Shaping the brain through experience: effects of stressful life events on hippocampal neurogenesis, morphology and function
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Chapter 5

Brief treatment with the GR antagonist mifepristone normalizes the reduction in neurogenesis after chronic stress.


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Abstract
In rodents, stress suppresses adult neurogenesis. This is thought to involve activation of glucocorticoid receptors in the brain. In the present study, we therefore questioned whether glucocorticoid receptor blockade by mifepristone can normalize the effects of chronic stress on adult neurogenesis. Rats received mifepristone on the last 4 days of a 21-day chronic unpredictable an inescapable stress regimen. Neurogenesis was analyzed by stereological quantification of adult-generated cell survival (BrdU), young neuronal survival (doublecortin) and cell proliferation (Ki-67). The results show that only 4 days of mifepristone treatment already normalized the stress-induced reductions in neurogenesis. Importantly, mifepristone by itself had no effect on neurogenesis. We conclude that -contrary to other compounds interfering with the effects of chronic stress on neurogenesis, like antidepressants- mifepristone’s normalizing effects on neurogenesis are rapid and particularly potent in a high stress environment. This neurogenic action of mifepristone could potentially contribute to its clinical mechanism of action.
Introduction

Stress activates the hypothalamo-pituitary-adrenal (HPA)-axis, which coordinates the release of adrenal glucocorticoids (GC). Upon their release, GCs bind to the high affinity mineralocorticoid receptor (MR) and the lower affinity glucocorticoid receptor (GR), that are both abundantly expressed in the hippocampus (Joels et al., 2004; de Kloet et al., 2005). Given the difference in affinity, the GR becomes extensively occupied only when plasma corticosteroid levels are elevated.

While acute stress generally facilitates adaptation, chronic stress can induce a dysregulation of the HPA-axis and significantly impact hippocampal function and structure (Joels et al., 2004; de Kloet et al., 2005). Chronic stress in animals impairs long term potentiation in the CA1 and causes dendritic retraction in the CA3 hippocampal regions (Magarinos et al., 1996; Alaszewicz et al., 2003). Reductions in hippocampal neurogenesis have also been observed after stress (Gould and Tanapat, 1999; Heine et al., 2004b; Mirescu and Gould, 2006). Given the repetitive activation of the HPA-axis under chronic stress conditions, it seems probable that GCs play an important role in the development of aberrant brain function after chronic stress, e.g. in suppression of neurogenesis.

Chronic stress is also considered as a risk factor for major depression in susceptible individuals (Fava and Kendler, 2000; Nestler et al., 2002; Caspi et al., 2003). Strong activation of the HPA-axis often occurs in a substantial part of depressed patients; symptoms include hypertrophy of the adrenals, escape of ACTH and cortisol release from dexamethasone suppression, elevated plasma cortisol levels and significant reductions in hippocampal volume (Hayes and Eti, 1983; Heuser et al., 1994; Rybakowski and Twardowska, 1999; Sheline et al., 1999; Dinan, 2001; Lucassen et al., 2001; Sheline, 2003; McEwen, 2005; Swaab et al., 2005). Consistent with a role of GCs in depression and psychosis, treatment with the GR-antagonist mifepristone was recently reported to ameliorate symptoms of psychotic depression (Flores et al., 2006). Of note, mifepristone was already effective after 4 days of treatment (Belanoff et al., 2001; Belanoff et al., 2002; Flores et al., 2006); for discussion see (Carroll and Rubin, 2006; DeBattista and Belanoff, 2006; Keller and Schatzberg, 2006). Whether or not the effectiveness of mifepristone involves alterations in neurogenesis is presently unclear.

The latter is of interest since, in rodents, many antidepressant drugs increase neurogenesis (Malberg et al., 2000), whereas depletion of neurogenesis nullifies the behavioural effects of current antidepressants (Santarelli et al., 2003). The time course of neurogenic effects in rodents also parallels the long-term time-to-effect of antidepressants of several weeks. These findings suggest a potential role for neurogenesis in antidepressant action and in the etiology of depression and/or psychotic depression (Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006). We therefore investigated whether a similar 4 day
treatment with the GR-antagonist mifepristone normalizes changes in adult neurogenesis induced after chronic unpredictable stress.

**Materials & Methods**

**Animals**

In this experiment 24 male Wistar rats (175-250 g) were used. Animals were housed in pairs, under controlled conditions, 12:12 h light/dark cycle (lights on 8:00 h) with food and water *ad libitum*. Temperature and humidity were within 20-22 °C and 50-55%. The local animal experimental committee of the University of Amsterdam approved the experiment.

