Differential responses of endogenous adult mouse neural precursors to excess neuronal excitation

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Abstract

Adult neurogenesis in the subgranular zone of the hippocampus (SGZ) is enhanced by excess as well as mild neuronal excitation, such as chemoconvulsant-induced brief seizures. Because most studies of neurogenesis after seizures have focused on the SGZ, the threshold of neuronal excitation required to enhance neurogenesis in the subventricular zone (SVZ) is not clear. Therefore, we examined the responses of SVZ precursors to brief generalized clonic seizures induced by a single administration of the chemoconvulsant pentylenetetrazole (PTZ). Cell cycle progression of precursors was analysed by systemic administration of thymidine analogues. We found that brief seizures immediately resulted in cell cycle retardation in the SVZ. However, the same effect was not seen in the SGZ. This initial cell cycle retardation in the SVZ was followed by enhanced cell cycle re-entry after the first round of mitosis, leading to precursor pool expansion, but the cell cycle retardation and expansion of the precursor pool were transient. Cell cycle progression in the PTZ-treated group returned to normal after one cell cycle. The numbers of precursors in the SVZ and new neurons in the olfactory bulb, which are descendants of SVZ precursors, were not significantly different from those in control mice more than 2 days after seizures. Because similar effects were observed following electroconvulsive seizures, these responses are likely to be general effects of brief seizures. These results suggest that neurogenesis in the SVZ is more tightly regulated and requires stronger stimuli to be modified than that in the SGZ.

Introduction

There are two neurogenic regions in the adult mammalian brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. Neural precursors, including stem cells and progenitors, reside in these regions and produce neurons throughout a mammal’s lifetime. There are at least three different precursor cell types in the adult SVZ: type B, relatively quiescent stem cells; type C, transitory amplifying cells undergoing active proliferation; and type A, proliferating neuroblast cells. After sequential differentiation from type B to C to A, precursors migrate to the olfactory bulb (OB) through the rostral migratory stream (Mori et al., 2005). It is known that the precursor properties, structures and microenvironments of neurogenic niches in the SVZ and SGZ are different. For example, the culturing conditions for and proliferation properties of these populations of cells (Walker et al., 2008), their cellular compositions (Doetsch et al., 1997; Kempermann et al., 2004) and the instructive cues required for precursor differentiation (Suhonen et al., 1996) are different. Endogenous precursors are a promising target for therapeutic strategies to replace neurons damaged by injury or neurodegenerative diseases. Understanding the mechanisms of cell cycle regulation and the differentiation of endogenous precursors is important, but these have not been thoroughly studied.

SGZ precursors in the adult hippocampus proliferate in response to both non-harmful and mildly harmful events, such as stress, ageing, learning, environmental enrichment and physical exercise, as well as strong pathological events, such as stroke, traumatic brain injuries and epileptic seizures (Fabel & Kempermann, 2008; Kernie & Parent, 2010). Epilepsy is the most common disorder of the brain and is triggered by abnormal neuronal excitation. In various animal models of epilepsy, such as kindling and status epilepticus, neurogenesis is enhanced in the hippocampus (Bengzon et al., 1997; Parent et al., 1997). These epilepsy models also essentially exhibit neuronal cell death (Cavazos et al., 1994; Kasof et al., 1995).

Pentylenetetrazole (PTZ) is a chemoconvulsant that acts as a gamma-aminobutyric acid (GABA)-A receptor antagonist. Systemic administration of PTZ induces various types of generalized seizures in a dose-dependent manner (Andre et al., 1998). Brief seizures induced by a single administration of PTZ are much milder than status epilepticus and kindling, and brief-seizure model animals show no apparent neuronal death (see references in Kasof et al., 1995). PTZ-induced brief seizures also dramatically enhance neurogenesis in the adult rat dentate gyrus of the hippocampus (Jiang et al., 2003).

Most studies of the relationship between epilepsy or seizures and neurogenesis have focused on the hippocampus (Bengzon et al., 1997; Parent et al., 1997). Although status epilepticus enhances neurogenesis...
in the SVZ–OB system (Parent et al., 2002), as it does in the SGZ, it is unclear whether brief seizures also have an effect on adult SVZ precursors. Therefore, the purpose of this study was to examine the responses of SVZ neural precursors to brief seizures in the short and long term.

