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Corticosterone-Induced Inhibition of Gliogenesis in Rat Hippocampus is Counteracted by Electroconvulsive Seizures

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Background: Volumetric changes and glial pathology have been reported in the central nervous system (CNS) of patients with depressive disorder, an illness often associated with elevated glucocorticoid levels. Glucocorticoids reduce gliogenesis in the adult rat CNS. Electroconvulsive seizure (ECS)-treatment, an animal model for the antidepressant treatment electroconvulsive therapy, can enhance proliferation of glial cells. This study examined glial cell proliferation in response to ECS in rats whose glucocorticoid levels were elevated to mimic the conditions seen in depression.

Methods: Rats were injected daily for seven days with either corticosterone or vehicle. ECS- or sham- treatment was given once daily during the first five days. Proliferating cells in the hippocampus were labeled with bromodeoxyuridine and analyzed for co-labeling with the glial cell markers NG2, O_x42, S-100 β and Rip.

Results: ECS counteracted the glucocorticoid-induced inhibition of NG2⁺, O_x42⁺ and Rip⁺ cell proliferation, and the gliogenesis rate was restored to baseline levels. Volumetric changes in rats treated with ECS were detected.

Conclusions: Our results show that ECS-treatment affects the proliferation of glial cells even in the presence of elevated levels of glucocorticoids. This result adds to an increasing number of studies suggesting that antidepressant treatment can counteract degenerative processes associated with major depression.

Key Words: NG2, electroconvulsive seizure, corticosterone, hippocampus, gliogenesis, oligodendrocyte

Several studies show that hippocampal volume is reduced in patients with major depression, compared with healthy controls (for review see Sheline et al 2003). Hippocampal volume is negatively correlated with the total duration of depression (Sheline et al 1999) and with depression-associated cognitive deficits such as memory impairment (Sheline et al 1996; MacQueen et al 2003). Although volume reduction can persist for decades following depressive episodes (Sheline et al 1996; Sheline et al 1999; Bremner et al 2000), a recent study demonstrates that it can be prevented by antidepressant treatment (Sheline et al 2003).

The precise mechanisms by which depression affects hippocampal volume are unknown, but several clinical studies suggest that the glucocorticoid cortisol may be involved. Up to 50% of depressed patients show elevated levels of circulating cortisol, which can be restored to normal levels with antidepressant treatment (Carroll et al 1976; Dinan 2001). Patients with Cushing's disease (an illness associated with hypercortisolemia) show high rates of depression (Sonino and Fava 2002) and hippocampal atrophy (Starkman et al 1992). Additionally, treatment with cortisol (Brown et al 2004) can lead to depression, and some antiglucocorticoid therapies have antidepressant-like properties (for review see Murphy 1997).

Similar findings have been observed in animal studies. Rats exposed to corticosterone (the cortisol homologue in rat) show depressive behavior (Gregus et al 2005) and significantly reduced hippocampal volume (Sousa et al 1998). Analogously psychosocial stress, which elevates cortisol levels, caused a

nonsignificant trend towards reduced hippocampal volume in tree shrews (Czeh et al 2001). This was reversed by the antidepressant tianeptine (Czeh et al 2001).

The mechanisms behind cortisol-induced hippocampal volume reductions are poorly understood. Previous studies focused on the impact of stress on neurons. It was shown that corticosterone-treatment and psychosocial stress down-regulate the neuronal proliferation known to occur in the dentate gyrus of adult rat hippocampus (Cameron and Gould 1994; Czeh et al 2001; McEwan 1999; Hellsten et al 2002; Malberg and Duman 2003), and that antidepressant drugs could counteract stress-mediated inhibition of neurogenesis (Malberg and Duman 2003; Czeh et al 2001). However, in postmortem studies of patients with a history of affective disorder, or high-dose steroid treatment, no reduction in the number of neurons could be demonstrated (Lucassen et al 2001; Muller et al 2001). This indicates that the tissue atrophy cannot be explained entirely by a stress-induced down-regulation of neurogenesis.

Interestingly, recent postmortem studies of depressed patients reveal a reduction in the number of glial cells in the amygdala and prefrontal cortex (Rajkowska 2000; Bowley et al 2002). These glial cells were identified as oligodendrocytes (Hamidi et al 2004; Uranova et al 2004). It is thus tempting to speculate that the reduction in hippocampal volume seen in depressed patients (Starkman et al 1992; Sheline et al 1996) may partly be due to glial cell loss.

We previously showed that corticosterone-induced inhibition of hippocampal neurogenesis in adult rats can be reversed by electroconvulsive seizure (ECS)-treatment, an animal model for the antidepressant treatment electroconvulsive therapy (ECT) (Hellsten et al 2002). We found that ECS-treatment, apart from inducing neurogenesis, stimulate proliferation of other cell types in rat hippocampus and amygdala. Many of these cells were identified as chondroitin sulphate proteoglycan NG2 (NG2)-expressing glial cells (Wennström et al 2003, 2004). Interestingly, a previous study showed that proliferation of NG2⁺ cells is also down-regulated by corticosterone (Alonso 2000).

NG2⁺ cells make up about 7.5% of all glia in the adult brain (Butt et al 1999); they are antigenically distinct from

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neurons, astrocytes, resting microglia and mature oligodendrocytes (for a review see Nishiyama et al 1999). As some NG2+ cells still have the capacity to divide, and many of them express markers for immature oligodendrocytes such as platelet-derived growth factor receptor alpha (PDGFR-alpha), and O-antigen 4 (O4) (Levine et al 1993; Nishiyama et al 1996; Reynolds and Hardy 1997), it has been suggested that these cells are oligodendrocyte progenitors. Another indication of a progenitor role is the finding that NG2+ cells differentiate into oligodendrocytes in vitro (Levine et al 1993). Because of the limited temporal overlap in the expression of progenitor and mature antigens, this has not been directly demonstrated in vivo. However, recent studies have shown that the promoters for 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Belachew et al 2001), and proteolipid protein (PLP) (Mallon et al 2002), both active in mature oligodendrocytes, are expressed in NG2+ cells. This evidence supports the view that these cells share a lineage relationship with oligodendrocytes.

