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Regeneration of Hippocampal Pyramidal Neurons after Ischemic Brain Injury by Recruitment of Endogenous Neural Progenitors

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Summary

The adult brain is extremely vulnerable to various insults. The recent discovery of neural progenitors in adult mammals, however, raises the possibility of repairing damaged tissue by recruiting their latent regenerative potential. Here we show that activation of endogenous progenitors leads to massive regeneration of hippocampal pyramidal neurons after ischemic brain injury. Endogenous progenitors proliferate in response to ischemia and subsequently migrate into the hippocampus to regenerate new neurons. Intraventricular infusion of growth factors markedly augments these responses, thereby increasing the number of newborn neurons. Our studies suggest that regenerated neurons are integrated into the existing brain circuitry and contribute to ameliorating neurological deficits. These results expand the possibility of novel neuronal cell regeneration therapies for stroke and other neurological diseases.

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Introduction

In adult tissues with high regenerative capacity, such as skin and liver, dead cells are replaced either by the proliferation of nearby cells or resident stem cells. Unlike such cases, it has long been believed that the adult mammalian central nervous system (CNS) is incapable of significant self-repair or regeneration (Björklund and Lindvall, 2000). Many lines of recent evidence have revealed, however, that progenitors with the ability to produce new neurons and glia remain in the adult CNS. In particular, neural stem cells defined with the characteristics of long-term self-renewal and multipotentiality have been shown to persist throughout life in various mammalian species including humans (Temple, 2001). The adult CNS also contains a range of progenitors with more limited capacities of growth and differentiation (Temple, 2001) (herein collectively called neural progenitors). Although these adult progenitors are known to be abundant in the periventricular areas, recent studies have revealed their widespread occurrence in the parenchyma of various CNS regions (Palmer et al., 1999; Yamamoto et al., 2001a, 2001b).

Consistent with these findings, continuous generation of new neurons has been detected in the adult (Temple, 2001; Gould and Gross, 2002). Although such persistent neurogenesis had originally been thought to occur in only a few restricted areas, more recent studies have demonstrated the production of new neurons in many other CNS regions (Rietze et al., 2000; Pencea et al., 2001; Gould and Gross, 2002). Furthermore, it has been shown that various insults stimulate the proliferation of endogenous progenitors either in known neurogenic sites (Gould and Tanapat, 1997; Liu et al., 1998; Fallon et al., 2000; Magavi et al., 2000; Jin et al., 2001; Yoshimura et al., 2001) or in regions where neurogenesis normally does not occur (Johansson et al., 1999; Yamamoto et al., 2001a, 2001b). In adult songbirds, however, induced death of neurons that normally turn over results in compensatory replacement of the same neuronal subtype, whereas normally nonreplaced types cannot be regenerated (Scharff et al., 2000). Thus, such a limitation in adult neurogenesis has been considered to be attributable to the lack of substantial regeneration after damage.

Current efforts toward curing various neurological diseases have mainly aimed at preventing neuronal cell death (Björklund and Lindvall, 2000; Horner and Gage, 2000). The above accumulating evidence, however, raises the alternative possibility that if dormant capacities of endogenous progenitors could be activated by certain manipulations, they may be recruited to generate new functional neurons and repair the damaged CNS. Although some recent studies have begun to address such an idea, regeneration of new neurons has been observed only at a very low frequency (Magavi et al., 2000) or not distinguished from simply the survival of existing neurons (Fallon et al., 2000; Yoshimura et al., 2001). Thus, the therapeutic values of the neuronal re-

placement strategies have not yet been evaluated intensively.

Here we sought to augment the regenerative capacity of endogenous progenitors upon focusing on the hippocampus. The adult hippocampus, a vital center for learning and memory, is extremely vulnerable to various insults such as ischemia. Although the hippocampus is known as an active neurogenic site, only one neuronal subtype, granule cells in the dentate gyrus (DG), are continuously generated, and the production of new neurons in other hippocampal regions appears to be very limited in the adult (Rietze et al., 2000). We show here that following ischemic brain injury, adult neural progenitors can be stimulated in situ by intraventricular infusion of growth factors to replace CA1 pyramidal neurons. These newly generated CA1 neurons form functional synapses and are integrated into the existing brain circuitry. In parallel to the massive induction of new hippocampal neurons, the growth factor treatment ameliorates deficits in hippocampal-dependent spatial cognitive functions in ischemic animals.

