

Reduced hippocampal neurogenesis in the GR^{+/-} genetic mouse model of depression

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Abstract Glucocorticoid receptor (GR) heterozygous mice (GR^{+/-}) represent a valuable animal model for major depression. GR^{+/-} mice show a depression-related phenotype characterized by increased learned helplessness on the behavioral level and neuroendocrine alterations with hypothalamo-pituitary-adrenal (HPA) axis overdrive characteristic of depression. Hippocampal brain-derived neurotrophic factor (BDNF) levels have also been shown to be reduced in GR^{+/-} animals. Because adult hippocampal neurogenesis has been implicated in the pathophysiology of affective disorders, we studied here the effects of the GR^{+/-} genotype on neurogenesis in vivo. In a 2 × 2 design, GR^{+/-} mice and GR^{+/+} littermate controls were

either subjected to 1 h of restraint stress or left undisturbed in their home cages after intraperitoneal injection of BrdU. Stress exposure and BrdU injections were performed once daily for 7 days and neurogenesis analyzed 4 weeks later. BrdU cell counts were significantly reduced as an effect of GR^{+/-} genotype and as an effect of stress. Majority of the BrdU+ cells showed co-labeling with mature neuronal marker NeuN or astrocytic marker S100β with no further significant effect of either experimental condition or of genotype. In sum, this results in reduced neurogenesis in GR^{+/-} mice which is further repressed by restraint stress. Our results, thus, reinforce the link between reduced neurogenesis, stress, neurotrophins, and behavioral symptoms of and susceptibility to depression.

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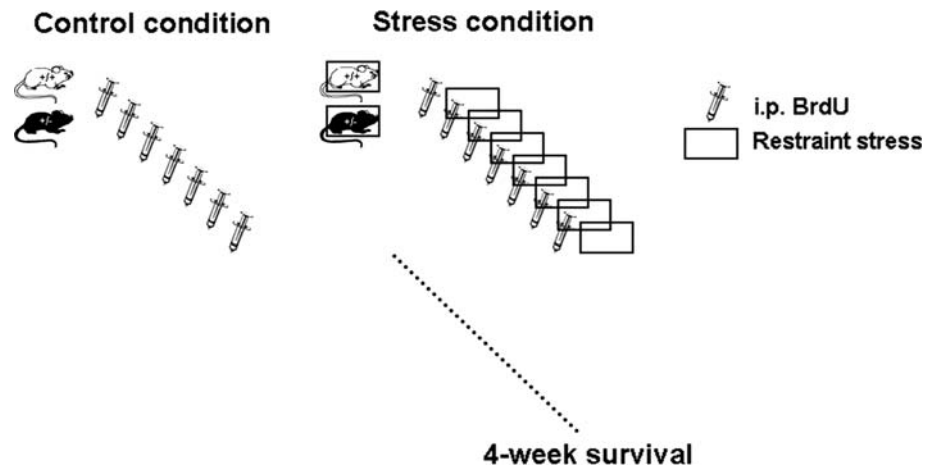
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Introduction

We have recently characterized a strain of glucocorticoid receptor (GR) heterozygous mice (GR^{+/-}) in which one GR allele has been ablated by a conventional knockout strategy, resulting in an approximately 50% reduction of GR protein levels in the brain [20]. These animals represent a valuable model for an endophenotype of severe major depression characterized by a disinhibited HPA-system due to altered GR function [11]. Similar to these severely depressed patients, GR^{+/-} mice demonstrate a non-suppression of corticosterone plasma levels to dexamethasone and a pathological combined DEX/CRH test. Furthermore, GR^{+/-} mice exhibit an altered neuroendocrine stress response with increased and prolonged corticosterone release after a stressor. Behaviorally, GR^{+/-} mice show increased learned

Fig. 1 Experimental paradigm: description of experimental procedures is given in “Materials and methods”. $N = 9$ –10 animals per group and condition



helplessness, a well-established analog of depression-like behavior in mice [4, 20].