The observation that oligodendrocyte numbers are reduced in patients with a history of major depression together with our previous results showing increased proliferation of putative oligodendrocyte progenitors after ECS-treatment prompted us to investigate whether ECS-treatment could counteract the reduction in the newly formed NG2-expressing oligodendrocyte progenitors, that is known to occur in corticosterone-treated rats. We furthermore measured the effects of corticosterone- and ECS-treatment on hippocampal volume.

Methods and Materials

Animals and Experimental Design

Adult male Wistar rats (Møllegaard breeding centre, Denmark), weighing 200 g at the beginning of the study, were used. Three rats were housed in each cage and kept on a 12 hour light–dark cycle with access to food and water ad libitum. Experimental procedures

were carried out according to the guidelines set by the Malmö-Lund ethical committee for the use and care of laboratory animals. To assess glial proliferation after corticosterone- and ECS-treatment, a short (S) and long (L) survival study was conducted. The rats (S: n = 23) (L: n = 24) were divided into following groups: (i) seven days of corticosterone injections and five ECS-trials (S: n = 6) and (L: n = 6); (ii) seven days of corticosterone injections and five sham-trials (S: n = 6) and (L: n = 6), (iii) five ECS-trials (S: n = 5) and (L: n = 6); (iv) seven days of vehicle and five sham trials (S: n = 6) and (L: n = 6) (Figure 1). The rats were weighed every third day in order to monitor the metabolic effects of corticosterone.

Administration of Electroconvulsive Seizures

For the first five days of the proliferation study, all rats were subjected once daily (10:00 am), to either a single ECS trial or sham treatment. Electric current was delivered via silver electrode ear clips (Somedic Sales AB, Sweden) (50 mA, .5 sec, 50 Hz unidirectional square wave pulses) (Figure 1). The rats were monitored after the ECS-treatment to ensure that clonic movements of the face and forelimbs occurred for 20–30 sec (indicative of limbic seizures). Control rats were sham-treated, that is, handled identically to the ECS-treated rats except that no current was passed.

Administration of Corticosterone

A stock emulsion of corticosterone (C2505; Sigma-Aldrich, St. Louis, Missouri) (33.3 mg/ml), was prepared daily by vortexing in sesame oil (Sigma-Aldrich) for 10 min, followed by 60 min of sonication. Before every injection, the emulsion was vortexed briefly and administered as subcutaneous injections in the neck (40 mg/kg) every 24 hours (9:00 am). This dose is adequate to elevate blood levels of corticosterone over a 24-hour period (Sapolsky et al 1985). Control rats received vehicle injections (sesame oil).

Proliferation study with short survival

	D0	D1	D2	D3	D4	D5	D6	D7
Group EC	E+C	E+C	E+C+BrdU	E+C+BrdU	E+C+BrdU	C+BrdU	C+BrdU	†
Group SC	S+C	S+C	S+C+BrdU	S+C+BrdU	S+C+BrdU	C+BrdU	C+BrdU	†
Group EV	E+V	E+V	E+V+BrdU	E+V+BrdU	E+V+BrdU	V+BrdU	V+BrdU	†
Group SV	S+V	S+V	S+V+BrdU	S+V+BrdU	S+V+BrdU	V+BrdU	V+BrdU	†

Proliferation study with long survival

	D0	D1	D2	D3	D4	D5	D6	D7	////		D27	D28
Group EC	E+C	E+C	E+C+BrdU	E+C+BrdU	E+C+BrdU	C+BrdU	C+BrdU		////			†
Group SC	S+C	S+C	S+C+BrdU	S+C+BrdU	S+C+BrdU	C+BrdU	C+BrdU		////			†
Group EV	E+V	E+V	E+V+BrdU	E+V+BrdU	E+V+BrdU	V+BrdU	V+BrdU		////			†
Group SV	S+V	S+V	S+V+BrdU	S+V+BrdU	S+V+BrdU	V+BrdU	V+BrdU		////			†

Figure 1. Experimental design and group assignment. Rats were divided into four treatment groups: electroconvulsive seizures and corticosterone (EC); sham and corticosterone (SC); Electroconvulsive seizures and vehicle (EV); and sham and vehicle (SV). Rats in groups EC and EV were subjected to a single electroconvulsive seizure-treatment (E) once daily from day 0 to day 4. Rats in groups EC and SC were subjected to one corticosterone injection daily (C) from day 0 to the day 6. Sham (S) and vehicle (V) animals were otherwise treated identically as those receiving ECS and corticosterone treatments, respectively. All rats received bromodeoxyuridine (BrdU) injections twice daily from day 2 to day 6, and were transcardially perfused 12 hours or 21 days after the last BrdU injection.

Administration of BrdU

The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (B5002; Sigma-Aldrich) was used as proliferation marker. Others have shown that this method detects cell proliferation rather than DNA repair (Palmer et al 2000; Cooper-Kuhn and Kuhn 2002). BrdU was dissolved in phosphate buffered saline and administered intraperitoneally. All rats in the study received 10 injections of BrdU (50 mg/kg) at 12-hour intervals (8:00 am and 8:00 pm), from day 2 to day 6 following the initial ECS treatment (Figure 1).

Tissue Preparation

Twelve hours (S) or three weeks (L) after the last BrdU-injection, rats were anesthetized with sodium pentobarbital (60 mg/ml). After the disappearance of nociceptive reflexes, rats were transcardially perfused with 100 ml .9% saline, followed by 250 ml 4% ice-cold paraformaldehyde. Following decapitation, the brain was removed from the skull and postfixed in 4% paraformaldehyde overnight at 4°C. The adrenal glands were dissected and weighed to assess the degree of atrophy as a measurement of the efficacy of the corticosterone treatment. The brains were left in 30% sucrose in PBS until they sank, and were then sectioned on a freezing microtome. Forty-micrometer thick coronal sections were cut, between 3.10 mm to 4.52 mm relative to the bregma (Paxinos and Watson 1986), and stored in cryoprotectant solution at -20°C until stained.

