Excitatory GABAergic Activation of Cortical Dividing Glial Cells

Adult neocortex contains dividing satellite glia population even though their characteristics and functions have still remained unknown. Nestin+ /NG2+ cells as major fraction of dividing glial cells express bicuculline-sensitive γ-aminobutyric acid A (GABA_A) receptors and receive GABAergic inputs. Due to their high [Cl]_i, GABAergic activation depolarized the cells and then induced Ca^{2+} influx into them. To assess an effect of this GABAergic excitation, we looked for the expression of neurotrophic factors. Among them, we detected the expression of brain-derived neurotrophic factor (BDNF) on the cells. The level of BDNF expression was elevated after cortical ischemia, and this elevation was blocked by bumetanide, an inhibitor for NKCC1 that blocks the GABAergic depolarization. Furthermore, performing a modified adhesive removal test, we observed that the treatment of bumetanide significantly attenuated the recovery in somatosensory dysfunction. Our results may shed a light on satellite glia population in the cortex and imply their roles in the functional recovery after ischemic injuries.

Keywords: BDNF, functional recovery, GABAergic depolarization, glial cells, neuron–glia interaction

Introduction

Adult neocortex contains a large number of dividing glial cells (Rakic 1985; Levi et al. 1999; Kornack and Rakic 2001; Dawson et al. 2003; Kocketsu et al. 2003). They never differentiate into neurons (Rakic 1985, 2002a, 2002b, 2006a, 2006b; Kornack and Rakic 2001; Kocketsu et al. 2003; Au and Fishell 2006; Bhardwaj et al. 2006; Breunig et al. 2007), but recent studies implies some roles of dividing glial cells in cortical circuitry (Spalding et al. 2005; Bhardwaj et al. 2006; Mandyam et al. 2007). Actually, NG2+ cells as dividing glial cells in adult neocortex possess supportive functions for axon growth (Yang et al. 2006) and cell survival (Stallcup 2002). Therefore, it is important to analyze how adult cortical dividing glial cells communicate with cortical circuitry.

Interestingly, NG2+ cells in the adult mouse hippocampus receive excitatory γ-aminobutyric acidergic (GABAergic) inputs (Lin and Bergles 2004). The concentration of GABA increased drastically at the injured hemisphere after the unilateral focal ischemia (Phillis et al. 1994; Matsumoto et al. 1996). It can be assumed that cortical NG2+ cells may receive excitatory GABAergic inputs and play some role in the functional recovery after ischemic injuries.

From these considerations, we supposed that adult cortical NG2+ glial cells produce some neurotrophic factor upon excitatory GABAergic stimulation. Among them, brain-derived neurotrophic factor (BDNF) is the most favorable molecules because it promotes axonal growth and guidance (Lykissas et al. 2007), neuronal survival, and behavior-related plasticity (Lipsky and Marini 2007). In this study, we focused on a NG2+ cell population that also expresses nestin (Lendahl et al. 1990) and observed that excitatory GABAergic inputs toward the dividing glial cells. In addition, our results may suggest that the increase in BDNF production from cortical dividing cells by excitatory GABAergic stimulation play some roles in the functional recovery from ischemic stroke.

Materials and Methods

Animals

Nestin-promoter-green fluorescence protein (GFP) (nestin-GFP) transgenic mice (Yamaguchi et al. 2000), adult male (≥8 weeks old) C57/B6.l mice (Sankyo Lab Service, Tokyo, Japan), and male Sprague Dawley rats (Sankyo Lab Service) were used in our experiments. Experimental procedures were carried out in accordance with animal experimentation protocols approved by the Animal Care and Use Committee at the University of Tokyo.

5-Bromodeoxyuridine Incorporation Assay

The DNA base analog 5-bromodeoxyuridine (BrdU; Wako, Osaka, Japan) was injected intraperitoneally (100 mg/kg body weight in 0.007 N NaOH in phosphate buffered saline [PBS]) 2 h before perfusion to label fast proliferating cells (short pulse). Alternatively, animals received BrdU through their drinking water (long pulse, 1 mg/ml) for 5, 10, or 30 days.

Immunohistochemical Staining

Immunohistochemistry on free-floating sections was performed as previously described (Koketsu et al. 2003), as was immunocytochemistry on cultured cells (Matsumoto et al. 2003). For NG2 staining, mice were perfused with PBS followed by 2% paraformaldehyde (PFA) in PBS. Brains were postfixed with the same solution for 4–6 h and then maintained for one day in 30% sucrose-PBS. The slices were blocked by PBS containing 5% normal goat serum for 30 min and incubated with a primary antibody in the same buffer overnight at 4 °C. For BrdU staining, the cryosections were incubated in 2 N HCl for 30 min at 37 °C. After neutralized by borated buffer (pH8.5) for 10 min, they were rinsed by Tris-buffered saline and blocked and incubated in BrdU antibodies (1:200; rat-IgG; Oxford Biotechnology, Oxford, UK) overnight.

Primary antibodies and dilutions used were as follows: GFP (1:1000, rat-IgG, Nakarai Tesque, Kyoto, Japan), 1:1000, rabbit-IgG, Molecular Probes, Carlsbad, CA); NeuN (1:1000, mouse-IgG, Millipore, Billerica, MA); nestin (1:200, mouse-IgG, BD Transduction Laboratories, Franklin Lakes, NJ); 1:200, mouse-IgG, Millipore; 1:200, rabbit-IgG, a generous gift from Dr Ron Mckay); NG2 (1:500, rabbit-IgG, Millipore); Ki67 (1:500, rabbit-IgG, Novocastra, Newcastle, UK); phospho-Histone H3 (pH3; 1:500, rabbit-IgG, Millipore); parvalbumin (PV; 1:500, mouse-IgG, Sigma, St Louis, MO); vesicular GABA transporter (VGAT; 1:500, rabbit-IgG, Synaptic Systems, Gottingen, Germany); BDNF (1:250; rabbit-IgG, Santa Cruz Biotech, Santa Cruz, CA); nerve growth factor (NGF; 1:500, rabbit-IgG, Millipore); vascular endothelial growth factor (VEGF;
1:200, rabbit-IgG, Santa Cruz Biotec); netrin-1 (1:50, goat-IgG, Santa Cruz Biotec); neurotrophin 3 (NT-3: 1:250, rabbit-IgG, Santa Cruz Biotec); MAP-2 (1:500, mouse-IgG, Sigma); or secondary antibodies: Alexa 488, Alexa 568-conjugated antibodies (Molecular Probes) were used at a dilution of 1:1000, and Cy5-conjugated antibodies (Jackson ImmunoResearch, West grove, PA) were used at a dilution of 1:250. Cultured cells were counterstained with the nuclear stain 4',6'-diamino-2-phenylindole (Sigma) at a dilution of 1:1000 in order to count the cell number. Images were taken using a confocal microscope (TCS SP2; Leica, Mannheim, Germany) fitted with individual filter sets for each channel. Image production was then performed using Adobe Photoshop (Adobe System, San Jose, CA).