As a molecular correlate—apart from reduced GR expression—for this ‘depressive’ phenotype we previously identified downregulation of BDNF at the protein level in hippocampus of $GR^{+/-}$ mice [20]. Compromised adult neurogenesis has also been implicated in the pathophysiology of hippocampal aspects of mood disorders [12]. Importantly, the ‘neurotrophin hypothesis’ of depression fits well with current concepts emphasizing the negative impact of stress on hippocampal structure [7]. Stress robustly impairs progenitor proliferation and neogenesis of granule neurons in the adult hippocampal dentate gyrus [5, 8, 9, 17]. Similarly, impaired BDNF signaling has recently been shown to result in impaired neurogenesis and refractory responses to antidepressants [15]. Deletion of the BDNF receptor *trkB* in adult progenitors also alters newborn neuron integration into hippocampal circuits and increases anxiety-like behaviors [1].

Here, we investigated net hippocampal neurogenesis in $GR^{+/-}$ mice either under control conditions or following repeated restraint stress. We speculated that neurogenesis may be compromised in this strain due to its depressive phenotype and concurrent BDNF downregulation.

Materials and methods

Animals and treatments

The generation of GR-heterozygous mice ($GR^{+/-}$) has been described in detail previously [20]. Briefly, the founders of $GR^{+/-}$ mice were developed by using homologous recombination in embryonic stem cells as described earlier [23]. $GR^{+/-}$ mice were generated by crossing heterozygous C57BL/6N males (backcrossed for >10 generations) with wild-type FVB/N females in order

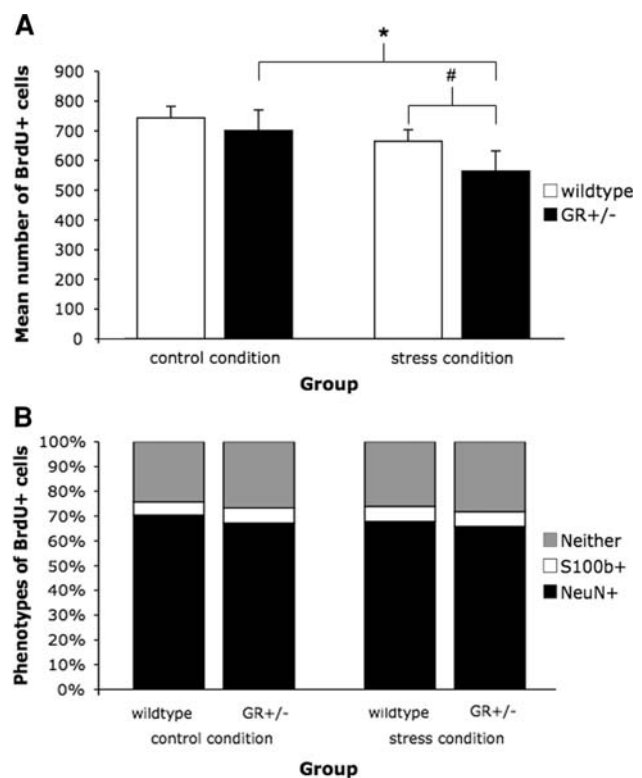
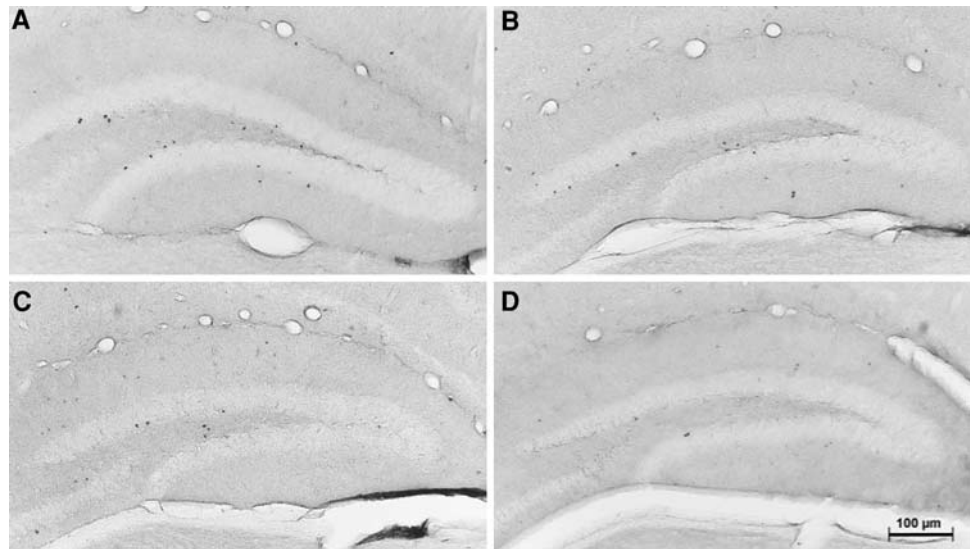