Antibodies

The following primary antibodies were used: rabbit anti-NG2 (1:500; gift from Dr. Bill Stallcup, The Burnham Institute, La Jolla, California) directed against the chondroitin sulfate proteoglycan NG2 expressed on glial progenitor cells (Stallcup and Beasley 1987), mouse anti-CD11b (Ox42) which recognizes microglia (Milligan et al 1991), rabbit anti-S100 β directed against astrocyte cellbodies (Boyes et al 1986), mouse anti-Rip (1:1000; Hybridoma Bank, Iowa City, Iowa) against myelin basic protein expressed by mature oligodendrocytes (Friedman et al 1989), rat anti-BrdU (1:100 Oxford Biotechnology, OBT 0030, Kidlington, United Kingdom) against bromodeoxyuridine and mouse anti-Ki67 (Novocastra, Newcastle, United Kingdom; cat no. NCL-Ki67-MM1) directed against a nuclear antigen expressed in all proliferating cells during late gap 1 (G1), DNA-synthesis (S), mitosis (M) and gap 2 (G2) phase of the cell cycle.

Immunohistochemistry

In order to analyze cell proliferation with Ki67 staining, sections were rinsed (3 \times 10 min) in .02 M potassium phosphate-buffered saline (KPBS) following exposure to blocking solution (KPBS + 5% normal goat serum (NGS) (S26 Chemicon, Temecula, California) + .25% Triton X-100) for one hour at room temperature. The sections were then incubated with mouse anti-Ki67 in blocking solution for 40 hours in darkness at 4°C and then rinsed in KPBS + .25% Triton X-100 (KPBS+) (3 \times 10 min) before incubation with Cy-3 conjugated goat anti-mouse 1:200 (Jackson, 115 165 003 Jackson Immuno Research, West Grove, Pennsylvania) in blocking solution for 24 hours in darkness at 4°C. After rinsing in KPBS (3 \times 10 min), sections were mounted on poly-L-lysine coated slides, air-dried, rinsed briefly in H₂O and cover-slipped with glycerol-based mounting medium.

For double immunofluorescence staining, sections were rinsed (3 \times 10 min) in KPBS and then incubated in blocking solution (KPBS + 5% blocking serum [NGS when staining for NG2 or S-100 β and normal horse serum (NHS) (Sigma Aldrich, St. Louis, Missouri) when staining for Rip or Ox42] + .25% Triton

X-100) for one hour at room temperature. Sections were then incubated with primary antibody in blocking solution for 24 hours at 4°C. Following this treatment, the sections were rinsed (3 \times 10 min) with KPBS + and subsequently incubated in blocking solution for 24 hours at 4°C, either with biotinylated goat anti-rabbit 1:200 (Vector BA-1000, Vector Laboratories Inc., Burlingame, California) as secondary antibody to detect NG2 or S-100 β , or with biotinylated horse anti-mouse 1:200 (Vector BA-2001, Vector Laboratories Inc.) as secondary antibody to detect Rip or Ox42. Sections were then rinsed (3 \times 10 min) in KPBS+ before incubation with Alexa 488 1:200 (Molecular Probes, Eugene, Oregon) in KPBS+ for 24 hours in darkness, at 4°C. After a KPBS wash (3 \times 10 min), the sections were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed (3 \times 10 min) in KPBS and then incubated in 1 M HCl at 65°C for 30 min. After further rinses in KPBS (3 \times 10 min), the sections were exposed to blocking solution (KPBS+ and 5% normal donkey serum (NDS) (Harlan Sera-Lab, Belton, United Kingdom)) for one hour in darkness at room temperature and then incubated with rat anti-BrdU in blocking solution for 40 hours in darkness at 4°C. The sections were then rinsed in KPBS+ (2 \times 10 min), and KPBS+, 2% NDS (1 \times 10 min) before incubation with Cy-3-conjugated donkey-anti-rat 1:200 (Jackson 712-165-153, Jackson Immuno Research, West Grove, Pennsylvania) in blocking solution for 24 hours in darkness at 4°C. After being rinsed in KPBS (3 \times 10 min), the sections were mounted as described above.

Data Quantification and Statistical Analysis

Coronal sections through the mid-dorsal hippocampus (-3.10 mm to -4.52 mm relative to the bregma) (Paxinos and Watson 1986) were analyzed by observers blind to the treatment conditions. Cell proliferation was assessed in the molecular layer (ML), the granule cell layer (GCL) and hilus of the dentate gyrus, with an Olympus AX70 fluorescence microscope fitted with a 40 \times objective. BrdU+ and Ki67+ cells were counted in the different dentate gyrus sub-regions. Every sixth section throughout the mid-dorsal hippocampus (six sections from each animal) was counted, and the values were averaged and expressed as mean cell number section and sub-region. The chondroitin sulfate proteoglycan NG2 is expressed on various cell-types, such as endothelial cells, monocytes, pericytes and oligodendrocyte progenitors. In this study, however, we only counted cells with the specific morphology previously attributed to oligodendrocyte progenitors (Levine et al 1993; Stallcup and Beasley 1987). Total cell numbers were determined using regular fluorescence microscopy. To confirm double staining for BrdU and glial markers, a Leica TCS SL, Spectral Confocal Microscope (Leica Microsystems, Mannheim, Germany), with a 100x oil immersion lens objective and Leica Confocal Software, version 2.61 (Leica Microsystems, Mannheim, Germany) was used. Cells were evaluated in z-series with a minimum of six consecutive optical sections (Figure 6). Data are presented as means \pm SEM and were analyzed with one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) post-hoc test. Statistical significance was set at $p \leq .05$.

Volume Measurement

The volume of the sub-regions of the dentate gyrus was estimated using the Cavalieri principle. The ML, GCL and hilus on every second section from each animal were delineated, using C.A.S.T.-GRID software (Olympus, Albertslund, Denmark) and an Olympus BH-2 microscope with a 10 \times objective and a

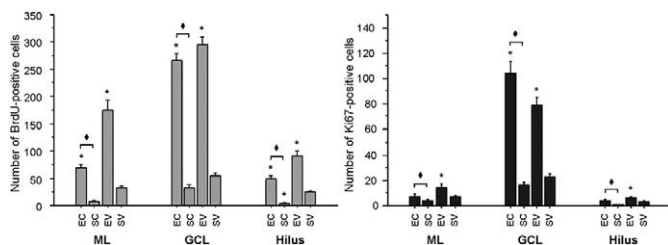


Figure 2. Bromodeoxyuridine (BrdU)+ and Ki67+ cells in the dentate gyrus of hippocampus. Sham/corticosterone-treatment (SC) significantly decreased the number of BrdU+ cells in the hilus of dentate gyrus. Electroconvulsive seizure/vehicle-treatment (EV) significantly increased the number of BrdU+ and Ki67+ cells in all dentate subregions compared to sham/vehicle-treatment (SV), and electroconvulsive seizure/corticosterone-treatment (EC) significantly increased the number of Ki67+ and BrdU+ cells compared to SC and SV. Asterisks indicate significant differences from controls. Diamonds indicate significant differences from SC. Data are presented as means \pm SEM as analyzed with analysis of variance (ANOVA) and Fischer's PLSD tests. Statistical significance was accepted at $p \leq .05$.