To check the specificity of the BDNF antibody, we performed control experiments. One was western blotting analysis. We confirmed the antibody only detected 14 kDa BDNF protein, not NGF or NT-3 proteins. The other was a control immunostaining experiment of cortical slices using the antibody preincubated with BDNF protein. In this case, we did not detect any fluorescent signal in the slices. From these experiments, we validated the specificity of this anti-BDNF antibody. Similar confirmations of the same antibody have been done by another group previously (Hayashi et al. 2001).

### Slice Preparation

Coronal (400-μm thick) brain slices from adult nestin-GFP+/- (homozygote) transgenic mice (a generous gift from Dr. Yamaguchi) were prepared as described previously (Fukuda et al. 2005; Tozuka et al. 2005). The ice-cold oxygenated solution containing the following (in millimoles [mM]): 125 NaCl, 2.5 KCl, 2 CaCl2, 1.5 MgCl2, 1.25 NaH2PO4, 0.1 t-aspartic acid, 25 NaHCO3, and 12.5 n-glucose (95% O2-5% CO2, pH 7.4). Slices were stored in the same solution at 37°C for 30 min and then transferred into artificial cerebrospinal fluid of the following composition (in mM).

**Patch-Clamp Recordings**

Patch-clamp recordings were made at room temperature from GFP+ cells located in the cerebral neocortex. Targeted patch recordings were performed as described previously (Fukuda et al. 2005). For whole-cell recordings, patch pipettes were filled with a K-gluconate-based solution containing (in mM) 120 K-gluconate, 6 NaCl, 6 CaCl2, 2 MgCl2, 2 MgATP, 0.3 NaGTP, 10 ethyleneglycol-bis(2-aminoethylether)-N,N,N’,N’-tetra acetic acid (EGTA), and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH7.2). Some experiments in this paragraph used patch-clamp interface (Axon Instruments, Foster City, CA) controlled by pCLAMP6 (Axon Instruments) software. Membrane current was sampled online at 4 kHz (PowerLab; AD Instruments, Grand Junction, CO) after filtering at 2 kHz. Data were recorded with Chart 4.0 software (AD Instruments) and stored on the hard disk of a personal computer for later off-line analysis with Igor Pro 4.01 (WaveMetrics, Lake Oswego, OR). For single-cell Ca2+ imaging, the Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan) was used as described previously (Imura et al. 2005).

After the recording session, the patch electrode was carefully removed from the cell and the slice was fixed for 4-6 h at 4°C in 4% PFA in PBS. Immunohistochemistry using antibody against NG2 was performed on free-floating slices as described before.

### Immunochemistry

Mice were perfused with PBS, followed by 4% PFA containing 0.1% glutaraldehyde in PFA at room temperature. Brains were postfixed overnight in 4% PFA in PBS at 4°C. Fifty-micron-thick vibratome sections of the hippocampus prepared as described above were incubated with rabbit anti-GFP (1:1000; Molecular Probes) for 48 h at 4°C and then with peroxidase-conjugated goat polyclonal anti-rabbit IgG (1:200; GE Healthcare, Buckinghamshire, UK) for 2 h. The sections were incubated with a 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma) solution for 15 min and then with a DAB solution containing 0.01% H2O2 for 15 min. Finally, sections were postfixed with 1% OsO4 in PB, dehydrated, and embedded in Epon 812. Thin sections (70-80 nm) were mounted on uncoated grids, stained with lead citrate, and examined by electron microscopy (EM; H7600, Hitachi, Tokyo, Japan) according to the precedent protocol (Tozuka et al. 2005).

### Measurement of the Amount of Secreted BDNF by ELISA

In the experiments of neurotransmitter response, GABA, N-methyl-D-aspartic acid (NMDA), and β-aminooxy-5-methylisoxazole-4-propionic acid (AMPA) were applied by focal application using a Picopump (PV820; World Precision Instruments, Sarasota, FL). Bicuculline methiodide, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 4-aminopyridine (4-AP), tetrodotoxin (TTX), kynurenic acid, and Cd2+ were bath applied.

Data were recorded with a CEZ-2400 amplifier (Nihon-Kohden, Tokyo, Japan) and Digidata 1200 interface (Axon Instruments, Foster City, CA) controlled by pCLAMP6 (Axon Instruments) software. Membrane current was sampled online at 4 kHz (PowerLab; AD Instruments, Grand Junction, CO) after filtering at 2 kHz. Data were recorded with Chart 4.0 software (AD Instruments) and stored on the hard disk of a personal computer for later off-line analysis with Igor Pro 4.01 (WaveMetrics, Lake Oswego, OR). For single-cell Ca2+ imaging, the Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan) was used as described previously (Imura et al. 2005).
Photothrombotic ischemia at sensory-motor area of adult mouse neocortex was produced according to precedent protocols (Schäbitz et al. 2004, 2007; Lee et al. 2007). Male C57/BL6 mice were first anesthetized by ketamine (50 mg/kg) and xylazine (5 mg/kg). Then they were intravenously administered 20 mg/kg of rose bengal (Sigma) for 5 min. For illumination, a fiber-optic bundle with a 1.0 mm aperture (white-light beam; Luminar Ace LA-150TX, Hayashi Watch Co. Ltd, Tokyo, Japan) was placed stereotaxically onto the skull (Paxinos and Franklin 2001). The mice were illuminated for 20 min to produce reactive oxygen from rose bengal. During the illumination, they were kept on a heating plate in order to keep their temperature at 37.0 ± 1.0 °C. Mice having higher or lower temperature during the illumination were excluded from the experiments, and all experimental instruments were covered to protect from the room light. Sham-operated animals underwent the same experimental procedures without the illumination.