**Chronic stress paradigm and mifepristone treatment**

Rats were randomly assigned to four different groups: handled controls (C), handled controls with mifepristone treatment (C+M), chronically stressed (S) and chronically stressed animals with mifepristone treatment (S+M). Control animals were handled and weighed daily, for 21 days.

Rats were subjected to an unpredictable and inescapable chronic stress paradigm for 21 days (Herman *et al.*, 1995) as described before (Alfarez *et al.*, 2003). Animals were subjected to two different stressors per day in an unpredictable order: immobilization, cold immobilization, vibration, isolation, crowding, swim stress and cold swim stress.

On days 18-21, approximately 30 minutes prior to the stress exposure, half of the stressed and handled control rats received mifepristone (5 mg/100g bodyweight; Sigma) dissolved in 15 µl ethanol/1.5 ml coffee cream (Campina) and administered through an oral syringe directly into the stomach; the remaining animals received only cream. Sixteen hours after the final treatment, animals were perfused. As reported earlier for a larger cohort of animals to which the present rats belonged, chronic stress reduced body weight gain and absolute thymus weight, while adrenal size was increased (Karst and Joels, 2007), which are all indices of a history of chronic stress and hypercorticism (Selye, 1998). This was not normalized by the 4 day treatment with mifepristone.

**BrdU labelling**

To study survival of the newborn cells, bromodeoxyuridine (BrdU) was injected at 200 mg/kg on the first day of the chronic stress regimen, 3 hours after the end of the first stressor.

**Brain tissue processing**

Animals were anaesthetized in the morning by pentobarbital sodium salt (Nembutal, 1mg/kg bodyweight; A.U.V. Cuijk, The Netherlands) and then perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. To prevent pressure artefacts, brains were additionally postfixed
overnight in the skull at 4°C. 40 μm thick sections were cut using a sliding microtome and collected in PB with azide.

Immunohistochemistry
Different stages of the neurogenic process were studied as described previously (Heine et al., 2004b; Mayer et al., 2006). Immunohistochemistry for BrdU (murine anti-BrdU, Roche Diagnostics, Netherlands, 1: 2000) was used to assess cell survival, Ki-67 (polyclonal rabbit α-Ki-67 (Novocastra, New Castle, UK, 1:2000) to assess proliferation and doublecortin (DCX) (polyclonal goat α-DCX, Santa Cruz, 1:800) to assess neurogenesis. Amplification was done with avidin-biotin (ABC kit; Elite Vectastain, Brunschwig Chemie, Amsterdam, 1:1000) and biotinylated tyramide (1:500, kindly provided by Dr. I. Huitinga, Neth. Institute for Neuroscience, Amsterdam). Chronogen development was done with diaminobenzidine (DAB; 20 mg/100 ml TB, 0.01% H2O2).

Quantification
DCX+ and BrdU+ cells were quantified stereologically by systematic random sampling in every 10th section using the Stereoinvestigator system (Microbrightfield, Germany). Because of the occurrence of clusters with this marker, all individual Ki-67+ cells were counted in every 10th hippocampal section (Zeiss microscope 200x magnification) and multiplied by 10 to estimate the total number of Ki-67+ cells per hippocampus.

Statistics
Data are presented as mean ± SEM. Immunohistochemical data were compared using a one-way ANOVA. When significant, pair wise comparison was performed with a post-hoc LSD test.

Results
Effects of chronic stress and mifepristone treatment on neurogenesis
Patterns of BrdU+, Ki-67+ and DCX+ neurons in the dentate gyrus were similar to those described previously (Mayer et al., 2006). Thus, BrdU+ cells prevailed in the subgranular zone, but were also found in the hilar zone and, in considerably lower numbers, in the granular cell layer. Qualitative analysis revealed no obvious difference in the distribution or migration pattern of the BrdU-labelled cells into the granular cell layer and they were therefore pooled with those from the subgranular zone. Somata of the DCX+ cells were located mainly on the inner border of the granular cell layer. Clusters of Ki-67 positive cells were found almost exclusively in the subgranular zone. For each experimental condition, representative photomicrographs of BrdU (Figure 1A-D), DCX (Figure E-H) and Ki-67 (I-L) are shown.