Materials and methods

Animals

Six- or 7-week-old male ICR mice were used in all experiments. Mice were supplied by Japan SLC Inc. (Hamamatsu, Japan). In total, 208 mice were used. The experimental protocols were approved by the animal ethics committee at Kansai Medical University and were performed in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Drugs

5-Bromo-2′-deoxyuridine (BrdU; Sigma, St Louis, MO, USA) and PTZ (Sigma) were dissolved in phosphate-buffered saline (PBS) at 10 mg/mL. Because of the lower solubility of 5-iodo-2′-deoxyuridine (IdU; Tokyo Kasei, Tokyo, Japan), it was dissolved in 0.014 M NaOH/PBS at 6 mg/mL. All drugs were administered intraperitoneally (i.p.). The dose of PTZ required to induce seizures in mice was determined before starting this study. PTZ was administered at a dose of 60 mg/kg (body weight), and only mice that exhibited brief generalized clonic seizures were used in experiments. BrdU (molecular weight = 307.1) and IdU (molecular weight = 354.1) were used to label proliferating cells. BrdU was administered at doses of 50 and 100 mg/kg for SVZ and SGZ analysis, respectively. IdU was administered at a dose of 115.3 mg/kg, equimolar to 100 mg/kg of BrdU, for SGZ analysis.

Experimental protocol 1

Cell cycle progression, migration and the long-term fate of SVZ precursors were analysed after PTZ-induced seizures using the following protocol. BrdU was administered 10 min after PTZ administration. An identical volume of PBS was administered to control mice. Mice were fixed at the following time points – 1, 2, 3, 4, 14, 16, 18 and 20 h, 3 and 7 days, and 4 weeks after BrdU administration.

Experimental protocol 2

The cell cycle kinetics of SGZ precursors were analysed by sequential administration of IdU and BrdU, as described previously (Burns & Kuan, 2005). Briefly, IdU was administered 10 min after PTZ administration, and an administration of BrdU followed 3 or 6 h later. Mice were fixed 30 min after BrdU administration. An identical volume of PBS substituting for PTZ was administered to control mice.

Experimental protocol 3

The number of proliferating precursors in the SVZ was analysed after PTZ-induced seizures using the following protocol. BrdU was administered 2 or 4 days after PTZ administration. Mice were fixed 2 h after BrdU administration. Control mice were administered an identical volume of PBS.

Experimental protocol 4

Surplus precursors were labelled by BrdU after PTZ-induced seizures using the following protocol. BrdU was administered 20, 24, 28 and 32 h (total four administrations per animal) after PTZ administration. Control mice were administered an identical volume of PBS. Mice were fixed 4 weeks after the last BrdU administration.

Experimental protocol 5

Electroconvulsive seizures (ECS) were induced by applying a single shock (80 V for 0.3 s) through the corneas without anaesthetic. After electrical stimulation, mice immediately exhibited generalized clonic or tonic clonic seizures for several seconds. BrdU was administered 10 min after the induction of ECS. Mice were fixed 2 or 20 h after BrdU administration.

Histological procedures

For histological analysis, mice were deeply anaesthetized with pentobarbital (50 mg/kg) and perfused transcardially with PBS followed by 4% formaldehyde in PBS. The brains were removed, post-fixed with the same fixative overnight and cryoprotected with 20% sucrose in PBS. Brains were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan), snap frozen on dry ice and cut transversely using a cryostat. For analysis of the SVZ and SGZ, brains were sectioned at a thickness of 30 or 16 μm as floating sections. For analysis of the OB, OBs were sectioned at a thickness of 12 μm and attached to glass slides. The sections were then processed for immunohistochemistry.

To detect BrdU, sections were pretreated with 2 M HCl for 30 min at room temperature followed by neutralization with 0.1 M boric acid (pH 8.5) and three washes in PBS. To detect Ki67, sections were heated at 99 °C in 0.01 M citrate buffer (pH 6.0) for 10 min. For double-immunostaining with anti-BrdU and another primary antibody, sections were incubated in a primary antibody cocktail. The following primary antibodies were used – rat monoclonal anti-BrdU (Clone BU1/75, 1 : 200; Abcam, Cambridge, UK), mouse monoclonal anti-BrdU (Clone B44, 1 : 200; BD Biosciences, San Jose, CA, USA), rabbit anti-phosphohistone H3 (PH3) (1 : 600; Millipore, Billerica, MA, USA), rabbit anti-Ki67 (1 : 1000; Novocastra, Newcastle, UK), mouse anti-NeuN (1 : 200; Millipore), mouse anti-GFAP (1 : 800; Sigma), guinea pig anti-DCX (doublecortin, 1 : 2000; Millipore), sheep anti-EGF receptor (EGFR, 1 : 50; Millipore), goat anti-Iba1 (1 : 400; Abcam) and rabbit anti-calretinin (CR, 1 : 100; Millipore). Primary antibodies were detected using species-specific donkey secondary antibodies conjugated to Cy2 or Cy3 (1 : 200; Jackson ImmunoResearch, West Grove, PA, USA). To visualize nuclei, stained sections were mounted onto glass slides using a medium containing 100 mM dithiothreitol (DTT), 50% glycerol and 5 μg/mL Hoechst 33258. Apoptotic cells were detected using a TUNEL kit (Roche, Mannheim, Germany). Fluorescence images were acquired using an epifluorescence microscope (BX-9000 with a Plan Apo 4×, NA 0.20 lens; Keyence, Osaka, Japan) or a confocal microscope (LSM510-Meta; Carl Zeiss, Oberkochen, Germany). For confocal microscopic images, a Zeiss C-Apochromat 40×/1.2w lens and pinhole aperture of 1 Airy unit was used. Voxel size was 0.45 × 0.45 × 1 μm. In total, 25–28 objective sections were acquired.