Fig. 2 Quantitative assessment of net neurogenesis. **a** The number of BrdU+ cells was analyzed 4 weeks after a 7-day series of once-daily BrdU injections. Two-way ANOVA yielded a significant effect of factor ‘treatment condition’ (control versus stress condition: $F_{1,33} = 10.2$, $p = 0.003$) and a significant effect of factor genotype ($F_{1,33} = 4.5$, $p = 0.04$) on BrdU cell counts with no significant interaction between factors (genotype \times treatment interaction: $F_{1,33} = 0.8$, $p = 0.39$). * $p < 0.05$ for the effect of stress within $GR^{+/-}$ genotype, # $p < 0.05$ for the effect of genotype within the ‘stress’ groups. **b** Phenotypic analysis of BrdU+ cells did not reveal differences in neuronal (NeuN) or astrocytic (S100 β) marker expression among experimental groups

to obtain F1 hybrid mice with exactly the same background as previously used for behavioral and neurochemical characterization studies [20].

Fig. 3 BrdU+ cells in the dentate gyrus are reduced as an effect of $GR^{+/-}$ genotype and as an effect of stress. The four images show BrdU-stained cells (DAB) in representative hippocampal sections of animals from the respective experimental groups. **a** $GR^{+/+}$ mice in the control condition. **b** $GR^{+/+}$ mice in the stress condition. **c** $GR^{+/-}$ mice in the control condition. **d** $GR^{+/-}$ mice in the stress condition



Male $GR^{+/-}$ mice and wild-type littermate controls were 4–6 months old at the beginning of experiments. Mice were housed individually in standard laboratory cages under a reversed 12 h:12 h light/dark cycle, with ad libitum access to food and water. All experiments were performed according to national and institutional guidelines and were approved by German animal welfare authorities. Thymidine analog BrdU (5-Bromo-2'-deoxyuridine; Sigma-Aldrich, Germany) was administered intraperitoneally at a concentration of 50 mg/kg body weight once daily. Restraint stress consisted of placing a mouse inside a plastic tube (inner diameter: 26 mm) for 1 h during the dark phase.

The experiment was structured in a 2×2 design (Fig. 1). Briefly, all animals received daily BrdU injections for a period of 7 days. Additionally, two groups were subjected to 1 h of restraint stress after BrdU injections. All animals were killed after an interval of 4 weeks. All experiments were performed according to the 'Principles of laboratory animal care' (NIH publication No. 86-23, revised 1985) and conformed to national and institutional guidelines.

Immunohistochemistry

Mice were deeply anesthetized with ketamine and transcardially perfused with 0.9% sodium chloride followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were dissected from the skulls, postfixed in 4% PFA at 4 °C over night, and transferred into 30% sucrose for dehydration. Brains were cut on a sliding microtome (Leica, Bensheim, Germany) in the coronal plane in 40- μ m-thick sections and cryoprotected. Sections were stained free floating with all antibodies diluted in Tris-buffered saline containing 3% donkey serum and 0.1% Triton X-100. For

