

Kindling-induced neurogenesis in the dentate gyrus of the rat

B.W. Scott^a, S. Wang^a, W.M. Burnham^{b,c}, U. De Boni^a, J.M. Wojtowicz^{a,*}

^aDepartment of Physiology, University of Toronto, Medical Sciences Building, Toronto, Ontario, Canada M5S 1A8

^bDepartment of Pharmacology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

^cBloorview Epilepsy Research Program, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Received 23 February 1998; accepted 21 March 1998

Abstract

Kindling, a form of neuronal plasticity produced by repeated low intensity electrical brain stimulation, leads to epileptic seizures. To address possible causes of this phenomenon, we have prepared amygdala-kindled animals and measured neurogenesis, by bromodeoxyuridine incorporation. Early, when focal seizures were present, there was no evidence of a change in the rate of hippocampal neurogenesis. In contrast, during the later phases of kindling, when secondary generalization was well established and motor seizures were present, neurogenesis was enhanced by 75–140%, depending on the hippocampal region. Double labelling with the neuron-specific marker TOAD-64 demonstrated the presence of numerous new-born granule neurons in the kindled animals. We propose that the newly-born neurons contribute to the cellular changes and behavioral symptoms associated with this type of epileptiform brain plasticity. © 1998 Elsevier Science Ireland Ltd. All rights reserved

Keywords: Epilepsy; Kindling; Neurogenesis; Hippocampus; Dentate gyrus; Granule neuron

The amygdala kindling model has been widely used for studies of epilepsy [8,16]. Repeated, temporally spaced, electrical stimulation of the amygdala leads to the development of secondarily propagated after-discharges in the hippocampus and the eventual onset of behavioral seizures.

The relevance of numerous reported physiological and anatomical changes associated with kindling to seizure development is still not well understood. The dentate gyrus of the hippocampal formation shows a number of these changes. Axonal sprouting from dentate granule neurons, for instance, results in the reorganization of hippocampal circuitry, with abnormal synapses being formed by mossy fibers in the molecular layer, hilus and CA3 [17]. These abnormal connections may ultimately lead to a lowered seizure threshold [12]. Parent et al. [14] have found that chemically-induced seizures or long, intense electrical stimulation of the hippocampus promote dentate granule cell proliferation, possibly as a compensatory response induced by tissue injury. This idea is supported by a study of Beng-

zon et al. [1] who has found that even a single episode of hippocampal electrical stimulation produces cell death in the granule cell layer, which in turn, is followed by cell proliferation. Given that adult neurogenesis in rats and other species can change in response to environmental and neural stimuli [9,10] we have measured neurogenesis during the traditional amygdala kindling procedure to determine whether enhanced proliferation of new neurons is one of the features of this kindling phenomenon.

Adult male Wistar rats (Charles River) were amygdala kindled according to the standard procedures described previously [3]. Age-matched control animals were surgically implanted with electrodes and handled identically but were not stimulated. In order to explore the relationship between progression of the kindling phenomenon and the neuronal proliferation, we divided the stimulated subjects into 'Early' and 'Late' groups. 'Early' stimulated subjects received bromodeoxyuridine (BrdU; 100 mg/kg) injections on the 2nd, 3rd and 4th stimulation days. 'Early' control subjects received matched BrdU injections. 'Late' stimulated subjects received BrdU injections on the days of their 2nd, 3rd and 4th stage 5 behavioral seizures as defined by Racine [15]. 'Late' controls received matched BrdU injections at

* Corresponding author. Tel.: +1 416 9782899; fax: +1 416 9784940; e-mail: martin@spine.med.utoronto.ca

these times. The number of cells which incorporated BrdU was used as an index of cell mitosis.

Animals were killed by decapitation 7 days after the third and final BrdU injection, the hippocampus ipsilateral to the electrode was dissected out and fixed. Hippocampi were cut into dorsal, middle and ventral thirds and sectioned at 15 μm . Six random, free-floating sections from each hippocampal third were processed for BrdU immunodetection [14].