CCD-IRIS color video camera. Values of the cross-sectional areas of these regions were obtained. Total volume of the region of interest was calculated as the mean cross-sectional area multiplied by the length of the region sectioned (1.4 mm).

Results

Corticosterone Decreases the Number of BrdU+ Cells in Dentate Gyrus

Rats given sham/corticosterone-treatment had significantly lower number of bromodeoxyuridine positive (BrdU+) cells in the hilus ($p = .004$) of the dentate gyrus compared to sham/vehicle-treated animals (Figure 2). In a two-group comparison (unpaired t -test) between sham/corticosterone-treated and sham/vehicle-treated groups the reduced number of BrdU+ cells was also significant in the molecular layer (ML) ($p = .003$).

ECS Counteracts Corticosterone-Mediated Reduction in BrdU+ Cell Number

Five electroconvulsive seizures (ECS) significantly increased the number of BrdU+ cells in vehicle-injected rats compared to sham/vehicle-treated rats. The increased cell proliferation occurred in all dentate gyrus subfields (ML ($p < .0001$); GCL ($p < .0001$); hilus ($p < .0001$)). ECS/corticosterone-treated rats showed a significant increase in the number of BrdU+ cells compared to rats that were sham/corticosterone-treated. In the granule cell layer, ECS-treatment completely abolished the inhibitory effect of corticosterone and also in ML and hilus the number of BrdU+ cells was significantly higher compared to sham/vehicle-treated rats (ML ($p = .009$); GCL ($p < .0001$); hilus ($p < .0001$)) (Figure 2).

BrdU+ Cell Numbers Correlate with Number of Cells Positive for Ki67

To determine whether the changes in BrdU+ cell numbers seen in ECS and/or corticosterone-treated rats were caused by a direct effect on proliferation, we stained tissue sections with antibodies directed against the endogenous proliferation antigen Ki67. Since this antigen is expressed only during cell division, the Ki67-staining detects only cells proliferating at the time of perfusion. The number of Ki67+ cells in sham/corticosterone-treated rats decreased in all dentate gyrus subregions. This result was only significant when the sham/corticosterone-treated group and the sham/vehicle-treated group were analyzed with un-

paired t -test (ML ($p = .036$); GCL ($p = .058$); hilus ($p = .024$)) and not when analyzed with multiple comparisons (ML ($p = .157$); GCL ($p = .367$); hilus ($p = .216$)) (Figure 2). ECS/vehicle-treatment significantly increased the number of Ki67+ cells in all subregions (ML ($p = .004$); GCL ($p < .001$); hilus ($p = .041$)). ECS-treatment also counteracted the corticosterone-induced inhibition of cell proliferation (ML ($p = .1318$); GCL ($p < .001$); hilus ($p = .059$)) (Figure 2).

Corticosterone Mediated Reduction of NG2/BrdU+ Cell Numbers is Counteracted by ECS-Treatment

In agreement with a previous report (Alonso 2000), sham/corticosterone-treatment significantly reduced the number of NG2+/BrdU+ cells in the ML ($p = .044$) and the hilus of the dentate gyrus ($p = .039$), compared with sham/vehicle-treated rats. The number of NG2+/BrdU+ cells in the GCL was not significantly reduced (Figure 3). ECS/vehicle-treatment significantly increased the number of NG2+/BrdU+ cells compared with sham/vehicle-treated rats. The increased proliferation occurred in all dentate gyrus sub-regions ($p < .0001$) (Figure 3). Furthermore, ECS-treatment counteracted the inhibitory effect of corticosterone on the NG2+ cell proliferation in the ML and hilus. The number of NG2+/BrdU+ cells was significantly increased compared with both sham/corticosterone-treated rats (ML ($p = .0001$); hilus ($p = .0002$)) and sham/vehicle-treated rats (ML ($p = .013$); hilus ($p = .020$)) (Figure 3). All NG2+ cells had small cell bodies and extensive processes, indicating a nonreactive state (Ong and Levine 1999).

Corticosterone and ECS Treatment Affect the Size of the NG2+ Cell Population

The total number of NG2+ cells decreased significantly after sham/corticosterone treatment in the ML ($p < .0001$) and hilus ($p = .0013$) of the dentate gyrus compared with sham/vehicle-treatment. ECS/vehicle-treatment increased the total NG2+ cell population in the ML ($p = .0001$), in the GCL ($p = .0483$) and in the hilus ($p = .0001$). The reduced numbers of NG2+ cells in the ML and hilus of rats treated with corticosterone was counteracted by ECS-treatment, and the population size did not differ from sham/vehicle-treated rats (Figure 4).

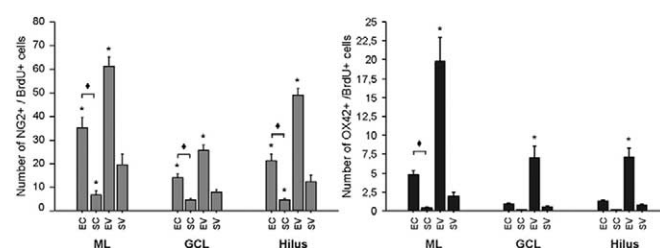


Figure 3. Proliferating NG2+ cells and microglia in the dentate gyrus of hippocampus. Sham/corticosterone-treatment (SC) reduced the numbers of proliferating NG2+ cells in the molecular layer (ML) and the hilus, compared with sham/vehicle-treated rats (SV). Electroconvulsive seizure/vehicle-treatment (EV) increased the number of BrdU-labeled NG2+ cells and Ox42+ cells in all dentate gyrus sub-regions compared with SV. Electroconvulsive seizure-treatment also counteracted the inhibitory effect of corticosterone (EC) and the number of proliferating NG2+ cells was significantly higher compared with SV, in all sub-regions of the dentate gyrus. Electroconvulsive seizure-treatment was also able to reverse the negative effect of corticosterone on BrdU+/Ox42+ cell numbers in ML. Asterisks indicate significant differences from SV. Diamonds indicate significant differences from SC. Data are presented as means \pm SEM as analyzed with analysis of variance (ANOVA) and Fischer's PLSD tests. Statistical significance was accepted at $p \leq .05$.

