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Electroconvulsive Seizures Induce Proliferation of NG2-Expressing Glial Cells in Adult Rat Amygdala

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Background: Volumetric changes and glial pathology have been reported in the amygdala in patients with major depressive disorder. Here we report an analysis of glial cell proliferation in response to electroconvulsive seizures (ECS), clinically used for the treatment of severe depression.

Methods: Male Wistar rats were subjected to five ECS-treatments and then injected with bromodeoxyuridine (BrdU) to detect cell proliferation in the amygdala. The animals were transcardially perfused either 12 hours or 3 weeks after the last BrdU injection. Tissue sections were double-stained for BrdU and the cell-type markers NG2, OX-42, RIP, S-100 β , Doublecortin, or NeuN.

Results: Electroconvulsive seizures dramatically increased the proliferation of amygdala cells expressing the oligodendrocyte progenitor marker NG2. Bromodeoxyuridine-labeled NG2-expressing cells were still present after 3 weeks of survival, and a small proportion of the proliferating cells had differentiated into mature oligodendrocytes.

Conclusions: Major depression has been associated with a reduction of glial cells. Our results show that ECS, an antidepressant treatment, significantly increases the number of NG2+ glial cells and mature oligodendrocytes in the adult rat amygdala.

Key Words: Major depression, electroconvulsive seizure, amygdala, gliogenesis, NG2, BrdU

The amygdala is a key structure in the regulation of emotional behavior and is a brain region that has been implicated in the pathophysiology of depression. Electrical stimulation of the human amygdala elicits feelings of fear and apprehension, and damage to the amygdala of experimental animals produces tameness (Iversen et al 2000). The amygdala is strongly interconnected with the prefrontal cortex, hypothalamus, and the brainstem, structures that also are involved in reactions to emotional stimuli (Iversen et al 2000). Several recent studies have indicated that the amygdala is structurally and functionally altered in mood disorders. Brain imaging studies on patients with major depression have revealed volume reduction (Mervaala et al 2000; Sheline et al 1998) as well as increased glucose metabolism and blood flow in the amygdala (Drevets 2000). The two latter findings have been positively correlated with depression severity and plasma cortisol levels in both unipolar and bipolar depression (Abercrombie et al 1998; Drevets et al 2002). The tissue alterations underlying the observed volume reduction is yet to be determined, but interestingly, postmortem studies of subjects with depression have revealed a reduction in the number of glial cells in the amygdala (Bowley et al 2002). In a recent study this glial cell loss was attributed to a decrease in the numbers of oligodendrocytes in the amygdala (Hamidi et al, in press).

We have previously shown that electroconvulsive seizure (ECS), an animal model for the antidepressant treatment electroconvulsive therapy, is associated with dramatically increased cell proliferation in adult rat hippocampus (Hellsten et al 2002; Madsen et al 2000), a limbic structure closely connected with the amygdala. We also recently found that a large proportion of the hippocampal cells proliferating in response to ECS are glial cells expressing the chondroitin sulfate proteoglycan NG2

(Wennström et al 2003). These cells are often referred to as oligodendrocyte progenitors (Levine et al 2001; Watanabe et al 2002).

Given the observation that oligodendrocytes are reduced in number in the amygdala of depressed patients, and the fact that ECS strongly increases the proliferation of NG2-expressing glial cells in adult rat hippocampus, we wanted to investigate whether glial cell proliferation also occurs in the adult rat amygdala in response to ECS.

Methods and Materials

Animals and Design of Study

Adult male Wistar rats (Møllegaard breeding center, Denmark) weighing 180 g at the beginning of the study were used. Rats were housed three per cage and kept on a 12-hour light/dark cycle with ad libitum access to food and water. 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 the increase in glial and neuronal precursor proliferation after ECS, a 12-hour survival study was designed, whereby the rats were assigned to the following groups: Control ($n = 6$) and 5 ECS ($n = 6$). A 3-week survival study was performed to determine the fate of the cells that proliferated in response to ECS. Rats were assigned to the following groups: Control ($n = 6$) and 5 ECS ($n = 6$) (Figure 1).

Administration of ECS

On days 0–4 of the 12-hour and 3-week studies all rats were subjected once daily (10:00 AM) to either a single ECS trial or a sham treatment. Electroconvulsive seizure was delivered via silver electrode ear clips (Somedic Sales, Hörby, Sweden) (50-mA, .5-sec, 50-Hz unidirectional square wave pulses) (Figure 1). The rats were monitored after ECS to ensure that clonic movements of the face and forelimbs occurred for 20–30 sec. Generalized seizures engaging the face and forelimbs were taken as an indication of limbic seizures. Control rats were sham-treated (i.e., handled identically to the ECS-treated rats except that no current was passed).

Administration of Bromodeoxyuridine

The thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, Missouri; cat. no. B5002) was used as a proliferation marker. Using similar protocols, others have shown

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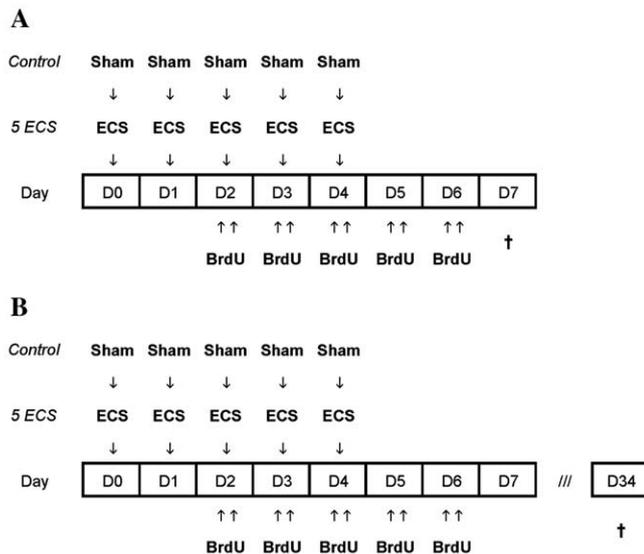


