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Hellsten, Johan

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Electroconvulsive Seizures Induce Endothelial Cell Proliferation in Adult Rat Hippocampus

Johan Hellsten, Malin Wennström, Johan Bengzon, Paul Mohapel, and Anders Tingström

Methods and Materials

Animals and Design of Study

The general layout of the experimental procedures performed is shown in Figure 1.

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 endothelial cell and neural precursor proliferation after ECS, a dose-response study was designed where the rats were assigned to the following groups: Control (n = 6), one ECS (n = 5), three ECSs (n = 6), and five ECSs (n = 6). The time course of proliferation of endothelial and neural precursor cells after one ECS was investigated in a time-course study where the rats were assigned to the following groups: Control (n = 4), day 0 (n = 6), day 2 (n = 6), day 4 (n = 6), day 6 (n = 5), and day 8 (n = 6). A survival study was performed to determine the fate of the endothelial cells and channeled cells in the SGZ proliferating in response to ECSs. Rats were assigned to the following groups: Control (n = 5) and five ECSs (n = 5).

Administration of Electroconvulsive Seizures

On the first day of the dose-response study (day 0), all rats were subjected to either a single ECS trial or a sham treatment at 1400. Electroconvulsive seizures were delivered via silver electrode ear clips (Bionic Sales AB, Sweden) (50 mA, 0.5 seconds, 50 Hz unidirectional square wave pulses). The rats were monitored after ECS to ensure that clonic movements of the face and forelimbs (indicative of limbic motor seizures) occurred for a minimum of 20 to 30 seconds. Rats receiving three ECSs and five ECSs trials were given the remaining treatments once daily on days 1 and 2 or 1 through 4, respectively. Control rats were sham-treated (i.e., Juan.

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Dose response study

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Administration of Bromodeoxyuridine

Bromodeoxyuridine (BrdU) (B5002; Sigma-Aldrich, St. Louis, Missouri) was dissolved in phosphate buffered saline and administered intraperitoneally. All rats in the dose-response and survival study received 10 injections of BrdU (100 mg/kg) in 12-hour intervals (900 and 2100), during days 2 through 6 after the initial ECS treatment. The rats in the time-course study were given four injections of BrdU (100 mg/kg) in 2-hour intervals (900, 1100, 1300, and 1500), either the same day or 2, 4, 6, or 8 days after the single ECS trial.

Tissue Preparation

Twenty hours (in the time-course study), 5 days (in the dose-response study), or 21 days (in the survival study) after the last injection of BrdU, the rats were anesthetized with sodium pentobarbital (60 mg/ml). In the absence of nociceptive reflexes, the rats were transcardially perfused with 5% saline for 2 minutes, followed by 4% ice-cold paraformaldehyde for 10 minutes. Following 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 50% sucrose in phosphate-buffered saline (PBS) until they sank. Coronal sections, 60 μm thick, were cut through the midlevel hippocampus (~3.50 mm to ~4.52 mm relative to bregma) (Paxinos and Watson 1998) and stored in antifreeze cryoprotectant solution at −20°C until the immunohistochemical procedure.

BrdU/Rat Endothelial Cell Antigen-1/Laminin and BrdU/Neuron-Specific Nuclear Protein Immunofluorescence Stainings

Brain sections were rinsed (5 × 10 minutes) in 0.2 mol/L potassium phosphate-buffered saline (KPBS) and then incubated in blocking solution (KPBS + 5% normal donkey serum [NDS] [Harlan Sera-Lab, Bella, United Kingdom] + 2% Triton X-100 [Sigma-Aldrich]) for 1 hour at room temperature. Sections were then incubated with 1:25 mouse anti-rat endothelial cell antigen-1 (RECA-1) (Serotec MCA 970, Oxford, United Kingdom) in KPBS and 1:100 rabbit anti-laminin (Sigma-Aldrich, St. Louis, Missouri) in KPBS for 24 hours in darkness at 4°C. After washing with KPBS, the sections were incubated in 4% paraformaldehyde for 10 minutes at room temperature, subsequently rinsed with 0.2% Triton X-100 (modified blocking solution) for 10 minutes and 1:200 Cy-3 Donkey-anti-Rat (Jackson 711-175-152, Jackson Immuno Research, West Grove, Pennsylvania) in KPBS for 24 hours in darkness at 4°C. After washing with KPBS, the sections were incubated in 0.1 mol/L hydrazine acid (HCl) at 65°C for 30 minutes. After rinsing in KPBS (3 × 10 minutes), the sections were then exposed to blocking solution (KPBS + 5% normal donkey serum) for 1 hour in darkness at room temperature, and then incubated with 1:100 rabbit anti-BrdU (Oxford Biotechnology, ORF0930, Kidlington, United Kingdom) and 1:100 rabbit anti-laminin (Sigma-Aldrich, St. Louis, Missouri) in blocking solution for 48 hours in darkness at 4°C.