Results

Degeneration and Recovery of Hippocampal Neurons Following Ischemia

Transient ischemia of the forebrain in rodents causes selective degeneration of hippocampal CA1 pyramidal neurons (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984). It remained unknown, however, whether regeneration of pyramidal neurons occurs late after ischemia. To address this issue, we have recently established a rat model in which CA1 neurons can be eliminated in a highly reproducible manner, yet leaves animals able to survive severe ischemic damage.

A notable feature of this animal model is delayed, extensive loss of CA1 pyramidal neurons in the dorsal part of the hippocampus. Immunostaining for NeuN, a marker for mature neurons, detected gradual loss of CA1 neurons 2 to 4 days after ischemia (DAI2-4). During this initial period, most of the CA1 pyramidal neurons expressed apoptosis-inducing activated caspase-3 (data not shown) and became TUNEL$^+$ (Figure 1L). Consequently, cells with pyknotic morphology occupied the degenerated CA1 (Figure 1G, arrow), and only a very few neurons remained at DAI7 (compare Figures 1B and 1B′ with 1A and 1A′, and see Figure 2B; 4 $\pm$ 1 NeuN$^+$ cells and 2 $\pm$ 1 cresyl violet-stained cells compared to 313 $\pm$ 24 and 370 $\pm$ 23 cells, respectively, in intact animals in the defined 0.15 mm^2 area; see Figure 2A and Supplemental Figure S1 at http://www.cell.com/cgi/content/full/110/4/429/DC1; mean $\pm$ SD, n = 6 animals, total 12 hemispheres). The extensive loss of pyramidal neurons was also evident at DAI14 (Figure 2B).

Despite such an extensive neuronal loss in the initial 2 weeks, we detected a small but significantly higher number of NeuN$^+$ cells at DAI28 (Figures 1C, 1C′, and 2B; 32 $\pm$ 7 cells, $p < 0.01$ in unpaired two-tailed t test). These cells were sparsely distributed within the destructed pyramidal layer and surrounded by the remnant of dead cells and gliotic fibers (Figure 1H, arrowhead). Their morphological features such as large nucleus, discrete nucleolus, and the size of their soma (diameter, 15.7 $\pm$ 2.6 μm in 4-μm-thick paraffin sections, total 183 cells examined, n = 4 animals) were reminiscent of intact pyramidal neurons (diameter, 16.5 $\pm$ 1.9 μm). Many of these neurons still survived 2 months after ischemia. Notably, β-tubulin type III$^+$ (TuJ1$^+$) and Hu$^+$ immature neurons, which are rarely detectable in normal adult animals, emerged after ischemia (Figure 2B).

The ischemia-induced loss and subsequent recovery of neurons were also detected in other regions of the hippocampus (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/110/4/429/DC1). The neuronal loss caused by global ischemia is generally less severe in the ventrolateral part of the hippocampus than in the dorsomedial part (Pulsinelli et al., 1982; Smith et al., 1984). However, we reproducibly observed significantly higher numbers of neurons at DAI28 than at DAI7 irrespective of the anteroposterior and dorsoventral positions of the hippocampus.

Enhanced Recovery of Hippocampal Neurons by Growth Factor Treatment

Ischemia has been shown to stimulate the proliferation of neural progenitors in the DG, which leads to increased production of new granule cells (Liu et al., 1998; Jin et al., 2001; Yoshimura et al., 2001). Thus, the generation of new neurons from endogenous progenitors could also occur in the CA1 pyramidal layer. If endogenous progenitors contribute to production of new neurons, expansion of their endogenous pool could augment their regenerative capacity.