Figure 1. Experimental design and group assignment. In the study with a 12-hour survival period (**A**), rats were given either five electroconvulsive seizures (ECS) or sham treatments. Rats were subjected to a single ECS (or sham treatment) once daily (10:00 AM) on days 0–4. All rats received bromodeoxyuridine (BrdU) injections twice daily (8:00 AM and 8:00 PM) at days 2–6 and were transcardially perfused 12 hours after the last BrdU injection. The study with a 3-week survival period (**B**) was conducted as the study with short survival with regard to the administration of the five ECSs and the BrdU injections. The rats were transcardially perfused 21 days after the last BrdU injection. D0–D34 represent days 0–34.

that this staining detects cell proliferation rather than deoxyribonucleic acid repair (Cooper-Kuhn and Kuhn 2002; Palmer et al 2000). Bromodeoxyuridine was dissolved in phosphate-buffered saline (PBS) 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) during days 2–6 after the initial ECS treatment (Figure 1).

Tissue Preparation

Twelve hours or 3 weeks after the last injection of BrdU rats were anesthetized with sodium pentobarbital 60 mg/mL. In the absence of nociceptive reflexes, the rats were transcardially perfused with 100 mL .9% saline, followed by 200 mL 4% ice-cold paraformaldehyde. After decapitation, the brain was removed from the skull and postfixated in 4% paraformaldehyde at 4°C overnight. Before sectioning on a freezing microtome, the brains were left in 30% sucrose in PBS until they sank. Coronal sections (40 μm thick) were cut through the mid-part of the rostral-caudal extension of amygdala (−2.65 mm to −3.40 mm, relative to bregma) (Paxinos and Watson 1986) and stored in antifreeze cryoprotectant solution at −20°C until immunofluorescence staining was performed.

Antibodies

The following antibodies were used: rabbit-anti-NG2 (gift from Dr. W.B. Stallcup) directed against the chondroitin sulfate proteoglycan NG2, expressed on glial progenitor cells (Stallcup 1981); mouse-anti-CD11b (OX-42) (Serotec, Oxford, United Kingdom; cat. no. MCA275), which recognizes microglia (Milligan et al 1991); rabbit-anti-S100β (Swiss Antibodies, Bellinzona, Switzerland) directed against astrocyte cell bodies (Boyes et al 1986); mouse-anti-RIP (Hybridoma Bank, Iowa City, Iowa) directed against myelin basic protein expressed by oligodendro-

cytes (Friedman et al 1989); goat-anti-Doublecortin (Santa Cruz Biotechnology, Santa Cruz, California; cat. no. sc-8066) directed against a protein expressed by migrating neurons (Gleason et al 1999); mouse-anti-neuron-specific nuclear protein (NeuN) (Chemicon, Temecula, California; cat. no. MAB 377) directed against a transcription factor that is expressed in the nucleus and cytoplasm of mature neurons (Mullen et al 1992); rat-anti-BrdU (Oxford Biotechnology, Kidlington, United Kingdom) directed against bromodeoxyuridine; and mouse-anti-Ki67 (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) phases of the cell cycle.

Double-Fluorescence Immunostaining

Sections were stained for neuronal and glial markers according to the protocols outlined in Table 1. Brain sections were rinsed (3 × 10 min) in .02 mol/L potassium PBS (KPBS) and then incubated in blocking solution (KPBS + 5% serum of the appropriate species + .25% Triton X-100) for 1 hour at room temperature. Sections were then incubated with primary antibody in blocking solution for 24 hours at 4°C. After this the sections were rinsed (3 × 10 min) with KPBS + .25% Triton X-100 (KPBS+) and subsequently incubated with secondary antibody in blocking solution, for 24 hours at 4°C. Sections incubated with biotinylated secondary antibodies were then rinsed (3 × 10 min) in KPBS+ before incubation with 1:200 Alexa 488 (Molecular Probes, Eugene, Oregon) in KPBS+ for 24 hours in darkness, at 4°C. After washing with KPBS (3 × 10 min), the sections were fixed in 4% paraformaldehyde for 10 min at room temperature, subsequently rinsed (3 × 10 min) in KPBS and then incubated in 1 mol/L hydrochloric acid at 65°C for 30 min. After rinsing in KPBS (3 × 10 min), the sections were then exposed to blocking solution (KPBS+ and 5% normal donkey serum [Harlan Ser-Lab, Belton, United Kingdom]) for 1 hour in darkness at room temperature and then incubated Rat-anti-BrdU (Oxford Biotechnology, Kidlington, United Kingdom; cat. no. OBT 0030) in blocking solution for 40 hours in darkness at 4°C. Sections were rinsed in KPBS+ (2 × 10 min), and KPBS+, 5% normal donkey serum (1 × 10 min) before being incubated with Cy3-conjugated donkey-anti-rat (Jackson Immuno Research, West Grove, Pennsylvania; cat. no. 712-165-153) in blocking solution for 24 hours in darkness at 4°C. After rinsing in KPBS (3 × 10 min) the sections were mounted on poly-L-lysine-coated slides, air-dried, rinsed briefly (10 sec) in water and coverslipped with glycerol-based mounting medium. Bromodeoxyuridine/NeuN and NG2/Ki67 stainings were performed according to slightly different protocols, as outlined in Table 1.