Quantification analysis

All animals were coded and the codes were not broken until the end of analysis to achieve unbiased quantification. Cell counting was performed on an epifluorescence microscope (×400 magnification, Cell cycle regulation of adult SVZ precursors
Results

PTZ-induced brief seizures

In a pilot experiment, the dose of PTZ necessary to induce brief, generalized clonic seizures with a twisted posture and falling that lasted for several seconds was determined. An i.p. injection of 60 mg/kg PTZ induced brief seizures in 69.1% of mice, and the mortality rate was 2.5% (n = 81). Brief seizures were induced within 5 min in 89.3% of the mice showing seizures (n = 56). Only mice that exhibited brief seizures of this type were used. There was no significant increase in the amount of neuronal death, as determined by the pyknotic nuclear morphology revealed by Hoechst 33258 nuclei staining (data not shown) and TUNEL staining in the SVZ of PTZ-treated mice (17.80 ± 2.71 in control vs. 27.40 ± 3.86 in the PTZ, t = 5, two-tailed Welch’s t-test to compare two groups, and one-way ANOVA to compare more than three groups. Statistical significance was set at P < 0.05. Statistical analyses were performed by R.

Cell cycle retardation of SVZ precursors immediately after brief seizures

Experimental protocol 1 was used to assess cell cycle progression immediately after brief seizures. S phase precursors are labelled by BrdU. When these cells enter the G2/M phase, they become positive for PH3, a G2/M phase-specific marker (Hendzel et al., 1997). The anti-PH3 antibody stains nuclei of mitotic cells, but the staining patterns are different between G2 phase (punctate signals in the nuclei) and M phase (intense and even signal in the nuclei or highly organized chromosomes) (Hendzel et al., 1997). In the present study, we counted only M phase cells to simplify quantification. Thus, the temporal change in the ratio of BrdU and PH3 double-positive cells among all PH3+ cells [(BrdU+, PH3+)/PH3+] indicates the progression of BrdU+ cells into the M phase (Fig. 1A–D).

First, BrdU labelling efficiency was comparable between the control and PTZ-treated groups at 1 h (1115.20 ± 53.66 cells in the control vs. 1112.20 ± 29.81 cells in the PTZ; t6,255 = 0.635, P = 0.549), and at 2 h (1789.40 ± 69.55 cells in the control vs. 1845.20 ± 67.20 cells in the PTZ; t7,991 = -0.577, P = 0.580) (Fig. 2A). Moreover average numbers of PH3+ cells were not significantly different at each time point between the control and PTZ-treated groups (t6.558 = 1.977, t6.800 = 1.263, t6.766 = 1.016, t7.815 = 0.131, t8 = -0.105, t7.615 = -0.836, t7.198 = 0.815 and t7.593 = -0.193, and P = 0.089, 0.242, 0.343, 0.899, 0.353, 0.427, 0.442 and 0.852 at 1, 2, 3, 4, 14, 16, 18 and 20 h, respectively) (Fig. 2B). The (BrdU+, PH3+)/PH3+ ratios were plotted against the interval between BrdU administration and fixation. In the control group, the (BrdU+, PH3+)/PH3+ ratio reached about 70% 2 h after BrdU administration (Fig. 2C) and continued to increase about 5% per hour.

In the PTZ-treated group, the (BrdU+, PH3+)/PH3+ ratio was significantly lower than in the control group at 1 h (13.76 ± 3.43% in the control vs. 8.52 ± 0.60% in the PTZ; t7,465 = 1.596, P = 0.155) or at 4 h (81.60 ± 0.58% in the control vs. 76.95 ± 2.60% in the PTZ; t8 = 1.728, P = 0.159) (Fig. 2C). This result shows that there is obvious cell cycle retardation in PTZ-treated mice immediately after seizures.