To test this idea, fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF), which act as mitogens for adult progenitors (Craig et al., 1996; Kuhn et al., 1997), were infused into the lateral ventricle by using osmotic minipumps from DAI2 to DAI5. Even after growth factor infusion, CA1 pyramidal neurons were progressively degenerated in the initial week (Figures 1D, 1D′, and 1I), and most of the cells became TUNEL$^+$ (Figure 1M). Consequently, the extent of neuronal loss was indistinguishable at DAI7 between treated and untreated animals (Figure 2C). Although a very few cells remained scattered among debris of dead cells (Figure 1I), electron microscopic analyses revealed that they exhibited destructed morphologies with broken cell membrane and nuclei (Figure 1O, arrowheads; Kirino and Sano, 1984; Colbourne et al., 1999b). Thus, neither FGF-2 nor EGF exhibited substantial neuroprotective effects on dying neurons.

Nevertheless, the number of neurons detected late after ischemia was dramatically increased by the treatment (Figures 1E, 1E′, and 1J). At DAI28, FGF-2 and EGF increased the numbers of NeuN$^+$ and cresyl violet-stained CA1 neurons 4.2-fold and 9.2-fold, respectively (Figure 2C; 134 $\pm$ 72 NeuN$^+$ and 101 $\pm$ 54 cresyl violet-stained cells, $p < 0.01$, n = 6 animals). Thus, the treatment led to 40% recovery of the total number of NeuN$^+$ cells lost by ischemia. Infusion of the vehicle solution alone did not cause such recovery.

These neurons were clustered along the original pyramidal layer and exhibited morphology highly reminiscent of intact pyramidal neurons (Figures 1J and 1P, arrowheads). They also expressed SCIP, a transcription factor expressed in adult CA1 neurons (Figures 1K and 1N). Many of these neurons survived for at least up to 6 months after ischemia (68 $\pm$ 9 NeuN$^+$ and 59 $\pm$ 16 cresyl

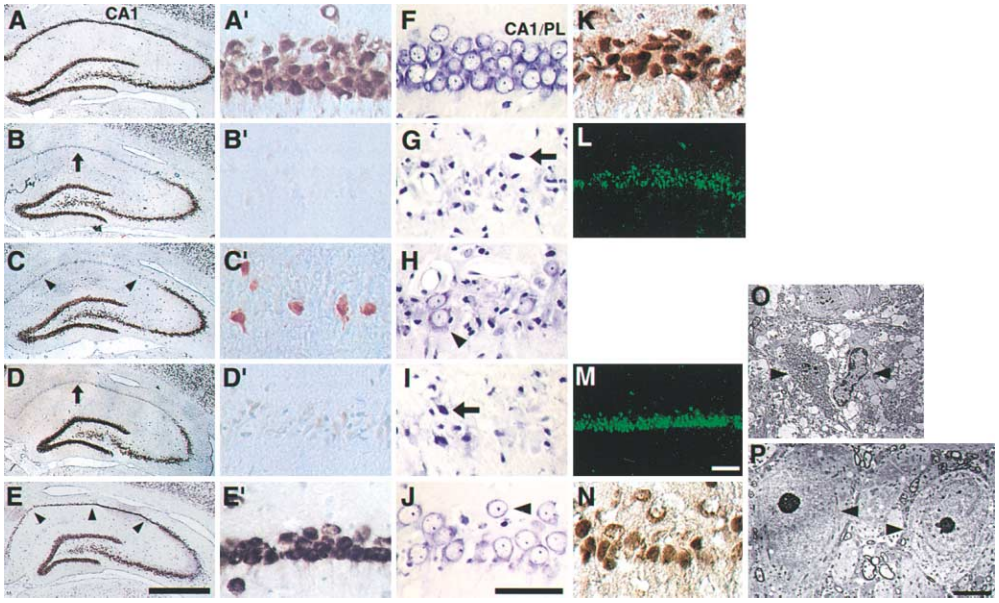

Figure 1. Degeneration and Subsequent Recovery of CA1 Pyramidal Neurons Following Ischemia

(A–J) NeuN (A–E′) and cresyl violet (F–J) staining of the CA1 pyramidal layer (CA1/PL) in intact (A, A′, and F), untreated/ishemic (B, B′, and G, at DAI7; C, C′, and H, at DAI28), and growth factor-treated (D, D′, and I, at DAI7; E, E′, and J, at DAI28) animals. (A′)–(E′) are higher magnification views of the CA1 shown in (A)–(E) (arrows). Arrows and arrowheads in (G)–(J) indicate cells with pyknotic and intact morphology, respectively.