Data Quantification and Statistical Analysis

Coronal sections through the mid-part of the rostral-caudal extension of amygdala (−2.65 mm to −3.40 mm, relative to bregma) (Paxinos and Watson 1986) were analyzed by observers blind to the treatments. The chosen coordinates give maximal cross-sectional diameter of the studied amygdala regions. To ensure that the sizes of the analyzed regions did not differ between animals or treatment groups, we quantified the cross-sectional areas of the four amygdala nuclei in NG2-stained sections from the studies with 12-hour and 3-week survival periods. None of the amygdala regions analyzed changed in size in response to ECS (data not shown). To identify the four main amygdala nuclei, we counterstained some sections with cresyl violet. In these sections, with the Paxinos and Watson (1986)

Table 1. Double Immunofluorescence Staining Protocols

Antigen	HCl	Block	Primary Antibody	Secondary Antibody	Avidin	PFA	HCl	Block	Primary Antibody	Secondary Antibody
NG2 BrdU		NGS	Rabbit-anti-NG2 1:500	Biotin-goat-anti-rabbit 1:200	Avidin-Alexa 488 1:250	X	X	NDS	Rat-anti-BrdU 1:200	Cy3-donkey-anti-rat 1:200
OX-42 BrdU		NGS	Mouse-anti-OX-42 1:100	Biotin-horse-anti-mouse 1:200	Avidin-Alexa 488 1:250	X	X	NDS	Rat-anti-BrdU 1:200	Cy3-donkey-anti-rat 1:200
RIP BrdU		NHS	Mouse-anti-RIP 1:1000	Biotin-horse-anti-mouse 1:200	Avidin-Alexa 488 1:250	X	X	NDS	Rat-anti-BrdU 1:200	Cy3-donkey-anti-rat 1:200
NeuN BrdU	X	NHS	Mouse-anti-NeuN 1:100	Biotin-horse-anti-mouse 1:200	Avidin-Alexa 488 1:250					
		NDS	Rat-anti-BrdU 1:100	Cy3-donkey-anti-rat 1:200						
S-100 β BrdU		NGS	Rabbit-anti-S-100 β 1:1000	Biotin-goat-anti-rabbit 1:200	Avidin-Alexa 488 1:250	X	X	NDS	Rat-anti-BrdU 1:200	Cy3-donkey-anti-rat 1:200
DCX BrdU		NHS	Goat-anti-DCX 1:1000	Biotin-horse-anti-Goat 1:200	Avidin-Alexa 488 1:250	X	X	NDS	Rat-anti-BrdU 1:200	Cy3-donkey-anti-rat 1:200
NG2 Ki67		NGS	Rabbit-anti-NG2 1:500	Biotin-goat-anti-rabbit 1:200	Avidin-Alexa 488 1:250					
		NGS	Mouse-anti-Ki67 1:100	Cy3-goat-anti-mouse 1:200						

Normal horse serum (NHS) obtained from Sigma-Aldrich, St. Louis, Missouri; normal goat serum (NGS) from Chemicon, Temecula, California (cat. no. 526); biotin horse anti-mouse (BA-2001), biotin goat anti-rabbit (BA-1000), and biotin horse anti-rabbit (BA-9500) all from Vector Laboratories, Burlingame, California. See text for other distributors/locations.

HCl, hydrochloric acid; PFA, paraformaldehyde; BrdU, bromodeoxyuridine; NDS, normal donkey serum; NeuN, mouse anti-neuron-specific nuclear protein.

atlas as a guide, it was easy to delineate the different amygdala subregions. After having become familiar with the gross anatomical landmarks, such as blood vessels and fiber tracts, in these cresyl violet stainings it was possible to correctly identify the four amygdala nuclei also in the fluorescence stainings (without the need for any counterstaining). Cell proliferation was assessed in the medial, central, basal, and lateral nuclei of the amygdala with an Olympus AX70 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a 40 \times objective. Proliferated BrdU+ cells were counted in the medial, central, basal, and lateral nuclei. Every sixth section throughout the mid-part of the rostral–caudal extension of the amygdala (three sections from each animal) was counted, and these values were averaged and expressed as mean number of cells per section and nuclei. The chondroitin sulfate proteoglycan NG2 is expressed by a number

of different cell types, such as endothelial cells, monocytes, pericytes, and oligodendrocyte progenitors. In this study we only counted cells with the specific morphology previous described as characteristic for oligodendrocyte progenitors (Stallcup 1981).

To exclude the possibilities of under- or overestimation of the cell number due to differences in cell size, we analyzed maximal cell cross-section area in 90 randomly picked BrdU-labeled cell nuclei in ECS-treated ($n = 6$) and sham-treated animals ($n = 6$). No significant difference could be detected, which suggests that no change in cell nuclear volume occurred in response to ECS. To confirm double-staining for BrdU and glial or neuronal markers, a Nikon confocal microscope (Nikon Tokyo, Japan) with a 40 \times objective and BioRad software (BioRad, Burlington, Massachusetts) was used. Cells were evaluated in z-series with a minimum of six consecutive optical sections. Data were analyzed