Next, we followed cell cycle progression after the first round of mitosis. In control mice, the (BrdU+, PH3+)/PH3+ ratio decreased to about 8% 14 and 16 h after BrdU administration. The ratio began to increase at 18 h, suggesting that BrdU-labelled precursors had entered a second M phase (Figs 1C and D, and 2C). In the PTZ-treated group, the (BrdU+, PH3+)/PH3+ ratio also began to increase at 18 h (Fig. 2C). However, at the 14-, 16-, 18- and 20-h time points, the (BrdU+, PH3+)/PH3+ ratios were not significantly different between the control and PTZ-treated groups (8.52 ± 0.60% in the control vs. 10.70 ± 0.78% in the PTZ; t7,533 = -2.211, P = 0.058 at 14 h, 8.77 ± 1.16% in the control vs. 7.56 ± 0.57% in the PTZ; t5,809 = 0.945, P = 0.381 at 16 h, 20.10 ± 3.14% in the control vs.

Axioplan2; Carl Zeiss). A Zeiss Plan-NEOFLUOR 40×/1.3 oil DIC lens was used. The SVZ was analysed at the level of the anterior SVZ (anteroposterior (AP) 0–1.0 mm from the bregma). Every tenth 30-μm-thick section of the SVZ was collected and a total of three sections per animal were analysed. For quantification of the total number of Ki67-immunopositive (+) cells in the SVZ, every third 30-μm-thick section was collected (AP = -1.3 to -2.7 mm from the bregma) and a total of 15 sections per animal were analysed. For quantification of the total number of Ki67+ cells in the SVZ, every other 30-μm-thick section was pooled (AP = -1.5 to -2.5 mm from the bregma) and a total of five sections per animal were randomly analysed. For analysis of the OB, every 25th section at the centre of the OB (AP 3.8–4.7 mm from the bregma) was collected and a total of three sections per animal were analysed. Stereotaxis coordinates are based on those of Paxinos & Franklin (2001). Percentages and total numbers are expressed as mean values per animal. All data are presented as the mean ± standard error of the mean (SEM). In all experimental conditions, more than three animals were analysed. Levels of significance were determined using the two-tailed unpaired Welch’s t-test to compare two groups, and one-way ANOVA to compare more than three groups. Statistical significance was set at P < 0.05. Statistical analyses were performed by R.

Fig. 1. Experimental design used to analyse cell cycle progression with immunohistochemical techniques. (A) Immediately after BrdU administration, S phase cells are BrdU+ (green), and G2/M phase cells are PH3+ (red). (B) After 3 h, almost all PH3+ cells become BrdU+ (arrows, green and red turn to yellow; an arrowhead, PH3-only+ cell). (C) After 16 h, almost all PH3+ cells are BrdU immunonegative (–) (arrowheads, red). (D) After 18 h, some PH3+ cells are BrdU+ (arrows, yellow; arrowheads, PH3-only+ cells). Merged and stacked confocal microscopic images of the dorsolateral SVZ region are shown. CC, corpus callosum; Stri, striatum; LV, lateral ventricle. For all images, scale bar = 50 μm.
Normal cellular composition of the SVZ after brief seizures

Brief seizures affect cell cycle progression of the SVZ precursors as shown above. Each type of the SVZ precursor, type A (DCX+), B (GFAP+) and C (EGFR+) cells, has different cell cycle length. Moreover, seizures can activate microglia (Iba1+) (Vezzani et al., 2011). Thus, we examined the cellular composition of the proliferating cells in the SVZ after brief seizures by calculating the ratio of the cell type marker and PH3 double+ cells among all PH3+ cells [(marker+, PH3+) / PH3+].

Because DCX and EGFR expressions partially overlapped each other (Fig. 3A, arrows), type A and C cells were stained with the same colour (red, Fig. 3B), and the ratio of (EGFR+ and/or DCX+, PH3+)/PH3+ was calculated. The vast majority of the proliferating cells were type A and C (total about 85%), and type B cells comprised about 5% (Fig. 3C and E), consistent with a previous report (Doetsch et al., 1997). There was virtually no proliferating Iba1+ microglia (Fig. 3D and E). The cellular composition of the proliferating cells in the SVZ did not change significantly between the control and PTZ-treated groups (EGFR, \( F_{4,20} = 1.285, P = 0.309 \); EGFR+DCX, \( F_{4,20} = 2.249, P = 0.100 \); GFAP, \( F_{4,20} = 0.757, P = 0.565 \); Iba1, \( F_{4,20} = 1.427, P = 0.262 \) (Fig. 3E). These data show that brief seizures do not change cellular composition of proliferating precursors in the SVZ.