Fluorescence Activated Cell Sorting Analysis
We performed intracellular nestin/BDNF staining and fluorescence activated cell sorting (FACS) analysis according to previous works (Raap et al. 2005; Noga et al. 2008) with some modifications. Adult male C57BL/6 mice undergone photothrombotic ischemia were perfused byPBS, and their brains were taken out immediately on the day 4–6 after the surgery. In some cases, the mice had been intravenously receiving 10% dimethyl sulfoxide (DMSO) in saline as vehicle or a NKCC1 antagonist, bumetanide at 1 mg/kg/day (Wako) dissolved in the same buffer. Cortical cells were dissociated from ipsilateral hemisphere. Importantly, we carefully cleared off the infralimbic area which can be recognized by its white color in the ipsilateral hemisphere. The cells were harvested in Hanks’ balanced salt solution with the enzyme as described above for 35 min at 37 °C. The cells were washed by PBS, incubated in 2.5 μg/ml ethidium monoazide bromide (EMA; Molecular Probes) in PBS for 15 min, and lighted for 10 min. They were next fixed by 2% PFA for 20 min. After PBS wash, they were first incubated in PBS containing 0.1% sodium azide (Wako), 0.1% BSA, and 0.1% saponin (Sigma) for 10 min and then blocked in the same buffer with addition of 5% FBS for 45 min. After spin, they were incubated in primary antibodies dissolved in the same buffer with serum for 30 min: mouse anti-nestin (1:1000; BD Pharmingen, San Diego, CA) and rabbit anti-BDNF (1:1000; Santa Cruz Biotech). After washed 2 times, they were incubated in secondary antibodies for 30 min as follows: anti-mouse IgG FITC (1:1000; DAKO), Glistrop, Denmark) and anti-rabbit IgG phycoerythrin (1:2500; Caltag, Carlsbad, CA). The cells were washed 3 times by the buffer without saponin.

We performed FACS analysis according to our previous works (Yoshida et al. 2003; Muramatsu et al. 2005) using FACS Caliber (Becton Dickinson, San Jose, CA). Cellular debris and dead cells were excluded by FSC/SSC scatters and the EMA expression. Cells only incubated with the secondary antibodies were used as control. For compensation, we used cells incubated in either nestin or BDNF antibody. We analyzed about 4000 nestin+ cells from each sample and made histograms of their BDNF intensity.

To acquire control images of BDNF staining, we followed all the method using the buffer not containing saponin. In this experiment, we could not detect any nestin or BDNF signals. Moreover, we also performed the same experiment by using cells kept in a refrigerator for a week after the PFA fixation. Also in this experiment, BDNF signal was not detected.

Behavior Tests
All behavior tests were conducted by an experimenter blinded to the study code. Adult male C57/BL6 mice were placed on a rotator cylinder (Model 7600; UGO Basile, Comerio, Italy), and the remaining time on the rod was measured. The trial ended if the mice fell off the rungs or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs (Schäbitz et al. 2004). The rotation speed was set at steady speed, 25 rpm. All mice were trained to achieve 200-s walking 3 days prior to the surgery. Mice failing this task were excluded from the subsequent experiment. The trained mice were administered sham or ischemic surgery according to the method described above.

For sensory measurement, a modified adhesive removal testing has been done according to the precedent protocol (Komotar et al. 2007) with slight adjustments to apply for mice. Mice first wrapped a 0.7 × 2.0 cm green tape around their cuff. The mice were put into the test box, and then, we waited for 10 s so as that they accommodated this environment. During the next 30 s, mice tried to remove the tape, such as biting the tape or shaking their hand, were measured by a stopwatch. The trials were repeated 4 times about each hand and the averages of the best 2 times were calculated everyday (for details, see Supplementary Videos online).

To analyze the influence of the adult cortical nestin+ cells onto neuronal recovery after the ischemia, ischemia-injured mice were intravenously administered vehicle as 10% DMSO or 1 mg/kg bumetanide (Wako) in 10% DMSO, an inhibitor for NKCC1 transporter (Dzhala et al. 2005; Wang and Kriegstein 2008) everyday.

Statistical Analysis
Data were expressed as mean ± standard error of the mean (SEM). The statistical significance of the difference between means was assessed using 2-sided Student’s t-test or Wilcoxon’s test, with the level of significance being set at *P < 0.05, **P < 0.01.

Results

Widespread Distribution of Dividing Nestin+/NG2+ Cells in the Adult Mouse Neocortex
The presence of nestin+ cells in the neocortex of the adult mouse brain was characterized by using nestin5-GFP transgenic mice (Yamaguchi et al. 2000) that express GFP under the control of the nestin promoter and its second intron (Zimmerman et al. 1994). This construct limits GFP expression to neural precursors even though nestin protein is also expressed in muscle precursors (Zimmerman et al. 1994). As shown in Figure 1(A–C), nestin5-GFP+ cells could be seen distributed throughout all cortical layers. These cells were typified by numerous, ramified thin processes radiating from a small cell body and were often observed adhering to cell bodies of pyramidal neurons (Fig. 1D), indicating that they are a type of satellite glia population (Rakic 1985; Kornack and Rakic 2001; Koketsu et al. 2003). Following the immunostaining of sections with anti-nestin antibody, all nestin5-GFP+ cells were positively stained with anti-nestin antibody (Fig. 1E). In this area, we did not detect any nestin5-GFP+ cells that also expressed GFAP (data not shown). To further assess whether nestin5-GFP+ cells also expressed NG2, we stained nestin5-GFP+ cells with anti-NG2 antibody. As shown in Figure 1(F), all nestin5-GFP+ cells were immunopositive for NG2. Conversely, more than half of the NG2+ cell population (56%) expressed nestin5-GFP (Fig. 1G,H). The data from triple immunostaining with anti-GFP, anti-nestin, and anti-NG2 were shown in Figure 1(I). These results strongly indicate that the nestin+ cells are a subpopulation of NG2+ cells in the adult neocortex. Interestingly, a part of nestin5-GFP+ cells (2%; 10 out of 500
Figure 1. Dividing nestin\(^\text{+}/\text{NG2}^+\) cells are widely distributed in the adult cerebral neocortex. (A–D) Nestin\(^\text{+}/\text{GFP}^+\) cells in the adult cerebral cortex. (A) GFP fluorescence. (B) Immunoreactivity for NeuN. (C) Merged image of (A) and (B). Note that nestin\(^\text{+}/\text{GFP}^+\) cells are widely distributed throughout the cortical layers. This picture shows pia surface (top line) and layer 1/2/3 of somatosensory cortex. We observed distribution of GFP\(^+\) cells in all cortical layers (data not shown) and all cerebral regions. (D) Nestin\(^\text{+}/\text{GFP}^+\) cells in close association with the cell body of a pyramidal neuron. Green, GFP; red, NeuN. (E) Nestin immunostaining of nestin\(^\text{+}/\text{GFP}^+\) cells. (E\(_1\)) GFP fluorescence. (E\(_2\)) Anti-nestin staining. (E\(_3\)) Merged image. Note that nestin protein is expressed in the processes and cell body of the nestin\(^\text{+}/\text{GFP}^+\) cell. (F) Most nestin\(^\text{+}/\text{GFP}^+\) cells express NG2. (F\(_1\)) GFP fluorescence. (F\(_2\)) Immunoreactivity for NG2. (F\(_3\)) Merged image. (G) A micrograph showing nestin\(^\text{+}/\text{GFP}^+\)/NG2\(^+\) cells (arrowheads). Graph showing the proportion of cell types in the NG2\(^+\) cell populations. 222 NG2\(^+\) cells from 2 animals were analysed. (I) Nestin and NG2 immunostainings of nestin\(^\text{+}/\text{GFP}^+\) cells. (I\(_1\)) GFP fluorescence. (I\(_2\)) Anti-nestin staining. (I\(_3\)) Anti-NG2 staining. (I\(_4\)) Merged image. (J) Nestin\(^\text{+}/\text{GFP}^+\)/NG2\(^+\) cells express an neuronal marker NeuN. (J\(_1\)) GFP fluorescence. (J\(_2\)) Anti-NG2 staining. (J\(_3\)) Anti-NeuN staining. (J\(_4\)) Merged. (K–N) Expression of intrinsic and extrinsic proliferative markers in nestin\(^\text{+}/\text{GFP}^+\) cells. (K\(_1\)) GFP fluorescence. (K\(_2\)) Immunoreactivity for Ki67. (K\(_3\)) Merged image. (L\(_1\)) GFP fluorescence. (L\(_2\)) Immunoreactivity for pH3. (L\(_3\)) Merged image. (M\(_1\)) GFP fluorescence. (M\(_2\)) Immunoreactivity for BrdU. (M\(_3\)) Merged image. Micrographs below and to the right of I\(_3\), J\(_3\), and K\(_3\) show z-stacks of the confocal images along the y-axis (right) and the x-axis (below). (W) Percentages of cortical nestin\(^\text{+}/\text{NG2}^+\) cells positive for intrinsic and extrinsic proliferation markers. When animals were administered BrdU for 2 h, 5 days, 10 days, or 30 days, the number of nestin\(^\text{+}/\text{NG2}^+\) cells incorporating BrdU gradually increased. Six hundred cells from 3 animals (200 cells per animal) were analysed for each group. Scale bars: 100 \(\mu\text{m}\) (C), 5 \(\mu\text{m}\) (D, E, I\(_4\)), 10 \(\mu\text{m}\) (F, J\(_3\), K\(_3\), L\(_3\), M\(_3\)), 50 \(\mu\text{m}\) (G).
nestin-GFP+ cells) weakly but significantly expressed NeuN, even though they showed morphologically multipolar glial phenotype (Fig. 1J).

