housing effect was observed. There was a significant day effect (p $<$ 0.001) with weight steadily increasing over the days and an interaction effect between day and treatment (p $<$ 0.001) (Greenhouse-Geisser correction). In contrast, in females chronic stress had no significant effects on the growth rate but here an effect of housing was observed ($F_{1,20} = 8.07$, p = 0.010), socially housed control rats showing a reduced growth rate ($F_{1,20} = 5.11$, p = 0.035) compared to individually housed control females. In females there was a significant effect of day (p $<$ 0.001) and interaction effect between day and housing (p = 0.034) (Greenhouse-Geisser correction).

Chronic stress had a significant effect on adrenal weight ($F_{1,32} = 10.26$, p = 0.003) (Table 1), socially housed but not in isolated male rats, showing adrenal hypertrophy after chronic stress exposure ($F_{1,16} = 6.85$, p = 0.019). In females, stress induced a significant increase in adrenal weight in individually housed rats ($F_{1,16} = 4.60$, p = 0.048) whereas in the socially housed females the adrenal weight was not increased. Besides stress effects, we found a gender difference in the relative adrenal weight. The female
adrenal was significantly larger than the male adrenal \(F_{1,32} = 116.43, \ p \leq 0.001\). Housing conditions alone had no significant effect on adrenal weight. Thymus weights were neither affected by stress nor by housing conditions.

Treatment \(F_{1,31} = 5.26, \ p = 0.029\), gender \(F_{1,31} = 106.58, \ p < 0.001\) and the interaction treatment\*gender \(F_{1,31} = 14.304, \ p < 0.001\) had a significant effect on the number of BrdU positive cells in the GCL of the DG (Figure 2B and 3). Females showed a significant effect of treatment \(F_{1,16} = 5.32, \ p = 0.035\) and a treatment\*housing interaction \(F_{1,16} = 8.08, \ p = 0.012\), whereas in the males only the treatment effect was significant \(F_{1,15} = 9.84, \ p = 0.007\). Chronic stress decreased the number of newly formed neurons in the granule cell layer in isolated males \(F_{1,15} = 6.85, \ p = 0.019\), whereas in socially housed males this effect was not significant. Isolated females, in contrast to isolated males, showed a stress-induced increase in the number of new neurons \(F_{1,16} = 11.05, \ p = 0.004\), while social housing prevented this increase. Stressed isolated females also had higher number of BrdU-positive cells than socially housed stressed females \(F_{1,16} = 5.42, \ p = 0.033\). BrdU-labelling in the hilus was not affected by stress, gender or housing conditions. Male rats, except the isolated stressed rats, had more BrdU labelled cells than females (indiv. control: \(F_{1,31} = 46.198, \ p \leq 0.001\), social control: \(F_{1,31} = 57.808, \ p \leq 0.001\), social stressed: \(F_{1,31} = 25.404, \ p \leq 0.001\)).

The majority of the BrdU-positive cells were double labelled with NeuN \(87 \pm 2.3\%\), only a small number of cells were double labelling for BrdU and GFAP \(3 \pm 1.7\%\), the remaining BrdU-positive cells were neither staining NeuN or GFAP. No group differences were found in the percentages of double labelling, so data were pooled (Figure 4).

Eight days of stress had no effect on cell proliferation in the dentate gyrus (isol. control: \(19.8 \pm 1.8\); isol. stressed: \(19.2 \pm 1.6\); social control: \(19.2 \pm 1.1\); social stressed: \(20.8 \pm 1.5\))(Figure 2A).
Discussion

Chronic stress exposure had different effects on individually housed male and female rats. Male rats did show the expected decrease in BrdU-labelling, whereas females unexpectedly showed an increase in BrdU-labelling. In males rats it has been shown that stress decreases cell proliferation, so likely the decrease found after 3 weeks of stress exposure in isolated males is a consequence of decreased cell proliferation. In females however we did not find an effect of housing conditions nor of acute stress on cell proliferation in the dentate gyrus, indicating that the differences found after 3 weeks of stress exposure are the result of changes in survival and not of increased proliferation. The absence of a acute stress effect on proliferation corresponds with data from Falconer et al. who showed no effect of acute predator odour stress on cell proliferation in female rats. The majority of BrdU-positive cells was also positively labelled with the neuronal marker NeuN, showing that most newly born cells became neurons, corresponding with other studies.

Treatments that are used for depressed patients, like antidepressant medication and electroconvulsive therapy have been found to increase neurogenesis in male rodents and hippocampal neurogenesis appears to be necessary for the behavioural effects of antidepressants to occur. Also treatment with the
antidepressant tianeptine prevents the stress-induced decrease in hippocampal cell proliferation and volume in tree shrews. Although we previously showed that social housing of males increased the adverse effects of chronic stress on behaviour and FOS expression, this more stimulating environment may have a slight ‘antidepressive’ effect and prevent a stress-induced decrease in neurogenesis in these males, or more likely contributes to increased survival of new neurons. Behavioural data, adrenal weights and limbic FOS-expression data indicated that isolated females were more affected by chronic stress, it is therefore unlikely that the increased neuronal survival signifies improved stress-coping. Experimentally induced DG damage has been shown to increase neurogenesis in the granule cell layer. It could be speculated that increased neurogenesis, as observed in the present study, in chronically stressed females is a consequence of pathological changes in the hippocampus, which is only observed in females due to higher stress sensitivity. The increase in survival of new neurons found in isolated females could therefore be a compensatory mechanism for this stress-induced neuronal damage.

It has been found that newly generated neurons in the GLC have axons extending into the CA3 region after 17-24 days and that are indistinguishable in shape and size from neighbouring neurons. Van Praag and co-workers also showed that newly born neurons form functional connections and are integrated in the hippocampal circuitry. These newly born neurons also respond to stimuli, as shown by FOS expression in these neurons, comparable with the expression in older granule cells.

Figure 4. Confocal image showing representative BrdU and NeuN double-labelled cells (white arrows) in the dentate gyrus of the hippocampus.
There are other reasons to assume that these new neurons may form functional connections in females, since it has been shown that chronic stress improves spatial memory, a hippocampal dependent learning task, in female rats,\(^1\) while in males neurogenesis is decreased \(^5\),\(^16\) and spatial memory impaired by chronic stress.\(^5\),\(^18\) Social housing of females may hypothetically provide a distraction that reduces the long-term impact of stress, among other things by preventing the incorporation of newly formed neurons into the neural circuitry that processes information related to the stressful events. Frodl and co-workers\(^11\) reported on a gender difference in hippocampal volume changes in first-episode depressed patients, with men showing a reduction in grey matter hippocampal volume, which was not observed in women. Stress-induced increased survival of new neurons might hypothetically provide a possible mechanism for this gender difference.

The present results demonstrate that chronic stress differentially affects neurogenesis in male and female rats and suggests that there are gender differences in the response to stressful events.

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