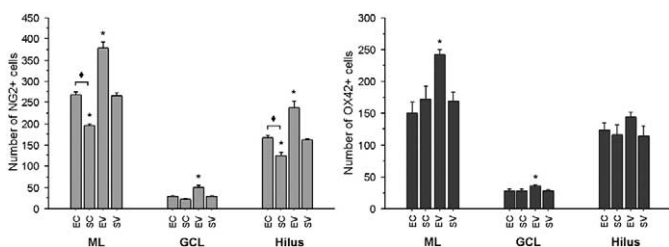


Figure 4. The total number of NG2+ cells decreased significantly in the molecular layer (ML) and hilus after sham/corticosterone-treatment (SC) compared with sham/vehicle-treatment (SV). This decrease was counteracted by electroconvulsive seizures (EC) and the cell numbers returned to normal. Electroconvulsive seizure/vehicle-treatment (EV) significantly increased the total number of NG2+ cells, whereas a significant increase in Ox42+ cells after EV were only detected in the ML. Asterisks indicate significant differences from SV. Diamonds indicate significant differences from SC. Data are presented as means \pm SEM as analyzed with analysis of variance (ANOVA) and Fischer's PLSD tests. Statistical significance was accepted at $p \leq .05$.

Corticosterone and ECS-Treatment Affect the Proliferation of Microglia but not Astrocytes

A trend towards decreased number of Ox42+/BrdU+ cells in the ML ($p = .440$) and hilus ($p = .421$) was detected in sham/corticosterone-treated rats. The reduction in BrdU-labeled microglia, however, reached significance when the groups were analyzed with unpaired t -test (ML ($p = .017$); GCL ($p = .023$); hilus ($p = .006$)). The number of Ox42+/BrdU+ cells was significantly increased in all the dentate gyrus subregions (ML ($p < .001$); GCL ($p < .001$); hilus ($p < .001$)) of ECS/vehicle-treated rats. ECS-treatments were also able to significantly increase the BrdU-labeled microglia in ML ($p = .0034$) of corticosterone-treated rats and the number of Ox42+/BrdU+ cells did not differ from sham/vehicle-treated rats. This was also seen in the GCL ($p = .002$) and hilus ($p < .001$) when groups were analyzed with unpaired t -test. The total number of Ox42+ cells in the ML ($p = .045$) and GCL ($p = .023$) increased in rats receiving ECS/vehicle-treatment compared to sham/vehicle-treated rats (Figure 4). The vast majority of the microglia had small cell bodies and a highly ramified appearance, indicating a nonreactive state (Streit et al 2002). A small fraction of the BrdU+ cells expressed the astrocyte antigen S-100 β (less than .1%). No significant differences between the groups could be detected (data not shown).

No Change in the Number of BrdU+ Mature Oligodendrocytes after Short Survival Time

Staining against the oligodendrocyte marker Rip showed that none of the mature oligodendrocytes were BrdU+ in any of the dentate gyrus sub-regions. The total number of Rip+ cell bodies did not significantly differ between the different treatment groups (data not shown).

Volume of DG Increases in Response to ECS Treatment

There was no significant reduction in the volume of either ML, GCL or hilus in sham/corticosterone-treated rats compared to sham/vehicle-treated rats. However, the ML ($p = .029$) and hilus ($p = .003$) in sham/corticosterone-treated rats were significantly smaller than those of ECS/vehicle-treated animals. Significant differences in ML ($p = .035$) and hilar ($p = .006$) volumes were also detected between rats receiving ECS/vehicle-treatment and rats receiving ECS/corticosterone-treatment. Hilar volumes in ECS/vehicle-treated rats were larger compared to sham/vehicle-treated rats ($p = .036$) (Figure 5).

Fate of Proliferating Cells

The fate of the proliferating cells was investigated by giving rats five ECS-treatments and/or corticosterone-treatment, with the same procedures as in the study described above, but with a survival time of three weeks. In agreement with previous findings (Wennstrom et al 2003), many of the NG2+/BrdU+ cells retained their NG2+ state. In rats subjected to ECS/vehicle-treatment, we still detected significantly elevated numbers of NG2+/BrdU+ cells compared with controls ($p < .0001$). In rats treated with ECS/corticosterone, the number of BrdU-labeled NG2+ cells was not significantly different from sham/vehicle-treated rats (Figure 6). The fraction of NG2+/BrdU+ cells out of all BrdU+ cells after three weeks was significantly lower in GCL in all treatment groups compared to the same fraction seen in rats with twelve hours survival. This fraction was also decreased in the ML and hilus of sham/corticosterone-treated rats and ECS/vehicle-treated rats (Table 1).