Sections were then rinsed with KPBS and subsequently incubated with 1:200 mouse anti-Neuron-Specific Nuclear Protein (Molecular Probes, Eugene, Oregon) in KPBS for 24 hours in darkness at 4°C. After washing with KPBS, the sections were incubated in blocking solution (KPBS + 5% normal donkey serum) for 1 hour in darkness at room temperature, and then incubated with 1:100 rat anti-BrdU (Oxford Biotechnology, ORF0930, Kidlington, United Kingdom) and 1:100 rabbit anti-laminin (Sigma-Aldrich, St. Louis, Missouri) in blocking solution for 48 hours in darkness at 4°C.

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sections were mounted on Poly-L-lysine coated slides, air dried, and coverslipped with glycerol-based mounting medium.

The BrdU/Neuron-Specific Nuclear Protein (NeuN) staining was performed as described above with regard to origin of antibodies (except for NeuN, below), rinsing, DNA denaturation, and blocking. The sections were exposed to the primary antibodies in blocking solution (1:100 Rat anti-BrdU + 1:100 Mouse anti-neuron-specific nuclear protein (NeuN) [MAB 377, Chemicon, Temecula, California]) for 40 hours at 4°C. After washing, the sections were incubated with the secondary antibodies in modified blocking solution (1:200 Cy-3 Donkey-anti-Rat + 1:200 Biotin Donkey-anti-Mouse) for 2 hours in darkness, at room temperature. Sections were then rinsed (5 × 10 minutes) in KPBS + before incubation with 1:200 Alexa 488 in KPBS for 2 hours in darkness, at room temperature. Sections were rinsed and mounted as previously described.

Endothelial Barrier Antigen Immunohistochemistry

Sections were rinsed in KPBS and incubated in blocking solution for 1 hour at room temperature. Sections were subsequently exposed to the primary antibody solution (blocking solution + 1:1000 Mouse anti-endothelial barrier antigen (EBA) [M7], Sternberger Monoclonals, Lutherville, Maryland) overnight at 4°C. After washing with KPBS+, the sections were incubated with the secondary antibody in modified blocking solution (1:200 Biotin Donkey-anti-Mouse) for 2 hours at room temperature. Sections were then rinsed (5 × 10 minutes) in KPBS+ before incubation with avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories Inc., Burlingame, California) in KPBS for 1 hour, at room temperature. After rinsing in KPBS, peroxidase detection was performed for 7 minutes (5 mg/mL diaminobenzidine [DAB], 5 mg/mL NCl, 0.1% hydrogen peroxide [H2O2]), followed by three rinses in KPBS, mounting, dehydration, and coverslipping.

Data Quantification and Statistical Analysis

Coronal 40 µm sections through the middorsal hippocampus (~–3.30 mm to +4.52 mm, relative to bregma) (Paxinos and Watson 1986) were analyzed by observers blinded to the treatments. Cell proliferation was assessed in the GCL (including the SGZ), hilus, and molecular layer of the dentate gyrus, using an Olympus AX70 fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan) with a 40 × objective. Proliferated endothelial cells were counted in the granule cell layer, hilus, and molecular layer. Cells lying within two cell diameters of the granule cell and hilar border were included in the granule cell layer count. Every 4th section throughout the middorsal hippocampus (averaging eight sections from each animal) was counted, and these values were averaged and expressed as means per section.

Endothelial cells in the GCL not associated with clusters were counted in a regular fluorescence microscope as described above; however, for cells in the subgranular zone appearing in tight clusters, no distinction was made between endothelial cells and other cell types, such as neural precursors, while counting in the fluorescence microscope. The percentage of the cluster cells (endothelial plus neuron) in the zero and five ECS groups from the dose-response study expressing endothelial cell markers was determined by confocal analysis using a Nikon confocal microscope (Nikon, Tokyo, Japan) with a 40 × objective and BioRad software (BioRad, Burlington, Massachusetts), in which 20 clusters per animal (four animals from each group) were analyzed for possible BrdU/RECA-1 double-labeling.