To assess the dividing activity of nestin+/NG2+ cells, we used 2 intrinsic mitotic marker antibodies, one against Ki67, a cell proliferation-associated nuclear protein expressed during the active cell cycle (G1, S, G2, and M phases) but absent in resting cells (G0 phase), and pH3, an M-phase marker. Small percentages of the total nestin-GFP+ cell number were immunoreactive for Ki67 (3.83 ± 0.93%) (Fig. 1K) and pH3 (0.50 ± 0.29%) (Fig. 1L), indicating that relatively few nestin+/NG2+ cells are actively cycling in situ. Furthermore, we estimated the proliferative ability of nestin+/NG2+ cells by means of BrdU-pulse labeling experiments. For short-term pulses, in which we labeled proliferating nestin+ cells by injecting the BrdU 2 h before analysis, 1.63 ± 0.26% of nestin+/NG2+ cells in the adult neocortex incorporated BrdU. For long-term pulses, where BrdU was supplied for 5 days, 10 days, or 30 days in the drinking water to label slowly dividing cells, the percentage of BrdU+ cells in the nestin+/NG2+ cell population was 19.98 ± 2.90%, 33.23 ± 1.42%, and 82.33 ± 3.28%, respectively (Fig. 1M,N). Based on these data, we can conclude that the widely distributed nestin+/NG2+ cells in the adult cortex are mitotically active cells.

**GABAergic Inputs into Nestin+/NG2+ Cells in the Adult Neocortex**

To examine whether any form of communication exists between nestin+/NG2+ cells and surrounding cortical circuits, we performed GFP-guided targeted electrophysiological recordings from nestin+/NG2+ cells in fresh cortical slices to determine possible neural efferent connections. After recordings of nestin-GFP+ cells with Alexa Fluor 568 in the pipette, we confirmed the expression of NG2 proteins on the surface of

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**Figure 2.** Adult cortical nestin+/NG2+ cells respond to GABA. (A–D) Expression of NG2 on recorded nestin+ cell as confirmed by postrecording immunohistochemistry. (A) GFP expression. (B) Fluorescence image of Alexa Fluor 568. (C) Immunoreactivity for NG2. (D) Merged images. Micrographs below and to the right of (D) show z-stacks of the confocal images along the y-axis (right) and the x-axis (below). Scale bar: 10 μm. (E) Membrane properties of typical nestin+/NG2+ cells. A K-gluconate-based patch pipette solution was used. (1) Membrane currents were evoked by 1-s voltage steps ranging from -100 to +30 mV from a holding potential of -70 mV. We observed a slowly inactivating outward current, reminiscent of the A current recorded from a nestin+/NG2+ cell. (2) Current-voltage curves obtained from current amplitudes at the beginning (black circle) and at the end of the voltage pulse (open circle) were plotted against the holding potential. (F) Responses of nestin+/NG2+ cells to different neurotransmitter receptor agonists. (1) Responses to GABA are mediated by the activation of GABAA-Rs. GABA (200 μM) elicits an inward current. Typical recordings obtained from one cell clearly demonstrate inhibition by the GABAA-R antagonist, bicuculline (Bic; 50 μM). Vi = -70 mV. (2) Current induced by the application of taurine (10 mM) and inhibition by bicuculline. Vi = -70 mV. (3) Current induced in response to the application of AMPA (0.5 mM) and its inhibition by the AMPA-R antagonist, CNQX (50 μM). Vi = -70 mV. (4) No detectable currents were induced by NMDA (1 mM). Responses at -30 and +30 mV are shown; 0.5 μM TTX and 0.1 mM CdCl2 were added to the bath solution to block voltage-gated sodium and calcium channels, respectively. (G) Affinity of GABAA-Rs to GABA in nestin+/NG2+ adult cortical cells. (1) GABA responses induced by increasing GABA concentrations. (2) Dose-response curve of GABA-induced currents in nestin+/NG2+ cells (n = 7). The data were normalized to the maximal response and were fitted by using the Hill equation. The half-maximal concentration and Hill coefficient were 55.04 and 1.228 μM, respectively. Vi = -70 mV.
all recorded GFP+ cells (n = 15/15; Fig. 2A–D). The basic membrane properties of the recorded nestin+/NG2+ cells showed that they were nonexcitable cells with a graded level of slowly inactivating outward current (Fig. 2EF), reminiscent of the A current. Their mean input resistance was 157.50 ± 30.51 MΩ (mean ± SEM, n = 48) and their mean membrane potential was −79.07 ± 1.28 mV (n = 48); these characteristics being similar to those of NG2+ cells existing in the postnatal neocortex (Chittajallu et al. 2004) and the postnatal hippocampus (Mangin et al. 2008; Kukley et al. 2008).