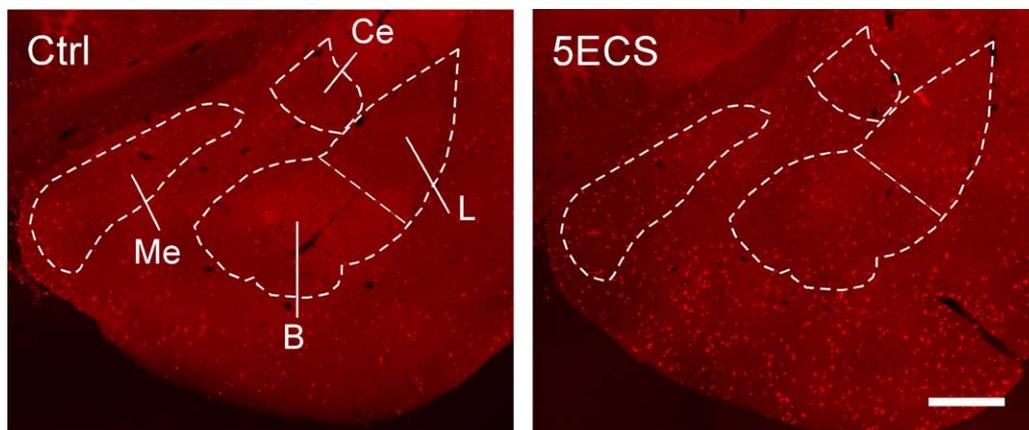


Figure 2. Immunofluorescence images showing rat amygdala (–3,14 relative to bregma) in sham-treated control animals (Ctrl) and electroconvulsive seizure-treated rats (5ECS). Five ECS increase the number of bromodeoxyuridine-labeled cells (red) in all amygdala subregions. Boundaries of the regions counted, the medial nuclei (Me), the central nuclei (Ce), the basal nuclei (B), and the lateral nuclei (L), are indicated by dashed lines. Scale bar = 350 μ m.

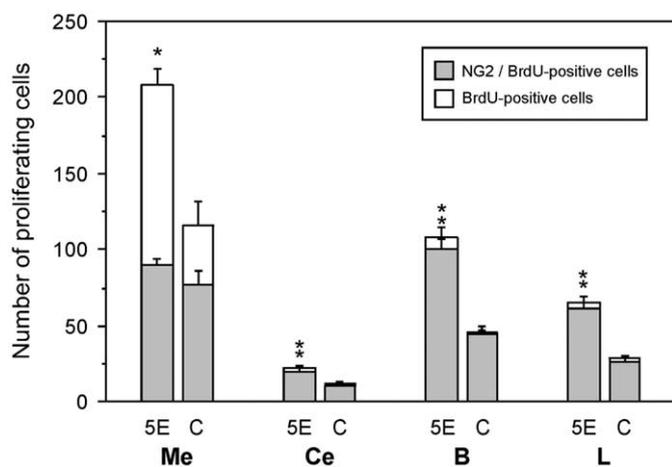


Figure 3. Study of cell proliferation in amygdala after a 12-hour survival period. Five electroconvulsive seizures (5E) increase the proliferation of bromodeoxyuridine (BrdU)-labeled cells (open bars) in all amygdala nuclei compared with control animals (C). Electroconvulsive seizures increase the number of BrdU+/NG2+ (gray bars) cells in the central nucleus (Ce), the basal nucleus (B), and the lateral nucleus (L) but not in the medial nucleus (Me). Asterisks indicate significant difference from control animals.

with analysis of variance and Dunnett test and are presented as mean ± SEM. Statistical significance was set to *p* < .05.

Results

ECS Increases Cell Proliferation in the Amygdala

The amygdala is a heterogeneous collection of nuclei (cell clusters) located in the limbic system. These nuclei are often referred to as the medial, central, lateral, and basal nuclei (Figure 2). Five ECS significantly increased the number of BrdU+ cells in the medial (80%), central (93%), lateral (135%), and basal (128%) amygdala nuclei relative to control animals (Figures 2 and 3).

Glial Cell Proliferation in Amygdala in Untreated Animals

To determine the phenotypic characteristics of the proliferating cell types found in the amygdala after ECS, we double-stained tissue sections with antibodies against the proliferation marker BrdU and various cell-type markers. Antibodies against

NG2 stained a considerable number of the BrdU+ cells in the medial, central, basal, and lateral nuclei of amygdala. In untreated animals the fraction of NG2+/BrdU+ cells of all BrdU+ cells was 93.18% ± .57% in the central, 93.28% ± .91% in the lateral, and 95.77% ± .80% in the basal amygdala nucleus, whereas it was only 68.49% ± 4.89% in the medial amygdala nucleus (Table 2, Figure 3). Staining against another cell-proliferation marker, Ki67, expressed during the cell cycle, showed that the NG2 proteoglycan is expressed already at this early stage (Figure 4). Staining against the microglial marker OX-42 showed that microglia are represented in the amygdala, but that the percentage of BrdU+ microglia in untreated animals was very low in the central, lateral, and basal nuclei (1.04% ± .51%, .00% ± .00%, and 1.06% ± .44%, respectively). In the medial nucleus, however, approximately 11.57% ± .81% of the BrdU+ cells were OX-42+ (Table 2). A small fraction of the BrdU+ cells expressed the astrocyte antigen S-100β (less than 1%), oligodendrocyte antigen RIP (less than 1%), or the neuronal progenitor antigen Doublecortin (less than 1%).

ECS Increases Proliferation of Glial Cells in the Amygdala

In response to five ECS, the number of BrdU+/NG2+ cells in the central, lateral, and basal amygdala nuclei increased by 84%, 129%, and 132%, respectively. These BrdU+/NG2+ cells represented 91.67% ± 1.81%, 95.23% ± .71%, and 93.12% ± .99% of all BrdU+ cells in the central, lateral, and basal amygdala nuclei, respectively. These figures did not differ significantly from what was seen in sham-treated animals (see above).