After the three-week survival period, we detected scattered Rip+/BrdU+ cells (oligodendrocytes) in ML and hilus. The number of BrdU-labeled oligodendrocytes was significantly elevated in ML and hilus of rats treated with ECS/vehicle, compared with sham/vehicle-treated rats ($p < .0001$), and significantly decreased in ML ($p = .006$) and hilus ($p = .0012$) of sham/corticosterone-treated rats, compared with sham/vehicle-treated rats. The number of BrdU+/Rip+ cells in ECS/corticosterone-treated rats was not significantly different from the number in sham/vehicle-treated rats (Figure 6). The number of Ox42+/BrdU+ cells was still significantly elevated after three weeks in all dentate gyrus subfields of ECS/vehicle-treated rats compared to sham/vehicle-treated rats (ML ($p < .0001$); GCL ($p < .0001$);

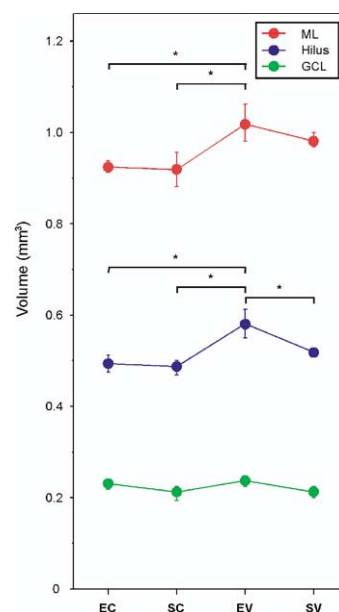


Figure 5. Volume in cubic millimeters of the dentate gyrus subfields; molecular layer (ML), granule cell layer (GCL), and hilus of dentate gyrus. Sham/corticosterone-treatment (SC) did not reduce the volume of any of the dentate gyrus subfields compared with sham/vehicle-treated rats (SV). The volumes of the ML and hilus in rats treated with sham/corticosterone (SC) or electroconvulsive seizure/corticosterone (EC) were significantly smaller than in rats treated with electroconvulsive seizures/vehicle (EV). Significantly increased hilar volume was seen in EV compared to SV. Asterisks indicate significant differences from EV. Data are presented as means \pm SEM and were analyzed with analysis of variance (ANOVA) and Fischer's PLSD tests. Statistical significance was accepted at $p \leq .05$.

Table 1. Percentage of Double-Labeled Cells

Study	Twelve Hours Survival				Three Weeks Survival			
	EC	SC	EV	SV	EC	SC	EV	SV
ML								
NG2+/BrdU+	48.53 ± 1.57	64.38 ± 7.19	33.15 ± 5.68	49.68 ± 4.79	39.97 ± 6.47	*5.00 ± 5.00	*17.67 ± 1.22	46.22 ± 4.36
Ox42+/BrdU+	6.90 ± .71	6.49 ± 3.12	11.27 ± 1.27	5.50 ± 1.17	*13.55 ± 2.37	5.35 ± 3.35	12.89 ± 1.36	*14.58 ± 1.07
Rip+/BrdU+	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	*2.30 ± .26	*1.00 ± 1.00	*1.26 ± .20	*2.72 ± .48
GCL								
NG2+/BrdU+	7.79 ± 1.20	10.09 ± 1.46	9.11 ± 0.65	15.24 ± 0.83	*.01 ± .13	*.00 ± .00	*1.61 ± .21	*.71 ± .28
Ox42+/BrdU+	.33 ± .05	.27 ± .12	1.97 ± .52	.83 ± .26	1.00 ± .23	.29 ± .21	*3.67 ± .27	*1.15 ± .13
Rip+/BrdU+	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00
Hilus								
NG2+/BrdU+	46.04 ± 3.74	66.57 ± 8.46	46.41 ± 3.82	46.83 ± 4.24	29.43 ± 6.38	*.00 ± .00	*29.50 ± 2.06	27.92 ± 5.00
Ox42+/BrdU+	2.62 ± .10	4.25 ± 7.75	6.86 ± 1.16	1.71 ± .73	*4.04 ± 1.00	10.00 ± 7.75	*11.58 ± 1.32	*7.96 ± 1.82
Rip+/BrdU+	.00 ± .00	.00 ± .00	.00 ± .00	.00 ± .00	*3.65 ± .44	*.00 ± .00	*2.82 ± .23	*3.66 ± .35

Values represent means ± SEM. Data were analyzed with ANOVA and Fischer's PLSD test ($p < .05$). Asterisks indicate significant decrease from the study with twelve hours survival in the respective region analyzed. ML, molecular layer; GCL, granular cell layer; EC, electroconvulsive seizures/corticosterone; SC, sham/corticosterone; EV, electroconvulsive seizures/vehicle; SV, sham/vehicle; ANOVA, analysis of variance; PLSD, -----.

hilus ($p < .0001$) and ECS-treatment led to significantly higher numbers of BrdU-labeled microglia in ML ($p = .020$) and GCL ($p = .004$) of corticosterone-treated rats compared to rats that only received sham/corticosterone treatment (Figure 6). This was also seen in ML ($p = .001$) when groups were analyzed with unpaired t -test. The fraction of Ox42+/BrdU+ cells out of all BrdU+ cells in sham/vehicle treated rats after three weeks was significantly lower in all dentate gyrus subregions compared to the same fraction in rats with twelve hours survival. This was also seen in the hilus of rats treated with ECS/corticosterone and ECS/vehicle and for the latter group also in GCL (Table 1).

Distribution of Glial Cells

The NG2+ cells in the hippocampus were located mainly in the molecular layer (ML), hilus and at the border of the GCL (the subgranular zone, SGZ), with the highest density in the hilus (Figure 2). Rip+ cells had a similar distribution pattern as the NG2+ cells. The oligodendrocyte cell bodies were found in the ML and hilus, but not in the GCL. The myelinated fibers were mainly found in the ML and hilus, with the densest distribution in the hilus. Ox42+ cells were evenly distributed over the three dentate gyrus subregions (data not shown).

Biological Efficacy of the Corticosterone Treatment

In comparison with rats treated with sham/vehicle, rats treated with corticosterone (40 mg/kg) for seven days displayed reduced weight gain and the ratio between the weight of the adrenal glands and body weight decreased (Table 2). The adrenal weight and adrenal weight/body weight ratio were still significantly lower in corticosterone-treated rats after three

weeks of survival, whereas the body weights were only significantly lower when the groups were analyzed with unpaired t -test (see Table 2).

Discussion

The observation that depression is associated with elevated cortisol levels (Carroll et al 1976; Dinan 2001) suggests that animals chronically treated with corticosterone can model some aspects of human depression. This model has been shown to increase depression-like behavior, as assessed by the forced swim test (Gregus et al 2005; Kalynchuk et al 2004). Additionally, it has been reported that corticosterone treatment causes an impairment of declarative memory, also seen in depressed patients (for review see Roozendaal 2002).