Positively labelled nuclear profiles were counted in the molecular layer, granule cell layer (GCL) and hilus of the dentate gyrus. Most of the labelled large nuclei (>5 μm diameter) located in the GCL and hilus were assumed to be nuclei of granule neurons [6]. Area measurements were combined with absolute cell counts to obtain cell density estimates for each hippocampal section. These estimates are likely to be valid when the averaged sizes of the counted nuclei are equal in the compared groups of subjects [7]. We have verified this assumption by direct measurements of nuclear diameters in control and experimental groups [11].

To achieve the double labelling of BrdU and a neuronal-specific marker, the Turned-On After Division 64kDa (TOAD-64) protein [13], the tissue was fixed in 4% phosphate buffered paraformaldehyde and later processed for both the protein immunocytochemistry and the BrdU detection [14]. TOAD-64 was chosen since it labels immature neurons. Other neuronal markers may not be expressed until later in development [6]. Sections were viewed under a confocal microscope with fluorescein isothiocyanate or Texas Red filter systems for visualization of TOAD-64 and BrdU immunolabelling, respectively.

In control animals, the number of BrdU-labelled nuclei was the largest in the granule cell layer (GCL), especially near the GCL/hilus border (Fig. 1). In stimulated animals, the density of labelled nuclei in the GCL was increased significantly when compared to controls in the 'late' but not in the 'early' group (two-way ANOVA, $P < 0.05$; Fig. 2). Area measurements showed that the hilar areas had significantly increased by 10–35% in the 'late' group, suggesting an overall growth of the dentate gyrus in association with kindling (two-way ANOVA, $P < 0.05$) as shown previously [2]. In contrast, in the GCL region, increases were not significant. High magnification of nuclei labelled with BrdU shows their correspondence in shape and size to surrounding neurons, in agreement with previous reports [6,14]. The identity of the labelled cells in the hilus and molecular layer is less certain. Most of the BrdU-labelled nuclei probably belong to glial cells since no TOAD-64 reactive cell bodies were seen in these regions (see below).

In consideration of previous reports of regional differences in cell proliferation [5], we also separately measured the number of labelled nuclei in the dorsal, middle and ventral thirds of the hippocampal regions. In control animals, the density of proliferating cells in the ventral GCL was about 50% lower in comparison to middle and dorsal