In the medial nucleus no ECS-induced proliferation of NG2-expressing cells could be detected (Table 2, Figure 3). The total number of BrdU-labeled cells in the medial nucleus, however, increased significantly, resulting in a relative decrease (to 43.08% ± 1.91%) of NG2+/BrdU+ cells. A substantial amount of the BrdU-labeled cells in the medial nuclei were OX-42+ (13.70% ± 1.28%), but in the central, lateral, and basal nuclei only 3.73% ± .63%, 1.40% ± .13%, and 4.54% ± .78%, respectively, of the BrdU+ cells were OX-42+. The vast majority of the microglia and all of the NG2-expressing cells had small cell bodies and extensive processes, which indicates a nonactivated state (data not shown). Proliferation of cells expressing the cell-type markers S-100β (astrocytes), RIP (oligodendrocytes), or Doublecortin (immature neurons) did not increase after ECS treatment.

Table 2. Proliferation Study with 20-Hour Survival after Last BrdU Injection

Staining	Number of Double-Labeled Cells		Number of BrdU+ Cells		Double-Labeled Cells (%)	
	5ECS	Control Animals	5ECS	Control Animals	5ECS	Control Animals
Medial Nuclei						
NG2/BrdU	89.20 ± 4.29	77.02 ± 8.85	208.13 ± 10.74 ^a	115.84 ± 15.97	43.08 ± 1.91	68.49 ± 4.89
OX-42/BrdU	28.14 ± 2.35 ^a	10.80 ± 1.37	211.02 ± 20.54 ^a	91.98 ± 6.42	13.70 ± 1.28	11.57 ± .81
Central Nuclei						
NG2/BrdU	19.72 ± 2.09 ^a	10.40 ± 1.46	21.55 ± 2.32 ^a	11.18 ± 1.58	91.67 ± 1.81	93.18 ± .57
OX-42/BrdU	1.02 ± .18 ^a	.22 ± .10	27.62 ± 1.55 ^a	17.60 ± 2.14	3.73 ± .63	1.04 ± .51
Lateral Nuclei						
NG2/BrdU	26.58 ± 2.04	26.58 ± 2.04	64.82 ± 3.88 ^a	28.48 ± 2.11	95.23 ± .71	93.28 ± .91
OX-42/BrdU	1.08 ± .12 ^a	.00 ± .00	77.27 ± 5.74 ^a	26.18 ± 3.37	1.40 ± .13	.00 ± .00
Basal Nuclei						
NG2/BrdU	100.14 ± 6.78 ^a	43.92 ± 2.89	107.69 ± 6.69 ^a	45.88 ± 3.09	93.12 ± .99	95.77 ± .80
OX-42/BrdU	4.08 ± .69 ^a	.35 ± .15	91.05 ± 7.31 ^a	30.35 ± 2.73	4.54 ± .78	1.06 ± .44

Values represent means ± SEM. Data were analyzed with analysis of variance and Dunnett post-hoc test (*p* < .05).

5ECS, rats receiving five electroconvulsive seizure treatments; BrdU, bromodeoxyuridine.

^aSignificant increase from the control group in the respective region analyzed.

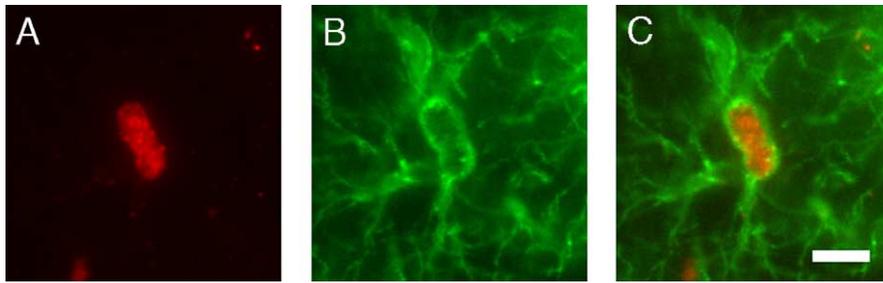


Figure 4. Double immunofluorescence staining for NG2 and the endogenous proliferation marker Ki67 (expressed during late Gap 1 (G1), DNA-Synthesis (S), Mitosis (M), and Gap 2 (G2) phases of the cell cycle). A large amount of the proliferating (Ki67+) cells in the amygdala expressed NG2, which indicates that the dividing cells are NG2+ also during the cell cycle. (A) Two closely opposed Ki67-labeled nuclei (possibly just after mitosis) belonging to NG2+ cells (B). (C) Red and green fluorescence channels are merged. Note the presence of cellular processes already at this early stage. Scale bar = 10 μm.

Expression of Glial Cell-Type Markers 3 Weeks after ECS Administration

To determine the fate of the proliferating cells, rats were given five ECS and then allowed to survive for 3 weeks. After this survival period we still detected significantly elevated numbers of BrdU-labeled cells in the medial, basal, and lateral nuclei of amygdala (Figure 5). The number of BrdU-labeled cells in the central nucleus was, after 3 weeks, no longer significantly different compared with control animals. The proliferating cells in amygdala still showed a high degree of colabeling with NG2. In control animals the fraction NG2+/BrdU+ cells (of all BrdU+ cells) were 87.22% ± 1.36% in the central nucleus, 91.13% ± 1.35% in the lateral nucleus, and 88.92% ± 2.23% in the basal nucleus. In ECS-treated animals the corresponding figures were 86.43% ± 3.77%, 85.55% ± 1.74%, and 84.33 ± .62%. The ECS-induced decrease seen in the lateral amygdala nucleus was significant. In the basal nucleus a trend for decrease was seen, but this did not reach significance. In the medial amygdala nucleus only 37.12% ± 3.64% and 69.64% ± 3.60% of the BrdU+ cells in ECS-treated and control animals, respectively, expressed NG2 (Table 3).