BrdU staining, DNA was denatured in 2 N HCl for 30 min at 37 °C. Sections were then rinsed in 0.1 M borate buffer and washed in Tris-buffered saline (TBS). Primary and secondary antibodies were diluted in TBS containing 0.1% Triton X-100 and 3% donkey serum (TBS+). Primary antibodies were applied in the following concentrations: anti-BrdU (rat, 1:500; Harlan Seralab, Indianapolis, IN), anti-NeuN (mouse, 1:100; Chemicon, Temecula, CA), anti-S100 β (rabbit, 1:2500; Swant, Bellinzona, Switzerland). Immunohistochemistry followed the peroxidase method with biotinylated secondary antibody (Jackson Immuno-Research Laboratories, West Grove, PA), ABC Elite reagent (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB; Sigma) as chromogen. For immunofluorescence FITC-, RhodRedX- or Cy5-conjugated secondary antibodies were all used at a concentration of 1:250. Fluorescent sections were coverslipped in polyvinyl alcohol with diazabicyclooctane (DABCO) as an anti-fading agent.

Quantification and imaging

Cell counts were determined in one-in-six series of sections covering the entire hippocampus in its rostro-caudal extension as described previously [13, 14]. Briefly, cells located in the granule cell layer and adjacent subgranular zone, defined as a two-cell body-wide zone of the hilus along the base of the granule cell layer were counted [2]. Cells in the uppermost focal plane were excluded to avoid oversampling. Phenotypic analysis of BrdU-labeled cells was performed using a spectral confocal microscope (TCS SP2; Leica, Nussloch, Germany). Appropriate gain and black level settings were determined on control slices stained with secondary antibodies alone.

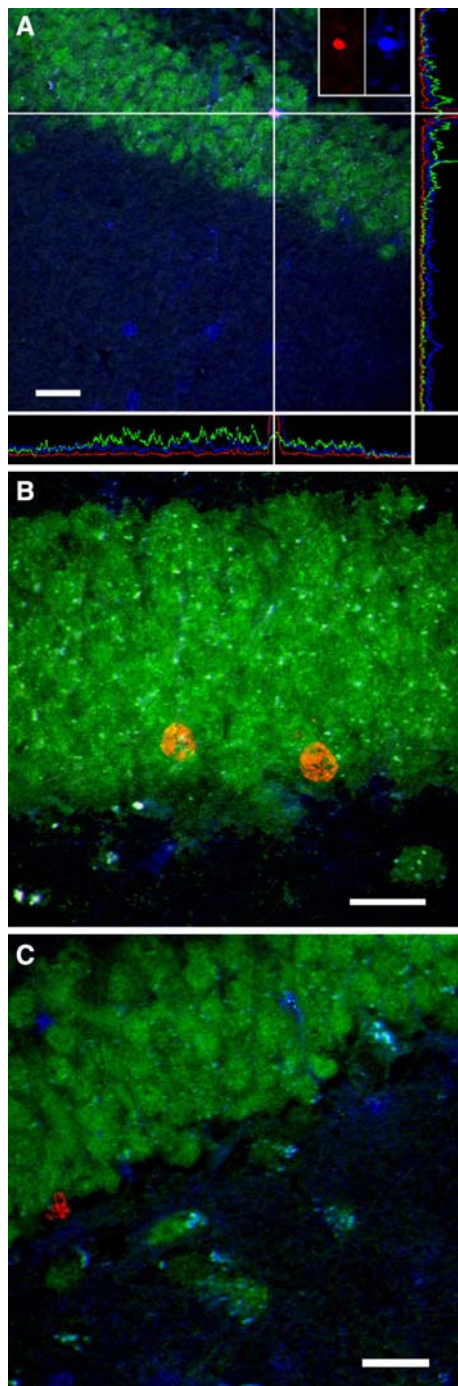


Fig. 4 Phenotypic analysis **a** Confocal image of a newly generated astrocyte in the granule cell layer expressing mature astrocytic marker S100 β (blue) and BrdU (red). Emission intensities measured along the lines drawn in the larger image (**a**) are shown in the *bottom* and *right* panels. Furthermore, insets in **a** show the cell investigated in single channels with separate wavelengths. **b** Newly generated neuron labeled with BrdU (red) and expressing NeuN-immunoreactivity (green). **c** BrdU-positive cell (red) expressing neither neuronal marker NeuN (green) nor astrocytic marker S100 β (blue). Scale bars in **a**, **b**, and **c** 25 μ m