It was shown in a previous study that corticosterone treatment suppresses the normal proliferation of NG2+ oligodendrocyte progenitors in rat hippocampus (Alonso 2000). In this study we confirm this finding and additionally show that corticosterone reduces the total number of NG2+ cells in the ML and hilus of the dentate gyrus. Since BrdU-labeling for five days reflects the sum of cells that have divided (and thereby taken up BrdU) minus newborn (BrdU-labeled) cells that have died, we verified the result of total BrdU+ cell number with a second proliferation marker - Ki67. This antigen is only expressed in proliferating cells and this method therefore gives an estimation of the number of proliferating cells at the time of perfusion. The results from the Ki67+ staining show that the reduction in BrdU+ cells seen after corticosterone-treatment reflects a reduction in cell proliferation and not merely death of BrdU+ cells.

Table 2. Adrenal Weight, Body Weight and the Adrenal: Body Weight Ratio

Treatment	EC	SC	EV	SV
S: Adrenal weight (mg)	*20.52 ± 1.17	*17.98 ± 1.10	*35.90 ± 2.00	31.83 ± .99
Body weight (g)	*225.00 ± 5.53	*225.00 ± 3.43	266.60 ± 4.08	271.00 ± 7.55
Adrenal weight : body weight	* $9.10 \times 10^5 \pm 4.81 \times 10^6$	* $8.03 \times 10^5 \pm 5.82 \times 10^6$	$1.34 \times 10^5 \pm 6.15 \times 10^6$	$1.17 \times 10^5 \pm 7.37 \times 10^6$
L: Adrenal weight (mg)	40.00 ± 2.34	*34.17 ± 2.46	49.67 ± 2.01	48.67 ± 4.60
Body weight (g)	349.17 ± 7.90	328.33 ± 6.01	351.67 ± .10	348.33 ± 5.73
Adrenal weight : body weight	$1.15 \times 10^5 \pm 8.04 \times 10^7$	* $1.04 \times 10^5 \pm 7.53 \times 10^7$	$1.41 \times 10^5 \pm 4.29 \times 10^7$	$1.39 \times 10^5 \pm 1.21 \times 10^6$

Values represent means ± SEM. Data were analyzed with ANOVA and Fischer's PLSD test ($p < .05$). Asterisks indicate significant increase from the control group in the respective region analyzed. S, twelve hours survival study; L, three weeks survival study; EC, electroconvulsive seizures/corticosterone; SC, sham/corticosterone; EV, electroconvulsive seizures/vehicle; SV, sham/vehicle; ANOVA, analysis of variance; PLSD, -----.

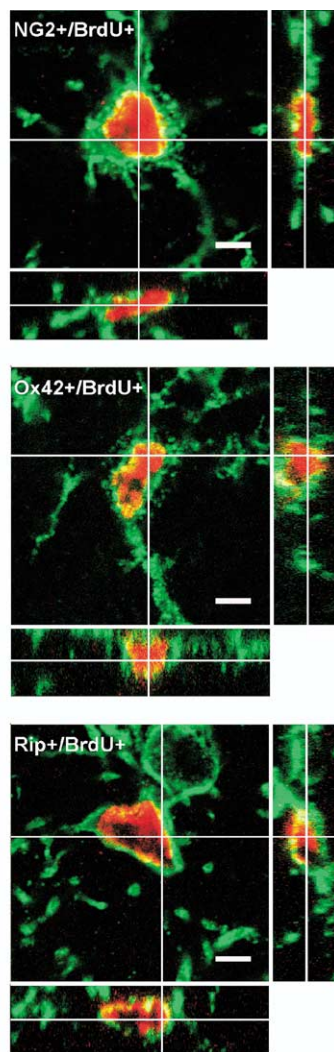
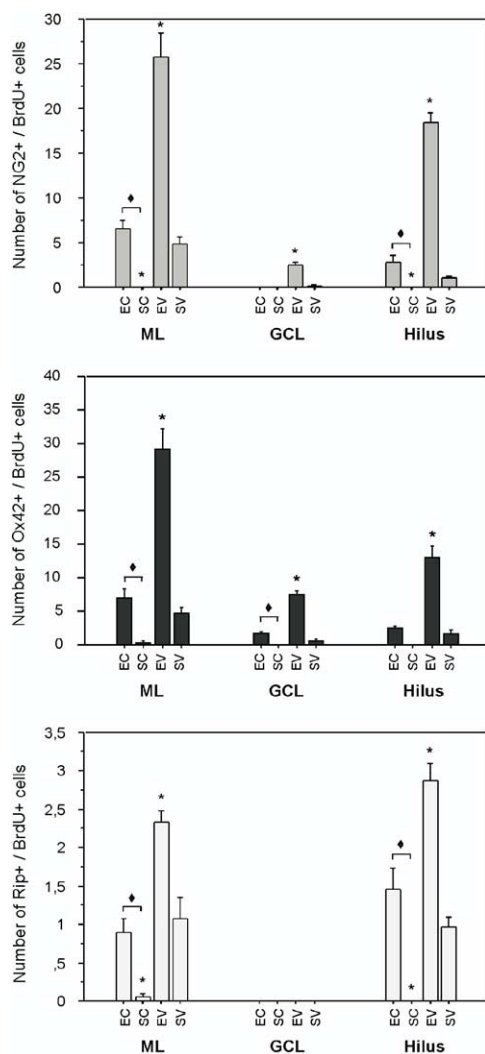


Figure 6. Bromodeoxyuridine (BrdU)+ glial cells after three weeks survival. Rats treated with electroconvulsive seizures/vehicle (EV) had significantly more NG2+/BrdU+ and Ox42+/BrdU+ cells in all three dentate gyrus sub-regions compared to sham/vehicle treated rats (SV). The number of Rip+/BrdU+ cells was significantly increased in the ML and hilus in EV compared with SV. The number of NG2+/BrdU+ and Rip+/BrdU+ cells in sham/corticosterone-treated rats (SC) was significantly lower in the ML and hilus compared with SV, and this effect was reversed by electroconvulsive seizure-treatment (EC). The number of Ox42+/BrdU+ cells in EC was significantly higher compared to SC and did not differ compared with SV. Asterisks indicate significant differences from SV. Diamonds indicates significant differences from SC. Data are presented as means \pm SEM and were analyzed with analysis of variance (ANOVA) and Fischer's PLSD tests. Statistical significance was accepted at $p \leq .05$. Confocal reconstructions of bromodeoxyuridine (BrdU)+ glial cells in the hilus of dentate gyrus are shown in the right panel. BrdU staining is shown in red whereas NG2, Ox42, and Rip stainings are shown in green. Three orthogonal (xy, xz and yz) planes are shown. Scale bar = 2.5 μ m.