Furthermore, the fraction NG2+/BrdU+ cells in basal and lateral nuclei was significantly lower in ECS-treated animals with long compared with short survival. No such decrease was seen in the sham-treated animals.

The fraction of BrdU+/OX-42+ cells in the medial nucleus after 3 weeks had not changed, and 13.47% ± .81% and 14.63% ± 1.27% of the BrdU+ cells in ECS-treated and control animals,

respectively, still expressed this microglial marker. A small portion of the BrdU-labeled cells in sham-treated animals (4.07% ± .64%, 9.61% ± 2.64%, 4.81% ± .79%, and 2.25% ± .78% in the medial, central, lateral, and basal nuclei, respectively) were positive for the mature oligodendrocyte antigen RIP. This portion was significantly increased in response to ECS in the lateral and basal nuclei (85% and 286%, respectively) but did not change significantly in the medial and central nucleus (Table 3). Also after 3 weeks survival very few of the dividing cells expressed the astrocyte antigen S-100β (less than 1%) or the neuronal marker NeuN (less than 1%).

Discussion

This study demonstrates that ECS strongly stimulates cell proliferation in the four main nuclei of the amygdala and that the response to the electroconvulsive stimulation differs between these regions. In the central, lateral, and basal nuclei a majority of the cells proliferating in response to ECS were glial cells expressing the chondroitin sulfate proteoglycan NG2; however, in the medial nucleus the increased proliferation was instead partly due to dividing OX-42+ microglia. In this region the proliferation of NG2+ cells was not affected by ECS.

The BrdU-labeled NG2-expressing cells and microglia were still present in all nuclei after 3 weeks of survival, but at this time point mature RIP+/BrdU+ oligodendrocytes were also seen. The number of RIP+/BrdU+ cells was significantly increased in the basal and lateral nuclei of ECS-treated animals.

Table 3. Proliferation Study with Three Weeks Survival after Last BrdU Injection

Staining	Number of Double-Labeled Cells		Number of BrdU+ Cells		Fraction of BrdU+ Cells (%)	
	5ECS	Control Animals	5ECS	Control Animals	5ECS	Control Animals
Medial Nuclei						
NG2/BrdU	50.00 ± 5.44	39.17 ± 4.21	134.24 ± 6.21 ^a	56.30 ± 5.08	37.12 ± 3.64	69.64 ± 3.60
OX-42/BrdU	21.42 ± 3.17 ^a	10.15 ± .83	159.50 ± 5.52 ^a	70.83 ± 6.75	13.47 ± .81	14.63 ± 1.27
Rip/BrdU	3.20 ± .21	2.07 ± .47	128.48 ± 9.04 ^a	62.58 ± 5.84	2.82 ± .32	4.07 ± .64
Central Nuclei						
NG2/BrdU	12.08 ± 1.00	9.02 ± .77	14.26 ± 1.97	10.37 ± .94	86.43 ± 3.77	87.22 ± 1.36
OX-42/BrdU	1.10 ± .22 ^a	.00 ± .00	13.47 ± 1.09 ^a	6.27 ± .45	9.11 ± 2.58	.00 ± .00
Rip/BrdU	.67 ± .33	.93 ± .37	10.65 ± 1.14	10.68 ± 1.26	7.26 ± 2.59	9.61 ± 2.64
Lateral Nuclei						
NG2/BrdU	54.52 ± 1.51 ^a	22.67 ± 2.46	64.00 ± 1.58 ^a	25.00 ± 2.89	85.55 ± 1.74	91.13 ± 1.35
OX-42/BrdU	3.12 ± .72 ^a	.20 ± .06	78.00 ± 12.44 ^a	22.77 ± 2.88	4.15 ± .98	.81 ± .29
RIP/BrdU	1.20 ± .20 ^a	.65 ± .12	35.03 ± 2.33 ^a	14.15 ± 1.56	3.85 ± .75	4.81 ± .79
Basal Nuclei						
NG2/BrdU	96.93 ± 4.54 ^a	37.47 ± 2.13	112.90 ± 5.80 ^a	42.08 ± 1.95	84.33 ± .62	88.92 ± 2.23
OX-42/BrdU	4.67 ± .56 ^a	.32 ± .15	96.68 ± 6.03 ^a	28.10 ± 1.40	4.77 ± .46	1.12 ± .55
RIP/BrdU	3.78 ± .45 ^a	.98 ± .42	91.43 ± 5.24 ^a	40.75 ± 3.29	4.14 ± .44	2.25 ± .78

Values represent means ± SEM. Data were analyzed with analysis of variance and Dunnett post-hoc test (p < .05).

5ECS, rats receiving five electroconvulsive seizure treatments; BrdU, bromodeoxyuridine.

^aSignificant increase from the control group in the respective region analyzed.