Data are presented as means ± SEM and were analyzed with analysis of variance (ANOVA) and Bonferroni/Dunn post hoc test. Statistical significance was set at p < .05.

Results

Vascular Cells Display an Endothelial Phenotype

Rat endothelial cell antigen (RECA-1) immunoreactivity is expressed on the luminal side of endothelial cells (Dai et al. 1992), while the basement membrane enclosing the entire vessel structure expresses laminin (Barsky et al. 1985); between these two layers lie endothelial cells and pericytes. The pericytes are completely surrounded by the basement membrane (Abbott et al. 1992), while the endothelial cells are limited by the basement membrane at the abluminal side and RECA-1 positive structures at the luminal side. Utilizing confocal microscopy, cell nuclei were denoted as being of vascular origin by defining them by the following morphologic criteria: flattened and slightly cupped, somewhat elongated, and in immediate contact with the RECA-1 stained capillary luminal surface. Using confocal microscopy and analyzing a subset (n = 100) of vessel-associated BrdU-stained nuclei, we noted that all cells defined as vascular cells, as described above, were enclosed by RECA-1 and laminin positive structures, and we therefore concluded that this cell type is indeed of vascular origin and of an endothelial phenotype (Figure 2).

ECS Increase Endothelial Cell Proliferation

A single electroconvulsive seizure increased the proliferation of endothelial cells in the GCL, hilus, and molecular layer (ML). Proliferation was further enhanced by three and five electroco-

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vulsive seizures (Figures 3 and 4). In agreement with an earlier study (Madsen et al 2000), this effect was also noted on clustered cells located in the SGZ (Figure 3 and 4), expressing either the early neuronal marker doublecortin (data not shown) or the endothelial marker RECA-1.

**Endothelial and Cluster Cells Proliferate in Concert in Response to ECS**

In response to a single electroconvulsive seizure, we detected a profound increase in endothelial cell proliferation in the GCL, hilus, and ML at day 2, with a very marked increase in the number of BrdU-labeled endothelial cells (Figure 5). No significant increases in endothelial cell proliferation relative to control could be detected at any of the other time points studied (Figure 5). In agreement with previously described results by Madsen et al (2000), the proliferation of cluster cells in the SGZ reached a maximum between days 3 and 5 (day 4) (Figure 5). At day 2, the rate of endothelial cell proliferation in all regions except the hilus was approximately 14 times higher than in the control animals, while the rate of proliferation of cluster cells in the SGZ at day 4 was approximately 3 times higher compared with control. We conclude that the nonclustered endothelial cells proliferate at a faster rate in response to a single ECS than the cluster cells located in the SGZ.

**Increased Fraction of Endothelial Cluster Cells after ECS**

The fraction of cells in the SGZ located in clusters, double-labeled with BrdU and RECA-1, was determined by confocal microscopy analysis. In four animals, each from the zero ECS and five ECS groups in the dose-response study, a total number of 87 ± 18.8 and 117 ± 23.2 cells, respectively, were analyzed per animal. The average cluster size in the zero ECS group was significantly smaller than in the five ECS group (4.6 ± 2 and 5.8 ± 2 cells per cluster, respectively).

**Cell Fate of the Proliferating Cells 3 Weeks after ECS**

To determine the fate of the cells proliferating after zero or five ECSs, rats were given BrdU at days 2 through 6 after the first ECSs. Twenty-one days after a series of five ECSs, we detected significantly elevated numbers of BrdU-labeled cells in the SGZ and GCL (Figures 7 and 8), of which approximately 80% colabeled with the neuronal marker neuron-specific nuclear protein (NeuN) (Figure 9). This finding is in direct agreement with what has previously been reported (Hellsten et al 2002; Madsen et al 2000). We also note that the number of BrdU/RECA-1-labeled cells in the GCL, hilus, and ML of rats receiving five ECSs are also still elevated compared to control (Figures 7 and 8).
We detected no differences in staining intensity of EBA between control and ECS-treated animals in either the time-course study (control and days 0 through 4) (Figure 10) or the dose-response study (zero and five ECSs) (data not shown). As a positive control for the staining, we used brain sections from rats subjected to 2 hours of middle cerebral artery occlusion (MCAO). These animals display extensive and profound loss of EBA expression in regions of the brain ipsilateral to the insult (Figure 10).