Normal cell cycling of SGZ precursors immediately after brief seizures

The SGZ precursors respond to proliferate after several days in various seizure models (Benzzon et al., 1997; Parent et al., 1997; Jiang et al., 2003; Bolteus & Bordey, 2004; Park et al., 2006), but their response is unclear immediately after seizures. The SGZ contains far fewer proliferating precursors than the SVZ, and the number of PH3+ cells is insufficient to analyse cell cycle stages precisely using protocol 1 (fewer than three PH3+ cells per 30-μm-thick section; data not shown). In a preliminary experiment, single administration of
100 mg/kg BrdU revealed about 12 BrdU+ SGZ precursors per 30-μm-thick section (data not shown). Thus, we quantified IdU- and BrdU-labelled SGZ precursors in 15 sections per animal using protocol 2 to analyse cell cycle progression in the SGZ after brief seizures. First, IdU was administered to label S phase cells. BrdU was then administered after 3 or 6 h. It is known that rat anti-BrdU antibody (clone BU1/75) recognizes BrdU, but not IdU, and that mouse anti-BrdU (clone B44) recognizes both BrdU and IdU (Burns & Kuan, 2005). Thus, the ratio of IdU-only+ cells among all+ cells (IdU-only+ cells, IdU and BrdU double+ cells, and BrdU-only+ cells) increases to approximately 50% until all IdU-labeled cells exit S phase (Fig. 4A and Fig. S2). Using this double thymidine analogue labelling method, Burns & Kuan (2005) estimated that the S phase of adult mouse SGZ precursors lasts for about 6 h. In the present study, the IdU-only+/all+ cell ratios were about 20 and 45% at 3 and 6 h, respectively, and they were consistent with previous report (Burns & Kuan, 2005). There was no significant difference between the control and PTZ-treated groups (20.63 ± 1.58% in the control vs. 22.02 ± 1.71% in the PTZ; \(t_{5.964} = -0.599, P = 0.571\) at 3 h, and 45.52 ± 1.02% in the control vs. 46.97 ± 2.53% in the PTZ; \(t_{5.964} = -0.532, P = 0.623\) at 6 h) (Fig. 4B). This result shows that there is no cell cycle retardation in the SGZ immediately after brief seizures, unlike in the SVZ.

### Enhanced precursor proliferation in the SGZ after brief seizures

Jiang et al. (2003) showed that precursor proliferation in the adult rat SGZ was enhanced between 3 and 14 days after brief seizures induced by a single administration of PTZ, and it peaked on the third day (Jiang et al., 2003). We also analysed precursor proliferation in the adult mouse SGZ by quantifying Ki67, a marker of proliferating cells, in the control vs. PTZ; \(t_{5.964} = 1.642, P = 0.144\) (Fig. 5A). At 3 days, the number of Ki67+ cells in the PTZ-treated mice was significantly higher than in the control mice (179.00 ± 13.18 in the control vs. 280.80 ± 15.05 in the PTZ; \(t_{5.966} = -4.090, P = 0.0035\)) (Fig. 5A and B). But at 7 days, the number of Ki67+ cells was not significantly different between the control and PTZ-treated group (165.00 ± 13.18 in the control vs. 136.80 ± 4.49 in the PTZ; \(t_{4.915} = 2.025, P = 0.099\)) (Fig. 5A). These data show that precursor proliferation in the adult mouse SGZ is also enhanced by brief seizures (Jiang et al., 2003).

### Transient expansion of the SVZ precursor pool after brief seizures

In general, strong pathological events that result in neuronal death, such as status epilepticus, stroke and certain brain injuries, expand the precursor pool and promote neurogenesis in the SVZ and the SGZ (Parent et al., 2002; Zhang et al., 2006). We examined whether PTZ-induced brief seizures, which are much less traumatic events than status epilepticus, also lead to expansion of the precursor pool. Importantly, as described above, cell cycle retardation occurs in the early phase.