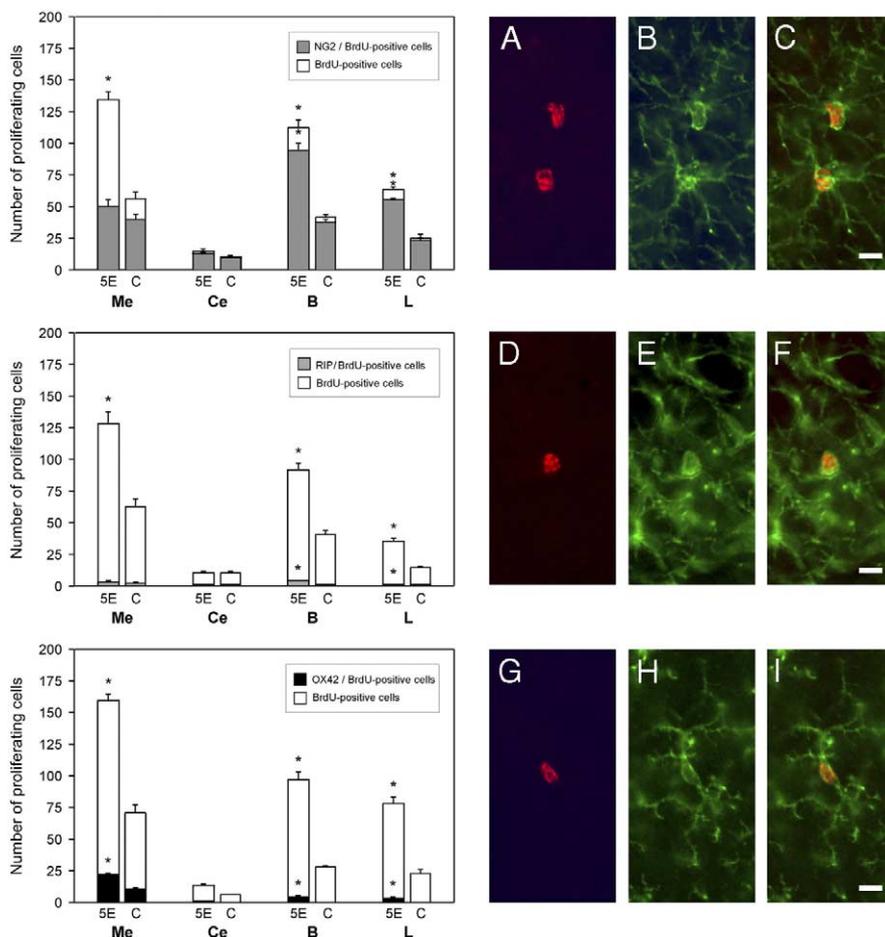


Figure 5. Study of cell proliferation in amygdala after a 3-week survival period. The number of bromodeoxyuridine (BrdU)-labeled cells (open bars) is still elevated in animals treated with five electroconvulsive seizures (5E) compared with control animals (C). In the basal nucleus (B) and the lateral nucleus (L) the number of BrdU+/NG2+ cells (dark gray bars), BrdU+/RIP+ cells (light gray bars), and BrdU+/OX-42+ cells (black bars) are significantly increased compared with control animals. The number of RIP+/BrdU+ and NG2+/BrdU+ cells did not increase in the central or medial nucleus; however, the number of OX-42+/BrdU+ cells significantly increased in both the central and the medial nuclei in response to five electroconvulsive seizures. Images of the various immunofluorescence stainings are shown at right. The cells are double-labeled with rat anti-BrdU antibodies (A, D, G) and rabbit anti-NG2 (B), mouse anti-RIP (E), or mouse anti-OX-42 (H) antibodies. (C, F, I) Red and green fluorescence channels are merged. The image of the NG2+ cell is captured from the basal nucleus, the RIP+ cell from the basal nucleus, and the OX-42+ cell from the medial nucleus. Scale bar = 8 μm.

Electroconvulsive seizure has previously been shown to induce cell proliferation in adult rat hippocampus (Hellsten et al 2002; Madsen et al 2000) and prefrontal cortex (Madsen, unpublished data), areas that have been suggested as important sites in the neuropathology of depression.

The exact molecular mechanisms underlying ECS-induced cell proliferation is yet to be determined, but a number of factors with known mitogenic effects, such as brain-derived neurotrophic factor, nerve growth factor, and basic fibroblast growth factor, are upregulated in response to ECS (Gospodarowicz 1976; Gwinn et al 2002; Nibuya et al 1995; Pencea et al 2001; Zetterstrom et al 1998). This study describes ECS-induced cell proliferation in yet another area important in depression, namely the amygdala.

The contribution of the amygdala to emotional processes has been extensively investigated, and a variety of functions have been attributed to the structure. The amygdaloid complex consists of a number of different regions, of which the medial, central, lateral, and basal nuclei are the most well known. The basal and lateral nuclei, often collectively referred to as the basolateral complex, receive afferent connections from sensory thalamus (Iversen et al 2000). The central nucleus sends efferent connections to brain areas involved in the control of emotional responses, such as hypothalamus and midbrain (Iversen et al 2000). The afferent pathways to the basolateral nucleus and the efferent pathways from the central nucleus are involved in emotional learning (LeDoux 2000). The medial nucleus receives sensory information from the olfactory bulbs and vomeronasal organ and is believed to facilitate the response to and assimila-

tion of sexually exciting stimuli (Newman 1999). The amygdala is a heterogenous brain region, and the different nuclei display distinctive cytologic and histochemical characteristics. The basolateral complex consists of 70% pyramidal neurons with a high glutamate activity (Ottersen et al 1986), whereas the medial and central nuclei have γ -aminobutyric acid-ergic activity (Swanson and Petrovich 1998). Our findings of distinctly different proliferation patterns after ECS between the medial nucleus and the central, basal, and lateral nuclei should be viewed in the light of the fact that these four amygdala regions have functional and structural differences, as described above.