Statistical analyses

All numerical analyses were performed using Statview 5.0.1. for Macintosh. Two-way ANOVA was followed by Fisher's post hoc test, where appropriate. All values are given as mean \pm standard error of the mean (SEM). *P*-values of <0.05 were considered statistically significant.

Results

Net neurogenesis in the hippocampus was analyzed 4 weeks after a 7-day course of single daily intraperitoneal injections of BrdU. In this particular injection paradigm, BrdU counts reflect a combination of initial proliferation and subsequent survival of newly generated cells. The experimental set-up is illustrated in Fig. 1. Briefly, GR $^{+/-}$ mice and GR $^{+/+}$ littermate controls were subjected either to the control condition (undisturbed in home cage) or to the stress condition (1 h of restraint stress directly after intraperitoneal injection of BrdU).

Two-way ANOVA revealed that BrdU cell counts were significantly reduced as an effect of stress and as an effect of GR $^{+/-}$ genotype (detailed results of the ANOVA statistics are presented in Figs. 2a and 3; analysis of 37 animals in total, $n = 9$ –10 animals/group). BrdU-labeled cells were subjected to further phenotypic analysis. The majority of cells showed co-labeling with mature neuronal marker NeuN or astrocytic marker S100 β (Figs. 2b and 4) with no further significant effect of either experimental condition or of genotype.

Discussion

Here we show that depression-prone GR $^{+/-}$ mice exhibit a significant net reduction of adult hippocampal neurogenesis. GR $^{+/-}$ mice represent a particularly valuable animal model of depression because they reflect HPA system dysregulation which is a hallmark of a subgroup of depressive disorders in humans [3]. Our observation is in line with the concept that neurogenesis has a role in the etiopathogenesis of mood disorders and contributes to the therapeutic actions of antidepressants [6, 16, 22]; but see also [10, 19, 24].

A number of current studies have provided novel insights into the link between depression-related behaviors and altered neurogenesis. In particular, recent work has shown that 'learned safety', as opposed to learned helplessness is associated with increased survival of new neurons and increased BDNF levels in hippocampus and that

conversely, ablation of neurogenesis retards safety learning [18]. Several other recent studies also demonstrate a link between BDNF signaling, neurogenesis, and behavior [1, 15, 21]. In particular, ablation of BDNF receptor *trkB* in neural precursor cells results in impaired neurogenesis. When exposed to chronic antidepressant treatment or wheel-running, no increase in neurogenesis has been observed in these mice [15]. Furthermore, ablation of *trkB* also renders the animals behaviorally insensitive to antidepressive treatment in depression- and anxiety-like paradigms [15]. Lack of *trkB* in adult progenitors also results in disturbed organization of basic synaptic connections of newly generated neurons and impaired neurogenesis-dependent long-term potentiation, accompanied by compromised survival of newly generated cells and increased anxiety-like behaviors [1]. Reduced net hippocampal neurogenesis in $GR^{+/-}$ mice as reported here fits well with these reports. Importantly, we have previously demonstrated a significant reduction of BDNF protein concentrations in hippocampus of $GR^{+/-}$ mice [20]. However, it should be noted that the reduction in neurogenesis in $GR^{+/-}$ mice which emerged in this comparatively large experiment was only moderate. This may support the notion that in addition to reduced neurogenesis, other mechanisms may also contribute prominently to the depression-related behavioral phenotype of $GR^{+/-}$ mice [6].

In summary, we here demonstrate reduced net adult hippocampal neurogenesis in $GR^{+/-}$ mice, a novel genetic mouse model of affective disorders. Our study provides further correlative evidence for a link between hippocampal neurogenesis, glucocorticoids, BDNF, and depression.

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