We compared the numbers of cells undergoing cycle re-entry between the control and PTZ-treated groups 20 h after BrdU administration by calculating the ratio of Ki67 and BrdU double+ cells among all BrdU+ cells [(Ki67+, BrdU+)/BrdU+] using experimental protocol 1 (Fig. 6A). Ki67 is expressed throughout all phases of the cell cycle and its protein expression level decreases rapidly after cells exit the cell cycle (Mori et al., 2009). The (Ki67+, BrdU+)/BrdU+ ratio in the PTZ seizure group was significantly higher than in the control group (63.86 ± 3.33% in the control vs. 77.02 ± 0.33%...
in the PTZ: \( t_{5,100} = -3.933, P = 0.011 \) (Fig. 6B). This result shows that approximately 1.2-fold more precursors re-entered the cell cycle in the first round of mitosis in the PTZ-treated group compared with the control group. Consistent with this data, the total number of Ki67+ cells was also about 1.2-fold higher in the PTZ-treated group than in the control group (4570.4 ± 201.36 cells in the control vs. 5330.0 ± 148.06 cells in the control vs. 3112.67 ± 271.27 cells in the control; \( t_{5,100} = 3.338, P = 0.0039 \)) (Fig. 6C). However, at 20 h, the number of PH3+ cells was not significantly different between the control and the PTZ-treated group (Fig. 2B). Because it suggests that surplus precursors are still in the G1 and S phases at 20 h, we followed the number of PH3+ cells after 20 h. The numbers of PH3+ cells were significantly higher in the PTZ-treated group than in the control group at 22 h (127.40 ± 7.10 cells in the control vs. 176.40 ± 12.85 cells in the PTZ; \( t_{5,237} = -3.338, P = 0.016 \)) and 26 h (227.00 ± 9.42 cells in the control vs. 160.40 ± 9.70 cells in the PTZ; \( t_{5,291} = -2.470, P = 0.039 \)). But the numbers of PH3+ cells were not significantly different between the control and PTZ-treated group at 30 h (136.60 ± 7.68 cells in the control vs. 149.80 ± 6.28 cells in the PTZ; \( t_{5,697} = -1.331, P = 0.220 \)) and 36 h (122.60 ± 6.71 cells in the control vs. 145.20 ± 9.88 cells in the PTZ; \( t_{5,044} = -1.892, P = 0.100 \) (Fig. 6D)).

Next, using experimental protocol 3, we examined whether SVZ precursor expansion is a long-term process. The total number of BrdU+ cells was plotted against the interval between PTZ and BrdU administration. There were no significant differences between controls and experimental mice at 2 days (1796.40 ± 37.39 cells in the control vs. 1818.00 ± 103.00 cells in the PTZ; \( t_{5,036} = 0.197, P = 0.851 \) and 4 days (1802.40 ± 97.19 cells in the control vs. 1670.20 ± 74.19 cells in the PTZ; \( t_{5,480} = 1.081, P = 0.315 \) after PTZ administration (Fig. 6E).

These data suggest that precursor pool expansion in the SVZ immediately after brief seizures is transient and does not continue after the first round of mitosis.

**Long-term effects of PTZ-induced seizures on adult neurogenesis in the SVZ–OB system**

The mouse SVZ precursors migrate to the core of the OB taking about 1 week (Lois & Alvarez-Buylla, 1994) and differentiate into mature granule or peri-glomerular neurons.

First, using experimental protocol 1, we addressed the possibility that brief seizures affect migration of precursors. There were few BrdU+ cells in the OB immediately after BrdU administration, but BrdU-labelled precursors migrated and accumulated in the core of the OB over time (Fig. 7A and B). After precursors reached the OB, they migrated radially and differentiated into mature neurons (Fig. 7C and F). The number of BrdU+ cells was not significantly different between the control and PTZ-treated groups at 3 days (728.67 ± 24.17 cells in the control vs. 756.70 ± 30.47 cells in the PTZ; \( t_{5,100} = 0.530, P = 0.561 \)) and 7 days after brief seizures (2245.67 ± 79.81 cells in the control vs. 2162.67 ± 107.73 cells in the PTZ; \( t_{5,100} = 0.619, P = 0.569 \) (Fig. 7D).

Second, we examined whether PTZ-induced brief seizures change the differentiation fate of precursors in the SVZ–OB system in mice killed at the 4-week time point. We examined neuronal differentiation of the SVZ precursors using protocol 1 by calculating the ratio of NeuN and BrdU double+ cells among all BrdU+ cells [(NeuN+, BrdU+)/BrdU+] at 2 and 4 days after seizures. Third, using experimental protocol 4, we labelled surplus precursors in the SVZ with BrdU and their final positions in the OB were examined. The total number of BrdU+ cells was not significantly different between the controls and PTZ-treated animals (533.00 ± 148.06 cells in the control vs. 523.00 ± 118.96 cells in the PTZ; \( t_{5,734} = 0.503, P = 0.960 \) (Fig. 8A)). And there was no significant difference in the [NeuN+, BrdU+]/BrdU+ ratio between the control and PTZ-treated groups (77.74 ± 2.26% in the control vs. 75.59 ± 0.45% in the PTZ; \( t_{5,926} = 0.932, P = 0.420 \) (Fig. 8B)).