(K–N) SCIP (K and N) and TUNEL (L and M) staining of CA1 neurons.

(O and P) Electron microscopic analysis of pyramidal neurons at DAI7 (O, arrowheads) and DAI28 (P, arrowheads) in growth factor-treated animals. Scale bar for (A)–(E) (shown in E) equals 1 mm; for (A′)–(E′), (F)–(K), and (N) (shown in J) equals 50 μm; for (L) and (M) (shown in M) equals 100 μm; and for (O) and (P) (shown in P) equals 4 μm.

violet-stained cells at DAI180-194, n = 2 animals, 4 hemispheres). We noticed slight enlargement of the ventricular lumen and deformity of the shape of the hippocampus in growth factor-treated animals (Figures 1D and 1E). However, significantly higher numbers of CA1 pyramidal neurons were detectable all along the anteroposterior axis of the hippocampus in the treated animals than in the untreated animals. These results suggest that growth factors induce substantial regeneration of hippocampal neurons after ischemia. In the following sections, we provide several lines of evidence supporting such an idea.

Generation of New Neurons by Endogenous Progenitors

To examine whether neurons detected after ischemia were produced by endogenous progenitors, 5-bromo-2′-deoxyuridine (BrdU), which selectively labels proliferative cells in vivo, was administered between DAI2 and DAI4. During DAI2-7, few $BrdU^+$ cells coexpressed neu-

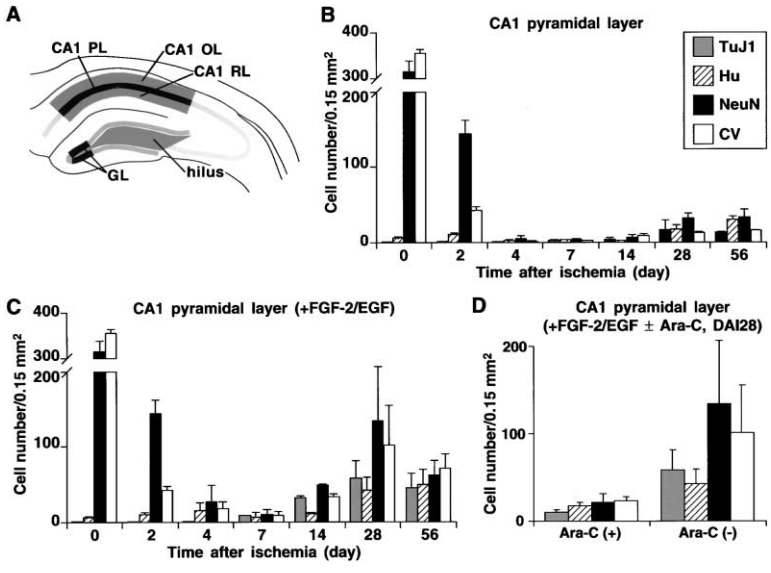

Figure 2. Quantification of the Loss and Recovery of Hippocampal Neurons

(A) Schematic representation of the areas defined to quantify neuronal numbers (see Supplemental Figure S1 for details). Abbreviations: GL, granule cell layer; OL, CA1 oriens layer; RL, CA1 radiatum layer.

(B and C) Changes in neuronal numbers in the CA1 pyramidal layer in untreated/ischemic (B) and growth factor-treated (C) animals (mean ± SD obtained from 6 animals, total 12 hemispheres).

(D) Reduction of regenerated CA1 neurons by Ara-C treatment (mean ± SD obtained from 4 animals, total 4 hemispheres). Bars in (B)–(D): shaded bars, TuJ1; hatched bars, Hu; filled bars, NeuN; open bars, neurons with intact morphology detected by cresyl violet staining.