No Reduction of EBA-Expression after ECS

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Discussion

We show that electroconvulsive seizures, which previously have been shown to induce a marked increase in proliferation of neural precursors residing in the SGZ, also induce a very pronounced increase in proliferation of hippocampal endothelial cells. The seizure-induced proliferation of endothelial cells and neural precursors occurs in concert and the effect is dose-dependent, meaning that the number of proliferating endothelial cells increases with the number of electroconvulsive seizures administered.

Electroconvulsive seizures are associated with an intense activity in hippocampal neural networks. Despite extensive studies, cell death, as regularly seen after prolonged epileptic seizures (i.e., status epilepticus) (Kondratyev et al 2001) has not been observed after ECS. Hallmarks of physiologic hippocampal activation, such as neurotrophin regulation, mossy fiber sprouting, synaptic remodeling, and neurogenesis, have all been described after ECS (Duman and Vaidya 1998; Madsen et al 2000; Stewart and Reid 2000). The magnitude of these molecular, subcellular, and cellular changes is generally greater after ECS than during normal physiologic hippocampal activity.

The causes and consequences of the dramatic endothelial cell proliferation reported in this study are not known, but it is tempting to speculate that increased neural network activity and remodeling, in response to ECS, may lead to a concomitant angiogenic response. Whether the endothelial cell proliferation is followed by an expansion of the vascular tree is yet to be determined. Quantitative stereological analyses to assess possible ECS-induced topological changes in the hippocampal vasculature are currently underway. It is, however, important to recognize that apart from the obvious role of angiogenesis in meeting an increased metabolic...
The number of BrdU-labeled endothelial cells detected in the GCL, hilus, and ML after five ECSs are still significantly elevated 21 days after the last BrdU injection. The number of BrdU-labeled cells in the SGZ is also still elevated. Values represent mean ± SEM number of cells detected per coronal section of the dentate gyrus. Data were analyzed with unpaired *t*-test (*, **, ***: p < 0.05, 0.01, 0.001, respectively). A substantial amount of the proliferating cells in the SGZ express the endothelial cell marker RECA-1. When analyzing clustered, newly proliferated cells within the SGZ, we found that in control animals approximately 9% of these cells are BrdU/RECA-1 positive. This fraction increased to 19% after a series of five electroconvulsive seizures. The vast majority of these BrdU-labeled cluster cells are not positive for RECA-1 but are instead grouped around and in close proximity to capillaries in the SGZ, still allowing for close contact between the vasculature and the neuronal progenitors (Figure 6). We believe that the increase in the fraction of RECA-1 positive cells per cluster noted after five ECSs is a reflection of the fact that, as compared to neutral precursors, endothelial cells proliferate at a higher rate in response to ECS.

It is important to emphasize that proliferating endothelial cells are not only seen in the SGZ but along the full extent of capillaries, originating from larger vessels bordering the ML and extending through ML, GCL, and into the hilus. Although most numerous in ML, proliferating endothelial cells are thus detected in all dentate gyrus subfields, indicating a mature neuronal phenotype are indicated by arrowheads. Scale bar, 45 μm. BrdU, bromodeoxyuridine; NeuN, neuron-specific nuclear protein.

**Figure 9.** Many cluster cells become neurons. Confocal image of BrdU-labeled (red) and NeuN-positive (green) cells in the granule cell layer of a rat surviving for 21 days after receiving five electroconvulsive seizures. Bromodeoxyuridine and NeuN double-labeled cells indicating a mature neuronal phenotype are indicated by arrowheads. Scale bar, 45 μm. BrdU, bromodeoxyuridine; NeuN, neuron-specific nuclear protein.
detect a fifteenfold to fiftyfold increase in endothelial cell proliferation in the various layers of the dentate gyrus analyzed after a series of ECSs. As described above, this increase in endothelial cell proliferation in the nonneuronal zones could also affect neuronal function in these regions. The ML harbors the afferent synapses of the dentate gyrus and the efferent synapses of hilar and granule cells. The hilus contains the dendrites of the granule cells, while the hilus contains axons from the dentate hilar neurons in the CA3 region.

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This work was supported by grants from 1) the Swedish Lundbeck Foundation, 2) the Spelvik Foundation, 3) the Guide- lines for the Conduct of Experimental Research and Ethics in the Lundbeck Research Council. We thank Professor Olle Lindvall for insightful comments on the manuscript.

Kastratos T, Stoll JM, Huguet M, Alon P, Vazquez M, et al. (2001): Endothelial cell proliferation detected in response to ECSs leads to an expansion of the vascular tree, alterations that could be important for the very profound increase in endothelial cell proliferation associated with major depression. Biol Psychiatry 47:1043–1049.

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