To assess whether these nestin+/NG2+ cells actually communicate with surrounding neural circuits, we investigated their responses to either GABA or glutamate. As shown in Figure 2(G), significant inward currents were detected in almost all nestin+/NG2+ cells (n = 34/37) following the focal application of GABA. These currents were completely inhibited (100% of all GABA-responsive nestin+/NG2+ cells; n = 10) by bicuculline, a selective antagonist of the GABA_A receptor (GABA_AR) (Fig. 2G1). The mean EC_{50} of GABA was 55.04 μM (Fig. 2H). In addition to GABA, taurine also activated GABA_A-mediated currents in nestin+/NG2+ cells (Fig. 2G2) (n = 8/8).

In relation to the activation of glutamate receptor-mediated currents, some nestin+/NG2+ cells (n = 6/13) responded only to a high concentration of AMPA/kainate (0.5 mM; Fig. 2G3), whereas none of the recorded cells responded to NMDA (n = 0/11) (Fig. 2G4). These results suggest that cortical nestin+/NG2+ cells make GABAergic connections with cortical neurons.

Previous reports have clearly demonstrated that NG2+ cells are contacted by functional GABAergic terminals in developing and adult hippocampus (Lin and Bergles 2004; Kukley et al. 2008; Mangin et al. 2008). Then, we next examined whether adult cortical nestin+/NG2+ cells also receive functional GABAergic inputs from surrounding neural circuits. We next examined whether nestin+/NG2+ cells actively receive functional GABAergic inputs from surrounding cortical neural circuits. Nestin+/NG2+ cells showed very low-frequency, spontaneous postsynaptic currents during prolonged recording times (1–2 events per 10 min of recording) (Fig. 3A–B). We estimated their rise time as being 4.7 ± 1.0 ms (n = 6) and the decay time as being 25.3 ± 5.7 ms (n = 6). This decay time was similar to values of previous studies about GABAergic synaptic inputs into NG2+ cells (Lin and Bergles 2004; Kukley et al. 2007), suggesting that these communications were from GABAergic neurons. To enhance the frequency of synaptic events, 4-AP (200 μM), a K+ channel blocker that augments transmitter release (Hennou et al. 2002) was bath applied. As shown in Figure 3(C–D), the frequency of spontaneous synaptic currents in nestin+/NG2+ cells was increased in the presence of 4-AP and almost completely blocked by bicuculline (n = 5). Next, we found that electrical stimulation of cortical GABAergic neurons elicited GABAergic currents in nestin+/NG2+ cells. These stimuli-evoked currents were completely abolished by either TTX (n = 7) (Fig. 3F,1,E4), or bicuculline (Bic; n = 9) (Fig. 3F,2,E4), whereas kynurenic acid, a broad-spectrum blocker of ionotropic glutamate receptors, had no effect on these inward currents (Kyn; n = 5) (Fig. 3F,3,E4). The reversal potential elicited by stimulation was close to that estimated for Cl− (Fig. 3F). It has demonstrated that NG2+ cells did not connect with surrounding neurons via gap junction coupling or receive tonic release of GABA (Lin and Bergles 2004; Kukley et al. 2008; Mangin et al. 2008). Based on previous reports and our current data, we can conclude that neural inputs on cortical nestin+/NG2+ cells are action potential-dependent GABAergic synaptic events.

To prove synaptic contacts between nestin+/NG2+ cells and GABAergic neurons, we performed a series of experiments in which both EM and confocal microscopy were used. In the EM analysis, we found that presynaptic structures, including vesicles, were closely apposed to the surface of nestin+/NG2+ cells (Fig. 4A–C). We used immunohistochemistry to examine whether GABAergic terminals make contact with the nestin+/NG2+ cells. For GABAergic synapse markers, antibodies against VgAT and gephyrin were used. VgAT+ terminals were observed to be in close apposition to nestin+/NG2+ cells. Many VgAT+ terminals were colabeled with PV, thus giving rise to one hypothesis that PV+ GABAergic interneurons make contact on nestin+/NG2+ cells (Fig. 4D–H). These anatomical results may imply GABAergic terminals on nestin+/NG2+ cells.

To determine the physical nature of the influence of GABAergic inputs on nestin+/NG2+ cells, we evaluated the short-term plasticity of the synaptic junctions they form. Paired-pulse facilitation and paired-pulse depression (PPD) are frequency-dependent short-term changes at GABAergic synapses, which have been suggested to depend on presynaptic neuronal activation. We performed paired-pulse stimulation as previously described (Lin et al. 2005) and found that the GABAergic responses of the nestin+/NG2+ cells showed PPD (Fig. 4I) in a manner similar to evoked inhibitory postsynaptic currents in PV+ fast-spiking neuron synapses (Galarreta and Hestrin 2002). Based on these electrophysiological and anatomical observations, these results strongly suggest that the connections between nestin+/NG2+ cells and PV+ GABAergic neurons may serve important functions in the maintenance and plasticity of the adult cortical neural network.

**GABAergic Excitation May Promote the Production of BDNF in Nestin+/NG2+ Cells**

In immature neuronal cells, the opening of GABA_A-receptor channels often triggers the efflux of Cl− (due to the elevated level of [Cl−]) and subsequent membrane depolarization (Ben-Ari 2002; Owens and Kriegstein 2002). To assess whether GABAergic activity depolarizes nestin+/NG2+ cells, we performed gramicidin-perforated patch-clamp recordings. The focal application of GABA depolarized nestin+/NG2+ cells held in current clamp mode (Fig. 5A). The evoked currents in nestin+/NG2+ cells reversed at −30.61 ± 3.70 mV (mean ± SEM, n = 10), that is, at a level more depolarized than their resting membrane potential (Fig. 5B). Based on this reversal potential, nestin+/NG2+ cells have a calculated [Cl−] of 44.67 ± 6.31 mM (mean ± SEM, n = 10), indicating that nestin+/NG2+ cells maintain a higher [Cl−] than mature cortical neurons as described previously (Yoshida et al. 2004). These data suggest that GABAergic inputs should have excitatory effects on nestin+/NG2+ cells in the adult neocortex.