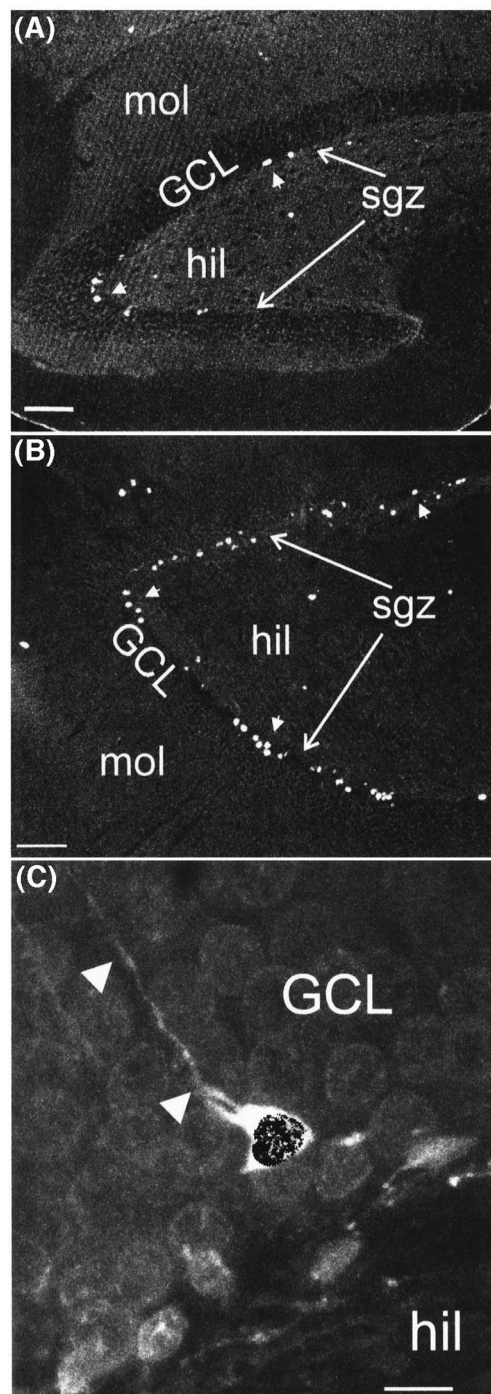


Fig. 1. Examples of BrdU positive nuclei in hippocampal sections from control (A) and kindled (B) rats, respectively. Most labelled nuclei (arrow heads) in control animals were near the border between the hilus (hil) and the granule cell layer (GCL) of the dentate gyrus. Kindling appeared to increase the rate of cell proliferation, as indicated by higher numbers of BrdU labelled nuclei, in GCL, particularly along the subgranular zone (sgz). Hilus and the molecular layer (mol) also showed a few labelled nuclei. Scale bar, 100 μm . (C) Double-labelled cell in a kindled animal showing BrdU immunoreactivity in the nucleus and TOAD-64, neuron specific, immunoreactivity in the cell body and the dendrite (arrow heads). Scale bar, 10 μm .

portions of the hippocampus. However, the proportional increase of cell density after kindling was the greatest in the ventral GCL.

The neuron-specific TOAD-64 immunoreactivity was dramatically stronger in sections obtained from kindled animals. The labelling was uniformly heavy and predominantly localized to the inner edge of the GCL, presumably corresponding to cell bodies of newly-born neurons. Sections double-labelled for TOAD-64 and BrdU revealed that many of the BrdU-labelled nuclei were localized within the band of granule cells labelled with TOAD-64. These cells could be unequivocally identified as immature granule neurons by their location, size and presence of dendritic processes (Fig. 1C). This is in agreement with previous studies showing that a majority of new-born cells in GCL are neurons and not glia [6,10,14].

Clustering and tight packing of fluorescent cell bodies and processes prevented us from counting the individual young cells. However, preliminary densitometric analysis of the fluorescent material performed on a subset of four stimulated and three control animals revealed a consistent

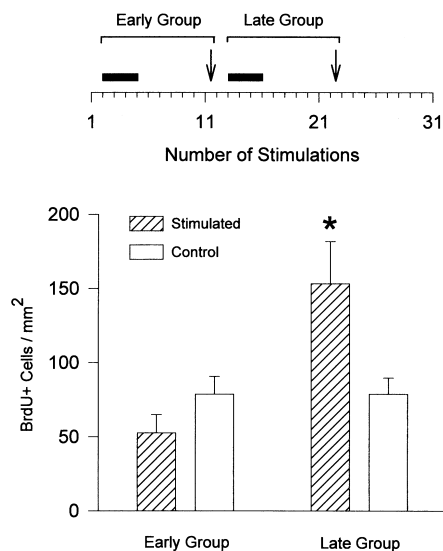


Fig. 2. Density of BrdU positive nuclei in granule cell layer (GCL) was significantly increased in the 'late' group of stimulated animals. The 'late' stimulated group ($n = 10$ animals) was different from the 'late' control ($n = 10$ animals), 'early' control ($n = 7$ animals) and 'early' stimulated ($n = 7$ animals) groups. Statistical significance was measured using two-way ANOVA and pairwise multiple comparison procedure at $P < 0.05$ (SE are also shown). Only the results from the middle hippocampal region are illustrated. Other regions also showed significant increases. The top graph illustrates the experimental protocol. Animals were stimulated once daily starting at day 1. Each animal in the 'early' group was injected with BrdU for 3 consecutive days (filled bar) 15 min prior to each kindling stimulation and all early animals were killed on day 11, 7 days after the last BrdU injection. In the 'late' group, animals were injected for 3 days starting on the day following the first stage 5 seizure. The time of the first such seizure varied in different animals but typically occurred on day 12. Animals in 'late' group were killed exactly 7 days after their last BrdU injection, i.e. approximately 24 h after the last recorded seizure.

increase after kindling. The average increase was $143\% \pm 60$ (SE) over the average control value. The measured intensity of immunoreactive cells may represent the increased number of neurons or the increase in the fluorescence per cell.

These results show that kindling-induced seizures produce marked changes in the rate of neurogenesis in the dentate gyrus. In the 'late' stimulated group, where the hippocampus would have experienced repeated episodes of reactive discharge, significantly increased neurogenesis was seen in the dentate gyrus. The mechanism responsible for the increase is not understood.