The mechanism by which corticosterone affects NG2+ cell proliferation is not well understood, but the presence of mineralocorticoid (MR) and glucocorticoid (GR) receptors on both immature and mature oligodendrocytes (Vielkind et al 1990; Bohn et al 1991) suggests that corticosterone can affect NG2+ cells directly.

This study is to our knowledge the first to demonstrate that ECS-treatment counteracts the inhibition of NG2+ cell proliferation by corticosterone, and that the total number of NG2+ cells is returned to baseline levels. The mechanisms responsible for these effects are not known, but either ECS-treatment can directly interfere with the suppressive effect of corticosterone by up-regulating NG2-cell-mitogens that are down-regulated by corticosterone treatment, or the two different treatments affect the proliferation of NG2+ cells through independent pathways.

This study also demonstrates that corticosterone treatment decreases the number of BrdU+ microglia. This corresponds well with a previous *in vitro* study showing that microglia express both GR and MR and that these two receptors regulate microglial cell proliferation (Tanaka et al 1997). The functional significance of the corticosterone-induced down-regulation of microglial proliferation is yet to be determined, but lately the importance of "resting" ramified nonreactive microglia has been discussed. Microglia in this state are thought to play a supportive

role of neuronal function and are known to secrete neurotrophic factors (for review see Vilhardt 2005). Previous studies have also shown that microglial processes make direct contacts with NG2+ cells (Nishiyama et al 1997). These observations are indicative of a functional interaction between microglia and NG2+ glial cells. Indeed, *in vitro* studies have demonstrated a role for nonreactive microglia in the survival and maturation of oligodendrocyte precursor cells (Nicholas et al 2001).

Postmortem studies of patients with a history of affective disorder show reductions in hippocampal volume. Additionally, attempts to mimic depressive disorders in animals have shown that corticosterone treatment leads to a significant reduction of all dentate gyrus subregions (Sousa et al 1998) and psychosocial stress, which is accompanied by raised cortisol levels, leads to small, but nonsignificant, hippocampal volume reductions in tree shrews (Czeh et al 2001).

In our study, we could not detect any reduction in overall volume of the dentate gyrus after corticosterone treatment. Because the volume reduction in depressed patients is correlated with the severity and duration of the illness, a plausible explanation for our result may be that the corticosterone treatment period (seven days) was too short to give detectable changes. However, ECS/vehicle-treated animals had significantly larger hilar volumes when compared with sham/vehicle-treated ani-

mals, and significantly larger ML and hilar volumes when compared to sham/corticosterone-treated animals.

The underlying mechanism of the hippocampal volume reduction in depressed patients remains unclear. A recent postmortem study on patients with a history of depression suggests that the hippocampal atrophy might be due to an increased packing density of neurons and glial cells (Stockmeier et al 2004). Another explanation, as mentioned in the introduction, could be that the volume reduction is, at least partly, caused by a reduction in oligodendrocyte numbers (Hamidi et al 2004). With respect to the latter hypothesis, it is interesting to note that the total number of NG2+ cells (thought to be oligodendrocyte progenitors) was reduced in corticosterone-treated rats in this study. It is also notable that the sub-region with the most pronounced volume reduction, the hilus, is also the sub-region with the densest distribution of NG2+ cells.

In agreement with other studies, we found that many of the NG2+ cells persist in their NG2+ state over a long period (three weeks) (Nishiyama 2001). NG2+ cells have both morphological and functional characteristics of a differentiated cell. The term synantocyte (Butt et al 2002) has been given to this fourth glial cell type distinguished from astrocytes, oligodendrocytes and microglia. Traditionally, as mentioned above, NG2+ cells are regarded as oligodendrocyte progenitors, but the details of the oligodendrocyte lineage are far from clear. Either NG2-positive synantocytes transdifferentiate and give rise to NG2-negative oligodendrocytes, or these two glial cell types have a common and presumably NG2-positive progenitor (Nishiyama et al 2002). In our study both the number of NG2+/BrdU+ cells and the fraction of NG2+/BrdU+ cells out of all BrdU+ cells are reduced after a three week survival period, compared with after twelve hours survival. This finding could be explained by death of some NG2+/BrdU+ cells or by differentiation into a cell type not expressing NG2. Our observation that a small number of mature Rip+/BrdU+ oligodendrocytes is detected in all of the hippocampal subregions after three weeks supports the explanation that some of the NG2+ cells had differentiated into oligodendrocytes.

Our study shows that the number of newly formed oligodendrocytes is significantly reduced already after seven days of corticosterone treatment. This result may be explained by the observation that corticosterone suppresses proliferation of NG2+ cells and thereby decreases the oligodendrocyte progenitor pool. Statistically significant changes after such short time could very well explain the oligodendrocyte reduction that has been observed in patients with many years of depressive illness. The reduction is counteracted by ECS-treatment, which implies that the loss of oligodendrocytes, as seen in postmortem studies of depressed patients, may be reversed by ECT. Interestingly a recent study shows that frontal white matter reductions in depressed patients are increased by ECT-treatment (Nobuhara et al 2004). Furthermore, one report also indicates that mood stabilizers can prevent the glial reduction found in patients with major depression (Bowley et al 2002).

We can thus conclude that a number of investigators have reported reduced volume of hippocampus and other brain regions in patients with depressive disorders. Postmortem studies have revealed a loss of oligodendrocytes in the amygdala and prefrontal cortex. Here we show for the first time in an animal depression model that reduced gliogenesis is counteracted by electroconvulsive seizures, and that this antidepressant treatment furthermore increases the volume of hippocampal dentate subregions. Glial cell dysfunction may be of central importance in

the pathophysiology of depression, and further knowledge on how antidepressant treatment affects proliferation, differentiation and survival of different glial cell populations may give clues to new therapeutic strategies.

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