Next, we tested whether GABAergic activity could trigger the elevation of [Ca2+]i in nestin+/NG2+ cells. In single-cell Ca2+-imaging experiments with whole-cell recordings, patch pipettes were filled with a Ca2+-free solution that contained 45 mM CaCl2 to mimic the intracellular environment. As shown in Figure 5(C), GABA application resulted in the elevation of [Ca2+]i in the nestin+/NG2+ cell soma, a response that was blocked by bath application of the voltage-gated calcium channel (VGCC) blockers Cd2+ and Ni2+. This transient elevation of Ca2+ was also observed in cell processes (Fig. 5D). It is likely that the GABA-induced, VGCC-mediated influx of intracellular Ca2+ thus
modifies an intracellular signaling pathway or triggers the synthesis or release of compound that acts extracellularly.

To assess whether excitatory GABAergic inputs on adult cortical nestin$^+$/NG2$^+$ cells would be beneficial for plasticity and repair in cortical circuits, we performed immunohistochemistry for BDNF, NGF, NT-3, VEGF, and netrin-1. Among these proteins with potentially beneficial effects on the regeneration of neural circuits, the robust expression of BDNF...
was observed in nestin+/NG2+ cells located over a wide range of sites including the sensory-motor area (Fig. 6A) and entorhinal cortex (Fig. 6B). In contrast, we were unable to detect immunohistochemically any significant expression of the other trophic factors or chemoattractant molecules on nestin+/NG2+ cells.

We next examined the possibility that BDNF secretion from nestin+/NG2+ cells could be induced by excitatory GABAergic stimulation. To quantify the BDNF secretion from nestin+/NG2+ cells, we isolated nestin+/NG2+ cells from adult rat neocortex by means of the Percoll gradient centrifugation method and then measured by ELISA the amount of BDNF secreted (Fig. 6C–D). We evaluated whether GABAergic stimulation induced the elevation of [Ca2+]i and the production of BDNF (Fig. 6E,F). As shown in Figure 6E, the rapid increase in [Ca2+]i was observed in cultured nestin+/NG2+ cells stimulated with GABA. For the evaluation of BDNF production and/or secretion, the culture supernatant from nestin+/NG2+ cells stimulated with GABA was collected and the amount of secreted BDNF was measured by ELISA. We detected the clear increase in BDNF secretion from the culture stimulated with GABA 9 h before in comparison to the control cultures (Fig. 6F). We did not observe the enhanced secretion of BDNF just after GABA stimulation (<30 min) (data not shown). These results may suggest that GABAergic stimulation promotes the production, but not secretion, of BDNF from cultured nestin+/NG2+ cells.
GABAergic Excitation and Increase in the Production of BDNF in Nestin+\textsuperscript{+}/NG2\textsuperscript{+} Cells after Ischemic Stroke

To assess whether the ischemic injuries stimulate the production of BDNF in nestin\textsuperscript{+}/NG2\textsuperscript{+} cells in the area beside, but apart from the peri-infarct area, we utilized a mouse photothrombosis model at sensory-motor areas (Fig. 6\textsuperscript{G},\textsuperscript{H}) and performed both immunohistochemical analysis and quantitative FACS analysis. The merit of this lesion model is highly reproducibility in size and location. In a pilot experiment, we detected the elevation of BDNF production in nestin\textsuperscript{+}/NG2\textsuperscript{+} cells on and after day 4, but not on day 2 after ischemia. In many of nestin\textsuperscript{+}/NG2\textsuperscript{+} cells located within 50–500 μm apart from the peri-infarct area at day 4, we detected the elevated production of BDNF proteins in cellular process areas as well as cell cytoplasm area (Fig. 6\textsuperscript{I},\textsuperscript{J}).

To quantify the BDNF levels, we performed FACS analysis after the intracellular staining of BDNF proteins in combination with anti-nestin staining. This method enables us to quantify the amount of BDNF proteins in each cortical cell. The detection of the intracellular BDNF proteins by FACS analysis has been performed elsewhere (Raap et al. 2005; Noga et al. 2008). We carefully dissected the cortical tissue from ipsilateral side of the neocortex, getting rid of the ischemic core and the peri-infarct area where reactive gliosis occurs. Then, we applied stained cells (ca. 500 000–800 000 cells) and evaluated the amount of the intracellular BDNF proteins in nestin\textsuperscript{+} cortical cells (Fig. 7\textsuperscript{A}). Subsequently, we performed the parallel FACS analysis using both ipsilateral and contralateral cortical cells. As shown in Figure 7\textsuperscript{B}, fluorescent intensities from...
BDNF-producing nestin

Micrographs below and to the right of each merged image in (A) show stacks of the merged image along the y-axis (right) and the x-axis (below). Green, nestin-GFP; red, BDNF. (B) Experimental procedures for analysis of BDNF secretion from adult cortical nestin+/NG2+ cells in vitro. Cerebral cortices from adult rats were enzymatically dissociated. Digested tissue was then suspended in Percoll solution and fractionated by centrifugation. Fractionated cells were then cultured for 4 days and then applied for BDNF-FACS analysis (Fig. 8). As a result, we observed that the increase in BDNF production after ischemia was completely blocked by this bumetanide treatment. Taken together with all these data, we can stress that GABAergic excitation may promote the production of BDNF from nestin+ glial cells after the ischemic injuries.

Block of the GABAergic Depolarization Causes the Attenuation of Functional Recovery in Sensory Functions

To assess an effect of excitatory GABAergic stimulation on the functional recovery after ischemic injury, we performed 2 behavior tests: rotarod to evaluate the recovery in general sensory-motor functions and adhesive removal test to evaluate the recovery in sensory function. For the adhesive removal test, we followed a protocol reported by Komotar et al. (2007). The advantages of this new sensory test are as follows. 1) This method can be evaluated by only sensory functions, not including motor functions because it is not necessary to move the impaired hand. 2) Drug influence onto uninjured network can be measured at the same time by analyzing the sensory activities of contralateral side. 3) Pretraining is not necessary, so this experiment is not time consuming. (Komotar et al. 2007; for details, see Supplementary Videos online).