Rats subjected twice daily to unpredictable stressors for 21 days (S) showed significantly reduced numbers of BrdU+ cells relative to control animals (C) (p<0.001) at one day after the last stress exposure (Figure 2). Treatment with
mifepristone (S+M) for only 4 days towards the end of the chronic stress paradigm fully normalized this reduction (Figure 2) to control levels. Interestingly, there was no effect of mifepristone alone (C+M) (ANOVA, F_{3,16} 10.93, p<0.001.

A similar pattern was observed for DCX+ new neurons (examples shown in Figure 1E-H). As shown in the graph in Figure 3, the number of DCX+ neurons was significantly reduced in the stress group (p= 0.03). Treatment with mifepristone during the last 4 days of the stress paradigm normalized the number of DCX+ neurons (figure 3). Mifepristone treatment alone had no effect (ANOVA, F_{3,17} 3.95, p =0.026; post hoc comparison S vs. C, p =0.008; S vs C+M p=0.042; S vs S+M p=0.009). Ki-67+ cell numbers were not different between the groups (group average ± SEM: C= 4476 ± 325; C+M= 4315 ± 429; S= 5240 ± 639; S+M = 5375 ± 413).
Figure 1. Immunohistochemistry. (A-D): BrdU+ cells (single arrow) are mainly located at the border of the granular cell layer (gel) and the hilus. (h). A. Control; B. Control + mifepristone; C. Stress; D. Stress + mifepristone. Stressed animals show less BrdU+ cells in the subgranular and granular layer (C) compared to controls (A). When treated with the GR-antagonist mifepristone (D), stressed animals show higher numbers of BrdU+ cells than in the untreated stressed group (C). Control animals treated with mifepristone (B) show no additional increase in BrdU+ cells. (E-H): Representative examples of doublecortin+ cells (single arrow) in the subgranular zone with processes extending through the granular cell layer (gel) into the molecular layer (double arrow). E. Control; F. Control + mifepristone; G. Stress; H. Stress + mifepristone. The arrow indicates a gap in doublecortin expression as was often found in the chronically stressed group. (G). A similar expression pattern can be found as in BrdU. The chronically stressed group (G), shows less DCX+ cells, which is normalized by mifepristone treatment (H). Mifepristone treatment in control animals (F) does not show an increase in cell number. (I-L): Representative examples of Ki-67+ cells (single arrow) in the subgranular zone in all 4 groups. I. Control; J. Control + mifepristone; K. Stress; L. Stress + mifepristone. Cells were mainly found in the subgranular zone (sgc).
Discussion

Stress is known to suppress neurogenesis in the dentate gyrus (Gould and Tanapat, 1999; Heine et al., 2004b; Mirescu and Gould, 2006). We presently found that the reduction in neurogenesis induced by 21 days of multiple, unpredictable stress is normalized already after 4 days of mifepristone treatment. The observation that the drug alone, i.e. in the absence of stress, did not affect neurogenesis, indicates that mifepristone is neurogenic specifically in a high corticosterone or stress environment. This differs from many
antidepressant drugs, dehydroepiandrosterone (DHEA) or brain-derived 
nootrophic factor (BDNF), which not only increase neurogenesis both under 
stress conditions but also in control animals (Malberg et al., 2000; Manev et al., 
2001; Karishma and Herbert, 2002; Scharfman et al., 2005). The specificity for 
a high stress environment may be due to mifepristone’s ability to directly bind 
and block GRs that are normally only fully occupied when circulating 
corticosterone levels are high. At low corticosteroid levels, GRs are not 
substantially occupied and blockage by mifepristone would then be less effective. 
An additional explanation could be that GR blockade may affect neurogenesis 
by promoting the function of the mineralocorticoid receptor (MR), which is 
implicated in tonic inhibitory control of the HPA axis and maintenance of 
neurogenesis (Gesing et al., 2001; Fischer et al., 2002; de Kloet et al., 2003). 
Interestingly, expression of the MR is induced following antidepressant 
treatment (Brady et al., 1991; Reul et al., 1993).