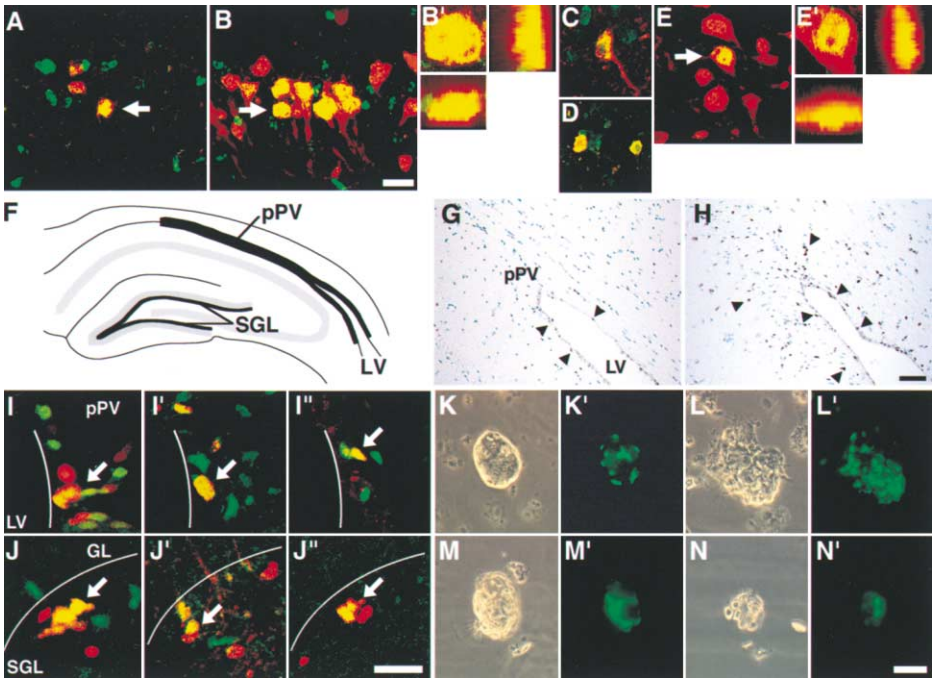

Figure 3. Regeneration of Hippocampal Neurons after Ischemia

(A–E′) BrdU$^+$ cells expressing neuronal markers. BrdU was administered to untreated/ischemic (A) and growth factor-treated (B–E′) animals during DAI2-4. At DAI28, BrdU$^+$ cells (green) expressing NeuN (A, B, B′, E, and E′), TuJ1 (C), and Hu (D) (red) are found in the CA1 pyramidal layer (A–D) and temporal cortex (E and E′). (B′) and (E′) show three-dimensional digital images of the cells indicated by the arrows in (B) and (E), respectively.

(F) Areas defined to examine the response of progenitors (for details see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/110/4/429/DC1). Abbreviations: pPV, posterior periventricle; LV, lateral ventricle.

(G and H) Distribution of Pax6$^+$ cells (arrowheads) in the posterior periventricular region near the hippocampus in the intact animals (G) and in the growth factor-treated ischemic animals at DAI7 (H).

(I–J″) Expression of Pax6 (I and J), Emx2 (I′ and J′), and Mash1 (I″ and J″) (green) in BrdU$^+$ (red) cells in the pPV (I–I″) and SGL of the DG (J–J″) (arrows) at DAI4.

(K–N′) Pax6$^+$ (K′), Emx2$^+$ (L′ and M′), and Mash1$^+$ (N′) cells (green) among neurosphere-forming adult progenitors derived from the pPV (K′ and L′) and SGL (M′ and N′). (K)–(N) are phase-contrast pictures of neurospheres shown in (K′)–(N′). Scale bar for (A)–(E) (shown in B) is 20 μm; for (G) and (H) (shown in H) is 50 μm; for (I)–(J″) (shown in J″) is 20 μm; and for (K)–(N′) (shown in N′) is 50 μm.