NG2+ cells in vitro. Cells were stimulated with 200 μM GABA for 9 h, and then production of BDNF was measured by ELISA. In an experiment, GABA caused an increase in BDNF production (control: 3.69 ± 0.36 pg/104 cells, n = 4; GABA: 7.07 ± 1.07 pg/104 cells, n = 4). These increases were inhibited by bicuculline (GABA/bicuculline: 4.29 ± 1.46 pg/104 cells, n = 3). BDNF productions in cortical nestin+/NG2+ cells beside the peri-infarct area. (G) A typical image of TTC staining on seventh day after the ischemic surgery. The white area in the gray matter indicates the ischemic core devoid of neuron at sensory-motor area of mouse cerebral cortex. (H) Schematic illustration of the cortical area where we focused on the following study (area of interest). (Left) The ischemic core and the peri-infarct area are shown by red. (Right) Enlarged image of the box depicted in the left panel. The blue indicates the area of interest, 50–500 μm apart from the peri-infarct area. (I, J) Expression of BDNF in nestin+/NG2+ cells, 50–500 μm apart from the peri-infarct area 4 days after ischemia. Note that, on the ischemic side of the neocortex, satellite glial-like nestin+/NG2+ cells expressed BDNF, and we detected BDNF-expressing nestin+/GFP+ cells in this area more frequently than in uninjured contralateral side of brain. Green, nestin-GFP; red, BDNF, and their merged image. Scale bar: 10 μm (A, H, J), 40 μm (D), 20 μm (I), 1 mm (G), and 5 mm (C).

Figure 6. Production of BDNF in cortical nestin+/NG2+ cells. (A, B) Two examples of BDNF-producing nestin+/NG2+ cells in (A) sensory-motor cortex and (B) entorhinal cortex. Note that we occasionally detected BDNF-producing nestin-GFP+ cells. Micrographs below and to the right of each merged image in (A) and (B) show z-stacks of the merged image along the y-axis (right) and the x-axis (below). Green, nestin-GFP; red, BDNF. (C) Experimental procedures for analysis of BDNF secretion from adult cortical nestin+/NG2+ cells in vitro. Cerebral cortices from adult rats were enzymatically dissociated. Digested tissue was then suspended in Percoll solution and fractionated by centrifugation. Fractionated cells were then cultured for 4 days and BDNF released in the medium measured by BDNF-ELISA. (D) Confocal images of cultured cortical nestin+/NG2+ cells: Green, nestin; red, NG2. (E) GABA-induced Ca2+ elevation in cultured cells and 200 μM GABA was applied. Summarized data of GABA-induced Ca2+ fluorescent changes (F/F0) was shown. Control (n = 5), GABA (n = 5). (F) GABAergic stimulation promotes BDNF production in cortical nestin+/ /
The presence of the microinfarction significantly impaired sensory-motor function in both rotarod and adhesive removal tests (Fig. 9). Next, to analyze whether or not the functional recovery was influenced by GABAergic stimulation, some mice were administered bumetanide. Although, there was a tendency for suppression by the bumetanide treatment on functional recovery as assessed in rotarod test, we did not detect the significant difference between the 2 groups (Fig. 9B). As shown in Figure 9(C), this administration significantly attenuated sensory recovery on the day 6, 7, and 8, as evaluated by the adhesive removal test (Fig. 9C and Supplementary Video 1 online). In addition, the results from the contralateral side indicated that the normal sensory function was not affected by this bumetanide administration (Fig. 9D and Supplementary Video 2 online). All these behavioral data suggest the significant attenuation of functional recovery in sensory functions by the bumetanide treatment.

Figure 7. BDNF-FACS analysis from nestin⁺ cells beside the peri-infarct area. All images were taken from Percoll-purified cells of cortical area beside the ischemic core from 2 mice underwent cortical ischemia 4 days before. (A) Two-dimensional contour line histogram from the purified cortical cells stained with anti-nestin and anti-BDNF antibodies (right panel). As a control, data from cells without these first antibodies is shown on the left. The x-axis shows the level of nestin expressions. The y-axis indicates the level of BDNF expressions. (B1) Histograms indicate the level of BDNF expressions in gated nestin⁺ cells (≥10 in nestin-FITC signal), evaluated from the control experiment, a vertical line shown in the right panel of (A). From cortical area beside the ischemic core (ipsilateral) or from contralateral cortex (contralateral; as a control). (B2) Histogram was from other equivalent experiment. Note that the level of BDNF expression was higher in ipsilateral cells than in contralateral cells.

Figure 8. BDNF-FACS analysis from ischemic mice treated with bumetanide. All images were taken from 3 mice underwent cortical ischemia 4 days before. (A) Two-dimensional contour line histogram showing the level of BDNF expression in cortical cells with graded amount of nestin proteins. From left to right: vehicle-treated mice and bumetanide-treated mice. (B, C) Histograms indicate the level of BDNF expressions in nestin⁺ cells (≥8 in nestin-FITC signal), evaluated from the control experiment as shown in Fig. 7A. In a day, we prepared 4 groups of cortical cells simultaneously to compare the level of BDNF expression precisely between vehicle-treated (n = 3) and bumetanide-treated (n = 3) mice. (B) Histogram showed the elevation of BDNF productions in cortical nestin⁺ cells ipsilateral to ischemic lesion, as shown in Figure 7(B1) and B2). (C) Bumetanide treatment blocked the elevation of BDNF production in cortical nestin⁺ cells ipsilateral to ischemic lesion. Equivalent data were obtained from other 2 independent experiments.
Discussion

Adult cortical tissue contains large number of dividing glial cells (Rakic 1985, 2002a, 2002b, 2006a, 2006b; Kornack and Rakic 2001; Koketsu et al. 2003), even though their detailed characteristics and functions have remained unclear. Very recently, it has been reported that in the dorsal root ganglia they affect pain-like sensory behavior (Vit et al. 2008) and neuron-glia communication (Zhang et al. 2007). A series of reports have shown that a particular subpopulation of NG2+ cells form synaptic junctions with GABAergic as well as glutamatergic neurons (Lin and Bergles 2004; Lin et al. 2005; Kukley et al. 2007; Ziskin et al. 2007). In this communication, we report that cortical nestin+ glial cells received GABAergic inputs.

Our histological results clearly demonstrate that PV+ interneurons (basket cells or chandelier cells) extend GABAergic fibers toward nestin+/NG2+ cells (Fig. 4). In addition, a short-term plasticity test showing paired-pulse depression at GABAergic synapses on nestin+/NG2+ cells showed similar characteristics to those shown by PV+ GABAergic synapses on cortical interneurons (Galarreta and Hestrin 2002). These experimental findings clearly indicate that PV+ GABAergic interneurons specifically innervate and make functional GABAergic synapses with nestin+/NG2+ cells.

Interestingly, we also discovered a part of nestin+GFP+ cells (2%) weakly but significantly expressed a neuronal marker NeuN, even though they belonged to NG2+ cells (Fig. 1J). Gallo et al. have also suggested that some of NG2+ cells expressed NeuN (Belachew et al. 2003; Aguirre et al. 2004) and possessed voltage-gated sodium channels (Chittajallu et al. 2004). We previously detected few BrdU+/NeuN+ cells in neocortex of adult nonhuman primates (Koketsu et al. 2003); however, these cells may belong to these glial cells, thereby the credibility of NeuN to detect neuronal cells remains ambiguous. It can be assumed that a subpopulation of NG2+ glial cells expresses NeuN. In turn, to evaluate neurogenesis in vivo, only double staining with NeuN and BrdU would not be sufficient. Further studies are definitely needed to characterize nestin+/NG2+ cells expressing NeuN.