Third, using experimental protocol 4, we labelled surplus precursors in the SVZ with BrdU and their final positions in the OB were examined. The total number of BrdU+ cells was not significantly different between the control and PTZ-treated groups (3079.33 ± 236.21 cells in the control vs. 3112.67 ± 271.27 cells in the PTZ; \( t_{5,926} = -0.093, P = 0.931 \)). Also the numbers of BrdU+ cells in the granular layer of the OB (GrO) (2529.33 ± 179.90 cells in the control vs. 2541.67 ± 231.90 cells in the PTZ;
Electroconvulsion has effects similar to PTZ-induced seizures on SVZ precursors

It is known that GABA-A receptor signals regulate precursor proliferation (LoTurco et al., 1995; Fiszman et al., 1999; Liu et al., 2005; Fukui et al., 2008). Because PTZ is an antagonist of the GABA-A receptor, it is possible that the cell cycle retardation and the expansion of the precursor pool we observed in PTZ-treated mice were driven by PTZ rather than by general effects of the seizures. To clarify this issue, using experimental protocol 5, we performed the same analyses following induction of an ECS. The (BrdU+ / PH3+)/PH3+ ratio in the ECS group was significantly lower than that in the control group at 2 h (71.39 ± 2.74% in the control vs. 57.96 ± 3.35% in the ECS; t2.577 = 3.100, P = 0.015) (Fig. 9A).

At 20 h, the (Ki67+, BrdU+/BrdU+) ratio in the ECS group was significantly higher than that in the control group (63.86 ± 3.33% in the control vs. 77.35 ± 1.26% in the ECS; t6.366 = -3.790, P = 0.007) (Fig. 9B). These results show that PTZ-induced seizures and ECS have similar effects on the cell cycle progression of SVZ precursors and that cell cycle retardation and expansion of the precursor pool are general effects of seizures.

Discussion

It is known that status epilepticus affects the proliferation of precursors in the adult SGZ (Bengzon et al., 1997; Parent et al., 1997), but few studies have focused on the SVZ (Parent et al., 2002). Additionally, there has been no study examining the responses of SVZ precursors to brief seizures, a much milder event than status epilepticus. In addition, there have been no analyses of early phase responses.

Cell cycle length in mouse SVZ precursors

Understanding cell cycle kinetics is the first step toward elucidating the mechanisms underlying adult neurogenesis. The mouse has become a more useful model animal for biological studies than the rat because of the many lines of transgenic mice that are now available. The cell cycle lengths of neuronal precursors in the adult rat SVZ and SGZ and the adult mouse SGZ have already been described by many research groups (Hayes & Nowakowski, 2002; Zhang et al., 2006; Mandyam et al., 2007), but there have been few studies of the adult mouse SVZ (Morshed & van der Kooy, 1992).

The present study found that the length of the cell cycle in adult mouse SVZ precursors is about 17 h. This is much longer than the 12.7 h estimated by Morshed & van der Kooy (1992). There are two
Early phase response of SVZ precursors after brief seizures

It is known that GABA-A receptor signals regulate precursor proliferation, but their effects are controversial. These signals can act as both positive (Fiszman et al., 1999; Fukui et al., 2008) and negative regulators (LoTurco et al., 1995; Liu et al., 2005). This inconsistency is likely to be dependent on the experimental conditions. Regardless, it is likely that irregular cell cycle progression after brief seizures is independent of PTZ and that it is a general effect of seizures because similar effects were observed both in PTZ-treated and in ECS groups.

Because cell cycle retardation occurs immediately after brief seizures, it is unlikely that de novo protein synthesis is responsible for cell cycle retardation. Depolarization induced by AMPA/kainate receptors negatively regulates the proliferation of embryonic cortical precursors (LoTurco et al., 1995). In addition, AMPA/kainite receptors and mGluR5 are expressed in migrating progenitors in the adult SVZ (Platel et al., 2008). Thus, it is possible that seizures enhance glutamate release from neurons and astrocytes and that the activation of glutamate receptors inhibits cell cycle progression. Moreover, nitric oxide (NO) generation is immediately upregulated following seizures (Kaneko et al., 2002). Because NO has an inhibitory effect on the proliferation of adult SVZ precursors (Moreno-Lopez et al., 2004), NO is another candidate molecule responsible for cell cycle retardation immediately after brief seizures.

Late phase responses of SVZ precursors after brief seizures

Expansion of the SVZ precursor pool after brief seizures is not dependent on PTZ, as discussed above. Moreover, because the half-life of PTZ is estimated to be about 2 h in vivo (see references in Park et al., 2006), late phase responses must be due to the general effects of seizures.

Because brief seizures did not affect migration of the precursors, transient expansion of the precursor pool could not be a result of a migration jam. Extracellular microenvironmental cues are important for proliferation and differentiation of precursor cells (Suhonen et al., 1996). Neuronal depolarization enhances the activity and gene expression of matrix metalloproteinase (MMP)-9 and several tissue inhibitors of matrix metalloproteinases (TIMPs), resulting in degradation and remodelling of the extracellular matrix (von Gertten et al., 2003; Gursoy-Ozdemir et al., 2004). Because MMPs induce precursor proliferation in vitro (Barkho et al., 2008), it is possible that microenvironmental changes lead to precursor pool expansion after brief seizures.