ronal markers, indicating that the labeling method used here did not detect repair reactions of damaged DNA (data not shown). At DAI28, however, many BrdU$^+$ cells were detected in the CA1, and some of them expressed the neuronal marker NeuN in both untreated (Figure 3A) and growth factor-treated (Figure 3B) animals. Some BrdU$^+$ cells also expressed TuJ1 and Hu (Figures 3C and 3D). Coexpression of these markers with BrdU in single cells was confirmed by three-dimensional digital reconstruction of the confocal images (Figure 3B′). In the above experiments, BrdU was administered for 2 days (DAI2-4), and hence labeled only a fraction of dividing progenitors. Nevertheless, 46% (412/890) of NeuN$^+$ neurons detected in the growth factor-treated animals were labeled with BrdU. Such double-labeled cells were also detectable in other hippocampal regions, and also outside the hippocampus including the temporal cortex (Figures 3E and 3E′).

The above analyses revealed marked effects of growth factors on the production of new neurons. The number of BrdU$^+$ cells detected in the CA1 was increased about 1.7-fold by the treatment (111 $\pm$ 38 and 66 $\pm$ 11 cells in treated and untreated animals, respectively, n = 3 animals, 6 hemispheres). Furthermore, the percentage of NeuN$^+$ neurons among these BrdU$^+$ cells in the treated animals (53%, 412 NeuN$^+$ cells in total 779 BrdU$^+$ cells, n = 3 animals) was much higher than that in the untreated animals (1%, 2/198). Thus, the short-term growth factor treatment appeared to stimulate not only the proliferation of endogenous progenitors, but also their differentiation into neurons.

We next sought to selectively ablate proliferative progenitors in vivo by the antimitotic drug cytosine β-D-arabinofuranoside (Ara-C) (Doetsch et al., 1999). Intraventricular infusion of Ara-C was started immediately after ischemia and continued for 7 days, while FGF-2 and EGF was infused between DAI2 and DAI5. Ara-C administered to the intact animals under the same condition could kill dividing cells around the lateral ventricle (H.N. and M.N., unpublished observation). As shown in Figure 2D, the Ara-C treatment significantly decreased the number of CA1 neurons (about 80% reduction) detected at DAI28 (21 $\pm$ 10 NeuN$^+$ and 23 $\pm$ 5 cresyl violet-stained cells, n = 4 animals, $p < 0.01$). This result is consistent with the idea that the massive increase in neuronal numbers by the growth factor treatment is attributable to increased cell divisions of endogenous progenitors.