Although mifepristone administered during stress strongly affected 
survival (BrdU) and neurogenesis (DCX), no effects were found on proliferation 
(Ki-67). Similar dissociations between different stages of neurogenesis have been 
observed before, including when mifepristone was administered for 4 days to 
animals treated with a high dose of corticosterone during 21 days (Pham et al., 
2003; Nacher et al., 2004; Mayer et al., 2006; Thomas et al., 2006; Xu et al., 
2006). Many studies in rat have found reductions in proliferation after stress 
(Falconer and Galea, 2003; Malberg and Duman, 2003; Heine et al., 2004a; 
Hill et al., 2006) but also clear exceptions have been reported (Pham et al., 2003; 
Nacher et al., 2004; Thomas et al., 2006; Thomas et al., 2007). Furthermore, as 
we confirm here, not only raised corticosterone levels but also stress affects 
survival and neuronal differentiation of the newly-formed cells (Ambrogini et al., 
2002; Montaron et al., 2003; Wong and Herbert, 2004; Mayer et al., 2006; 
Wong and Herbert, 2006). In a related paradigm of chronic mild stress, cell 
proliferation was found to be reduced (Alonso et al., 2004; Jayatissa et al., 2006), 
whereas others used the same paradigm and found survival and not the 
proliferation phase to be reduced (Lee et al., 2006; Thomas et al., 2007).

One study found reductions in proliferation and survival of the newly 
generated cells after chronic psychosocial stress in rats (Czech et al., 2002). 
However, proliferation was studied using BrdU and not Ki-67 immunohistochemistry. 
Since Ki-67 detects all cells engaged in cell cycle and in fact reflects the effects of the last stressor only 16 hours later, this represents a 
different population than the one studied with the birth date marker BrdU (Kee 
et al., 2002). The stress paradigm used by (Czech et al., 2002) further consisted of 
daily exposure to social defeat, a psychosocial and uncontrollable, yet rather 
predictable stressor associated with strong increases in plasma corticosterone 
and ACTH levels. Our present design of mixed physical and psychosocial 
stressors applied twice daily in an unpredictable manner differs considerably 
from this study and is associated with moderate rises in basal corticosterone 
levels (Herman et al., 1995; van Gemert and Joels, 2006). Taken together, the
discrepancies in literature most likely result from differences in experimental design and the nature and timing of the stressor.

Alternatively, stress effects on the different stages of the neurogenic process are influenced by the dynamic GR-expression in adult generated cells yielding a variable sensitivity to the local corticoid environment (Garcia et al., 2004; Wong and Herbert, 2004, 2005). Proliferating cells in particular, have low GR-levels which may contribute to the observed lack of effect on proliferation, as also found by others (Nacher et al., 2004; Thomas et al., 2006). By contrast, survival of older cells - with high GR-levels - (Garcia et al., 2004), will be strongly influenced by stress and mifepristone as also shown by others (Garcia et al., 2004; Wong and Herbert, 2004, 2005; Montaron et al., 2003) in different experimental designs and not after chronic stress. Clearly, given the differences in controllability and predictability of the stressor and the differential sensitivity of the newborn cells to the local corticoid environment, it is difficult to compare stress effects on selective stages of the neurogenic process between studies as discussed in detail elsewhere (Lucassen et al., 2007).

Given the timing of mifepristone treatment, the changes in both BrdU and DCX indicate that mifepristone affected survival of cells from 17 days of age onwards. This suggests a “rescue” effect within the maturing neuronal population. Although many of the newly generated cells die between 6 and 28 days (Dayer et al., 2003), a recent paper on chronic corticosterone application reported that post-mitotic corticosterone levels regulate neuronal survival and differentiation. This action was most prominent from 19-27 days after the cells were born, suggesting the presence of a sensitive period (Wong and Herbert, 2006). Also, DCX+ cells without dendrites can still divide and expand four- to fivefold within a few days (Kempermann et al., 2004). Hence, the observed reversal back to control levels after mifepristone treatment around this time period could very well be accomplished after a few additional divisions of a significant portion of the young neuronal population starting from day 17 onwards. Whether the same holds for BrdU labelled cells, or whether indirect effects on e.g. the local environment surrounding the new cells are involved (Heine et al., 2005), requires additional stress experiments including the analysis of intermediate time points, which was beyond the scope of the present study. It is interesting to note here that the exact same mifepristone treatment regime also rapidly normalized various electrophysiological properties of hippocampal cells induced after chronic stress (Krugers et al., 2006; van Gemert and Joels, 2006; Karst and Joels, 2007).

The time frame we observed for mifepristone’s cellular effects on neurogenesis after chronic stress, parallels its effects in the clinic, where four days of treatment was reported to relieve symptoms of psychotic depression (Belanoff et al., 2001; Belanoff et al., 2002; Flores et al., 2006). Many antidepressants typically take 3-4 weeks to relieve depressive symptoms, which also matches their time-to-effect on neurogenesis. Our data are hence consistent with the assumption that medication for depression and/or psychotic depression share a similar feature in that they may act by normalizing a stress-induced
impairment of structural plasticity (Manji et al., 2003; Fuchs et al., 2004; Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006).

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