Stressful experience and manipulation of cortisol levels have been shown to reduce granule cell production [4,9]. It is therefore unlikely that stress associated with seizure is acting to cause the increase in granule cell proliferation found here. In preceding studies, using pilocarpine-induced status epilepticus [14] and strong electrically-induced discharges in the hippocampus [1], the enhanced neurogenesis may have been the result of the transient cell damage or apoptotic death. In our study, however, the stimulated animals experienced only a single amygdala stimulation per day and experienced only a few behavioral seizures during the time of BrdU injections. Extensive excitotoxic tissue damage is thus unlikely and none was evident in the tissue samples, although a subtle tissue damage cannot be ruled out.

In summary, the increased proliferation of granule neurons during kindling confirms that neurogenesis in the dentate gyrus is responsive to a variety of neural stimuli. In the conditions of excessive neuronal activity, such as in epilepsy, new-born granule neurons may contribute to abnormal reorganization of synaptic circuitry and aggravate the extent of seizures through the formation of ectopic axonal collaterals and excitotoxic synapses.

We wish to thank Mr. A. Mendonca for technical support, and MRC of Canada and the Bloorview Childrens Hospital Foundation for financial assistance. The TOAD-64 anti-bodies were generously donated by Dr. S. Hockfield, Yale University, New Haven, CT, USA.

- [1] Bengzon, J., Kokaia, Z., Elmer, E., Nanobashvili, A., Kokaia, M. and Lindvall, O., Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures, *Proc. Natl. Acad. Sci. USA*, 94 (1997) 10432–10437.
- [2] Bertram, E.H. and Lothman, E.W., Morphometric effects of intermittent kindled seizures and limbic status epilepticus in the dentate gyrus of the rat, *Brain Res.*, 603 (1993) 25–31.
- [3] Burnham, W.M., Primary and 'transfer' seizure development in the kindled rat, *Can. J. Neurol. Sci.*, 2 (1975) 417–428.
- [4] Cameron, H. and Gould, E., Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus, *Neuroscience*, 61 (1994) 203–209.
- [5] Cameron, H., McEwen, B. and Gould, E., Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus, *J. Neurosci.*, 15 (1995) 4687–4692.
- [6] Cameron, H.A., Woolley, C.S., McEwen, B.S. and Gould, E.,

- Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat, *Neuroscience*, 56 (1993) 337–344.
- [7] Coggeshall, R.E. and Lekan, H.A., Methods for determining numbers of cells and synapses: a case for more uniform standards of review, *J. Comp. Neurol.*, 364 (1996) 6–15.
- [8] Goddard, G.V., Development of epileptic seizures through brain stimulation at low intensity, *Nature*, 214 (1967) 1020–1021.
- [9] Gould, E., McEwen, B.S., Tanapat, P., Galea, L.A.M. and Fuchs, E., Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation, *J. Neurosci.*, 17 (1997) 2492–2498.
- [10] Kempermann, G., Kuhn, H.G. and Gage, F.H., More hippocampal neurons in adult mice living in an enriched environment, *Nature*, 386 (1997) 493–495.
- [11] Kuhn, H.G., Dickinson-Anson, H. and Gage, F.G., Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation, *J. Neurosci.*, 16 (1996) 2027–2033.
- [12] McNamara, J.O., Pursuit of the mechanisms of kindling, *Trends Neurosci.*, 11 (1988) 33–36.
- [13] Minturn, J.E., Geschwind, D.H., Fryer, H.J.L. and Hockfield, S., Early postmitotic neurons transiently express TOAD-64, a neural specific protein, *J. Comp. Neurol.*, 355 (1995) 369–379.
- [14] Parent, J.M., Yu, T.W., Leibowitz, R.T., Geschwind, D.H., Sloviter, R.S. and Lowenstein, D.H., Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus, *J. Neurosci.*, 17 (1997) 3727–3738.
- [15] Racine, R., Kindling: the first decade, *Neurosurgery*, 3 (1978) 234–252.
- [16] Sato, M., Racine, R.J. and McIntyre, D.C., Kindling: basic mechanisms and clinical validity, *Electroenceph. clin. Neurophysiol.*, 76 (1990) 459–472.
- [17] Sutula, T., Xiao-Xian, H., Cavazos, J. and Scott, G., Synaptic reorganization in the hippocampus induced by abnormal functional activity, *Science*, 239 (1988) 1147–1150.