Another possibility is that seizures enhance the expression of some growth factors responsible for precursor proliferation and/or survival. In the hippocampus, status epilepticus, kindling and synaptic activation upregulate expression of several growth factors including bFGF, BDNF (Isaakson et al., 1991; Indulekha et al., 2010) and growth hormone (Kato et al., 2009). These growth factors are involved in the neurogenesis, proliferation and survival of precursors (Kuhn et al., 1997; Benraiss et al., 2001). However, there was no significant increase in the number of BrdU+ cells in the OB after brief seizures. Because brief seizures are much milder than status epilepticus and kindling, these microenvironmental changes and upregulation of growth factors could be transient.

Under normal conditions, many precursors in the SVZ–OB system are eliminated by programmed cell death (PCD) before they completely mature (Biebl et al., 2000). Surplus precursors generated by brief seizures might also die, although no obvious cell loss was observed by TUNEL staining. There are at least three possibilities. (i) Surplus precursors are eliminated by apoptosis gradually, making it difficult to detect significant increases in the number of apoptotic cells in PTZ-treated mice by TUNEL staining. (ii) They die by autophagy, a type of PCD that occurs normally during development (Fimia et al., 2007). Indeed, growth factor withdrawal induces autophagy in cultured hippocampal neural stem cells (Yu et al., 2008). Thus, transient upregulation of growth factors after brief seizures could lead to autophagy of surplus precursors. (iii) Brief seizures might enhance
phagocytic activity of DCX+ cells, a recently identified function of DCX+ precursors (Lu et al., 2011). Unfortunately, autophagy and phagocytosis cannot be detected by TUNEL staining. Further studies are needed to elucidate the mechanisms underlying cell cycle regulation and survival after brief seizures.

Seizures and neurogenesis – differential responses between the SVZ and the SGZ

We detected cell cycle retardation in the SVZ but not in the SGZ immediately after seizures. In addition, in a similar experiment, Jiang et al. (2003) showed that brief seizures enhanced precursor proliferation in the SGZ that peaked on the third day after induction of seizures, and an increased number of BrdU+ mature neurons in the dentate gyrus. Moreover, neuronal excitation induces neuronal differentiation of adult hippocampal neural precursor cells in vitro culture system (Deisseroth et al., 2004; Babu et al., 2009). These findings are in sharp contrast to the transient SVZ precursor pool expansion demonstrated in the present study. These differences between the SVZ and the SGZ might arise from differences in the precursor properties or the microenvironments of the two neurogenic regions. Indeed, there is some evidence to suggest that these factors vary between the SVZ and the SGZ (Suhonen et al., 1996; Walker et al., 2008).

It is still unclear whether neuronal death is necessary for enhanced neurogenesis after seizures. Several studies suggest that neuronal death might not be required for enhanced neurogenesis in the SGZ after repeated seizures and ischemia (Jin et al., 2001; Park et al., 2006). On the other hand, our results suggest that, in the adult SVZ, strong events that can induce obvious neuronal death might be needed for the induction of differentiation and survival of precursors. Indeed, status epilepticus enhances neurogenesis in the SVZ–OB system (Parent et al., 2002).

In summary, we demonstrated that brief seizures resulted in cell cycle retardation in the adult SVZ immediately after stimulation. This inhibition was followed by a transient expansion of the precursor pool. Brief seizures did not increase the number of new neurons in the OB. Moreover, SVZ and SGZ precursors responded differently to brief seizures, and neurogenesis in the SVZ–OB system was tightly regulated.

Supporting Information

Additional supporting information may be found in the online version of this article:
Fig. S1. PTZ-induced brief seizures do not significantly induce apoptosis.
Fig. S2. Cell cycle analysis with double thymidine analogue labelling in the SGZ.
Fig. S3. BrdU-labelled precursors differentiate into mature neurons in the OB after 4 weeks.

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Abbreviations

BrdU, 5-bromo-2′-deoxyuridine; CR, calretinin; ECS, electroconvulsive seizures; GABA, gamma-aminobutyric acid; Gl, glomerular layer; GrO, granular layer of the OB; IdU, 5-iodo-2′-deoxyuridine; MMP, matrix metalloproteinase; NO, nitric oxide; OB, olfactory bulb; PBS, phosphate-buffered saline; PCD, programmed cell death; PH3, phosphohistone H3; PTZ, pentylenetetrazole; SGZ, subgranular zone; SVZ, subventricular zone.

References