In this study, we have clearly demonstrated by BDNF-FACS analysis that, upon ischemic injuries, the productions of BDNF from nestin+/NG2+ cells was elevated. This elevation might be due to the excitatory GABAergic stimulation after ischemic injuries (Fig. 8). The quantification of cytokines using FACS was performed mostly in the area of immunology. However, recently, this method has become applicable for quantification of neurotrophins such as BDNF and NT-3 (Raap et al. 2005; Noga et al. 2008). This FACS quantification has various advantages. First, we can detect the presence of intracellular BDNF. Second, we can identify the cell type simultaneously. Third, we can quantify the amount of BDNF precisely.

Figure 9. Bumetanide treatment attenuated the recovery in somatosensory dysfunctions after the focal ischemia. (A) Experimental procedures for behavior tests. 10% DMSO as vehicle or 1 mg/kg (body weight) bumetanide, an inhibitor for NKCC1 transporter, in 10% DMSO was intravenously administered to the ischemic damaged mice everyday. Sample numbers: vehicle n = 6, bumetanide n = 6. (B) Results of the daily rotarod test. There was no difference between the 2 groups. (C) Results of the adhesive removal test when the tape was attached to the left cuff (the affected injured side). Administration of bumetanide delayed the sensory recovery on the day 6, 7, and 8 after the focal ischemia. Asterisks indicate significant difference in score between the vehicle and the bumetanide-treated groups. *P < 0.05, **P < 0.01. (D) Results of the adhesive removal test when the tape was attached to the right cuff (the contralateral side). No decrease of scores was observed, suggesting that this dose of bumetanide did not affect the normal sensory functions.
we can perform the experiment using fewer cells than other methods as western blotting.

The activity-dependent production of BDNF from neurons had been reported previously, in which calcium signaling promoted the elevation of cyclic adenosine 3',5' monophosphate and then triggered the production of BDNF (Obrietan et al. 2002). Because nestin^+/NG2^ cells are an immature cell type--like nestin^+^ progenitor cells in adult dentate gyrus (Tozuka et al. 2005), GABA in these cases acts as an excitatory neurotransmitter, initiating the elevation of [Ca^{2+}]_i. It is reasonable to postulate that nestin^+/NG2^ cells, closely apposed to neighboring neurons, serve as a site of BDNF secretion, supported by the results from Komitova et al. (2006). BDNF promote neuronal survival (Lipsky and Marin 2007), dendrite development (Horch and Katz 2002), synaptic plasticity (Chakravarthy et al. 2006), and neuronal differentiation (Scharfman et al. 2005). However, NG2 knockout mice did not influence on adult neurogenesis in dentate gyrus and hippocampal-dependent behavioral tasks (Thallmair et al. 2006) and they had no difference of development compared with the wild-type mice (Grako et al. 1999). These results suggest that the BDNF expression from adult cortical glial cells may influence on the plasticity rather than the general development of cortical circuitry.

After the injuries, GABA and glutamate increased in the peri-infarct area (Phillis et al. 1994; Matsumoto et al. 1996), and glutamate excitotoxicity leads to cellular death (Leach et al. 1993; Lyden and Lonzo 1994; Zipfel et al. 2000). Conversely, glutamate excitotoxicity leads to cellular death (Leach et al. 1993; Lyden and Lonzo 1994; Zipfel et al. 2000). GABA agonists (Schwarz-Bloom and Sah 2001), and taurine (Molchanova et al. 2006; Wang et al. 2007) attenuated ischemic damages, suggesting the role of GABAergic network on the protection and repair of neural circuit after ischemic injuries. In addition, recent studies have well suggested that BDNF plays a crucial role in repairing neural circuits. Ectopic expression of BDNF by either the infusion or the viral transfer into the damaged circuit promoted the functional recovery after brain injuries, such as stroke or spinal cord injuries (Schäbitz et al. 2004, 2007; Girard et al. 2005). In addition, the endogenous production of BDNF promoted the functional recovery after brain injuries (Griesbach et al. 2004).

In this study, we also clearly demonstrated that an inhibitor for NKCC1 transporter, bumetanide, suppressed sensory recovery after focal ischemia (Fig. 8C). NKCC1 maintains high [Cl^-]_i and causes cellular depolarization after the opening of Cl^- channels. At early postnatal stage, very recently, the depolarization of cortical neurons by GABAergic stimulation had been reported (Khirug et al. 2008). Others reported the robust expression of NKCC1 in NG2^+^ cells (Price et al. 2006). [Cl^-]_i of nestin^+/NG2^ cells was extremely high as 45 mM, in which E_GABA is around -30 mV. Therefore, it is reasonable to assume that GABAergic depolarization by NKCC1 could occur in such immature nestin^+/NG2^ glial cells like neuronal cells during postnatal stage (Wang and Kriegstein 2008).

From the point of satellite glial cells, their activity greatly affects pain-like sensory mechanism of ganglia through secreting cytokines (Scholz and Woolf 2007; Vit et al. 2008). Following inflammation, activation of satellite glial cells promoted the secretion of IL-1 and this factor enhanced excitability of ganglion sensory neurons (Takeda et al. 2007). Not only neuronal cells but also satellite glial cells intervene in the regulation of sensory functions by secreting some neuronal factors, and same phenomenon can arise in the ischemic damaged neocortex. In addition to satellite glial cells, oligodendrocytes, astrocytes, or microglia secrete such growth factors. Actually, we detected few mature glial cells (APC^-^) expressing BDNF, like a previous study by Dai et al. (2003) in the area 50--500 µm apart from the peri-infarct area (indicated by blue color in Fig. 6H). We focused on this area because we can speculate reorganization of damaged neural circuit. In this area, the density of nestin^+/NG2^ cells coexpressing BDNF was extremely higher than that of APC^-^ cells. In addition, we observed the clear enhancement of BDNF production in nestin^+/NG2^ cells in response to the ischemic injury. Therefore, these results imply that nestin^+/NG2^ satellite glia may also be involved in the functional recovery after ischemic injuries. Further works must be necessary to elucidate the effect of enhanced BDNF production from adult cortical satellite glial cells on circuit protection and repair. We hope that our results provide additional cues to understand the function and plasticity of adult neocortex and to create a new therapeutic application for ischemic injuries.

Supplementary Material

Supplementary videos can be found at: http://www.cercor.oxfordjournals.org.

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Notes

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