A main finding in this study is that the majority of the proliferating cells in the amygdala express the chondroitin sulfate proteoglycan NG2. Although this proteoglycan can be expressed by several cell types, the vast majority of NG2-expressing cells in our study had a characteristic appearance, with irregularly shaped cell bodies and extensive arborization of their cell processes. Because this particular cell type has been found to express platelet-derived growth factor α receptor and O-antigen 4 (Nishiyama et al 1996; Reynolds and Hardy 1997), it has been suggested to be an oligodendrocyte progenitor. Indeed, it has been shown that some NG2+ cells in the spinal cord can downregulate their NG2 expression and differentiate into mature oligodendrocytes (Watanabe et al 2002). Interestingly, it seems that a majority of these cells do not differentiate further but remain in the NG2+ state for a significant time (Butt et al 2002; Nishiyama 2001), which suggests that these cells might have another fate than becoming mature oligodendrocytes. Even though the function of these glial cells is not well established, it

has been shown that processes from NG2-expressing cells are juxtaposed to neurons at pre- and postsynaptic structures (Bergles et al 2000; Ong and Levine 1999), and direct synaptic connections between NG2-expressing cells and neurons have been described (Bergles et al 2000). These contacts are believed in part to be mediated by the NG2-proteoglycan, which shares certain structural features with cell adhesion molecules (such as neural cell adhesion molecules) (Stegmüller et al 2003). Furthermore, it has been suggested that NG2+ cells can regulate synaptic diffusion of glutamate (Ong and Levine 1999). Thus, it is plausible that NG2-expressing cells can have an important role in regulating synaptic plasticity and function.

In our study many of the NG2+ cells remained in their NG2+ state 3 weeks after ECS; however, the fraction of NG2+/BrdU+ cells of all BrdU+ cells at this time point in the ECS group was significantly lower in the basal and lateral amygdala nuclei compared with the fraction after 12 hours' survival. A reduced fraction could be due to death of some NG2+ cells and proliferation of other cell types but also to differentiation into a cell without NG2 expression. The latter explanation is supported by our finding that a small number of mature RIP+ oligodendrocytes were detected in all amygdala nuclei 3 weeks after ECS, with a significant increase in the basal and lateral nucleus. Further support comes from the finding that the fraction of NG2+/BrdU+ cells of all BrdU+ cells was lower in the basal and lateral nuclei after 3 weeks in the ECS-treated animals when compared with sham-treated animals (the decrease reached significance only in the lateral nucleus). It is thus likely that some of the NG2-expressing cells have differentiated into oligodendrocytes.

A substantial number of the proliferating cells in our study were positive for the microglial marker OX-42. Microglia play an important role in host defense against invading microorganisms, immunoregulation, and tissue repair. In response to injury, such as ischemia or status epilepticus, microglia proliferate and become reactive, indicated by a swollen cell body and shortened processes (Gehrmann et al 1992). Activated microglia are known to participate in the phagocytosis of debris and facilitate regenerative and neuroprotective processes (for a review see Streit 2002). In this study we report a significant increase in the number of proliferating microglia in all amygdala nuclei after ECS, but the vast majority of these cells show no morphologic signs of reactivity. The function of resting microglia is poorly understood, but because they are able to release trophic factors (such as basic fibroblast growth factor, nerve growth factor, and NT-3) and express purinergic receptors these cells are most likely to influence and communicate with surrounding cell types (for a review, see Hansson and Ronnback 2003). Recent studies have shown that microglia are directly associated with NG2+ cells. The two cell types are often closely apposed to each other, and cell–cell signaling seems to be conducted through microglial processes contacting NG2+ cells (Nishiyama et al 1997). Factors secreted from microglial cells have been shown to influence the proliferation of NG2+ cells in vitro (Filipovic, unpublished data), and it has been speculated that microglia might influence the differentiation of oligodendrocytes (Nishiyama et al 1997). The most profound proliferation of microglia in our study was seen in the medial amygdala nucleus. In this region the numbers of NG2+ cells and RIP+ cells were not enhanced by ECS treatment. This result would thus indicate that proliferation of microglia does not automatically lead to proliferation and differentiation of oligodendrocytes.

Abnormalities of structure and function, similar to what is

seen in the amygdala of patients with major depressive disorder, have also been described in other areas relevant for the regulation of emotion. Magnetic resonance imaging studies of patients with major depression and patients with bipolar disorder have revealed volumetric changes in the prefrontal cortex and in the hippocampus (Drevets 2000; MacQueen et al 2003; Sheline et al 1996). Furthermore, an alteration in glial cell numbers has been reported in the prefrontal cortex (Ongur et al 1998; Rajkowska 2000), and the glial cells affected were in part identified as oligodendrocytes (Uranova et al 2001). Patients with major depression often have a disturbed hypothalamic–pituitary–adrenal axis, with elevated levels of the glucocorticoid cortisol. Immature and mature oligodendrocytes contain receptors for both glucocorticoid and mineralocorticoid receptors (Bohn et al 1991; Vielkind et al 1990), and studies have shown that chronic corticosterone treatment negatively affects the proliferation of NG2+ cells in the rat central nervous system (Alonso 2000). Furthermore, previous studies have indicated that mood stabilizers can prevent the glial reduction found in patients with major depression (Bowley et al 2002). Our data, which show that ECS causes structural changes to the amygdala by increasing the proliferation of glial cells, add to the growing numbers of studies suggesting that antidepressant treatment can counteract degenerative processes associated with affective disorders.

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