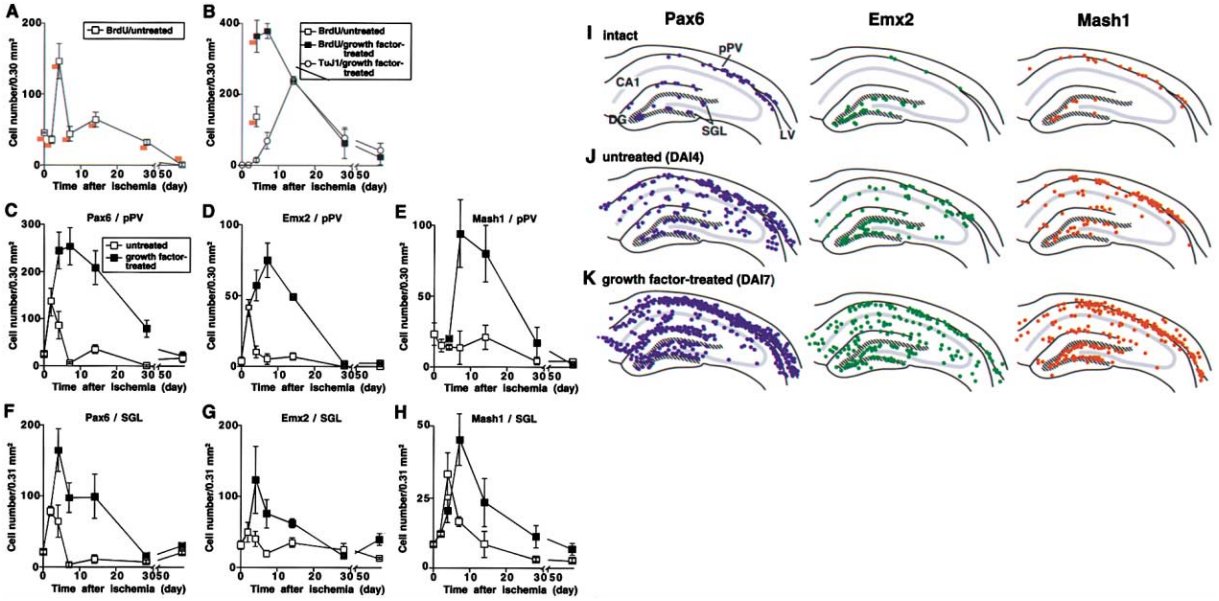

Figure 4. The Kinetics of Progenitor Activation in and around the Hippocampus Following Ischemia

(A) Increases in the number of BrdU$^+$ cells in the pPV of untreated ischemic animals at DAI2, 4, 7, 14, 28, and 56. Red bars below the symbols indicate the periods of BrdU administration (48 hr).

(B) Changes in the numbers of BrdU$^+$ (filled squares, labeled during DAI2-4) and TuJ1$^+$ (open circles) cells in the pPV of growth factor-treated animals. An open square indicates the number of BrdU$^+$ cells in untreated animals at DAI4 for comparison.

(C–H) Responses of Pax6$^+$ (C and F), Emx2$^+$ (D and G), and Mash1$^+$ (E and H) cells in the pPV (C–E) and SGL of the DG (F–H) in untreated (open squares) and growth factor-treated (filled squares) animals. All data in (A)–(H) are mean ± SD obtained from 6 animals (total 12 hemispheres).

(I–K) Distribution patterns of Pax6$^+$ (left), Emx2$^+$ (middle), and Mash1$^+$ (right) cells in the intact (I), untreated/ischemic (J, DAI4), and growth factor-treated (K, DAI7) animals. Each dot indicates the position of a cell expressing the respective factor in a representative section.

Activation of Endogenous Progenitors In Situ

The next important issues are where progenitors responsible for generating new neurons reside and how they respond to ischemia. In the rostral forebrain, the periventricular region is the source of dividing progenitors (Doetsch et al., 1999). Near the hippocampus, such dividing cells were also detectable around the posterior periventricle (pPV in Figure 3F). In the intact brain, the number of BrdU$^+$ cells in the pPV was much lower than in more anterior parts of the forebrain. We found, however, that their proliferation was markedly increased following ischemia (Figures 3I–3I″), as in the SGL of the DG (Figures 3J–3J″) (see below).

The BrdU-labeling method detects many cell types other than neural progenitors, particularly following ischemia, and thus hampers detailed analyses of the spatio-temporal regulation of endogenous progenitors. Thus, we next combined specific molecular markers with BrdU. Pax6, Emx2, and Mash1 are transcriptional regulators expressed in the developing hippocampus, and each factor distinguishes a specific subpopulation of neural progenitors. BrdU$^+$ cells in both the pPV and SGL of the DG expressed these markers in ischemic animals (Figures 3I–3J″). The expression of these markers was also detected when adult progenitors were isolated and cultured in vitro (Figures 3K–3N′).

These molecular markers revealed the dynamic response of endogenous progenitors. Although BrdU labeling detected a transient increase in cell divisions in the pPV at DAI4 (Figure 4A), the numbers of Pax6$^+$ and Emx2$^+$ cells increased as early as DAI1 and reached the maximum during DAI2-4 (Figures 4C and 4D and data not shown). Thus, the proliferative response of progenitors appeared to precede neuronal loss in the hippocampus. Furthermore, exogenous FGF-2 and EGF induced a 2.4-fold increase in the number of BrdU$^+$ cells at DAI4 (Figure 4B) and also increased both the maximum level and duration of the responses of progenitors (Figures 4C–4E). Similar changes were also observed in the SGL of the DG (Figures 4F–4H).

The spatial response patterns of endogenous progenitors in and around the hippocampus were further examined (Figures 3G, 3H, and 4I–4K). The majority of Pax6$^+$, Emx2$^+$, and Mash1$^+$ cells were confined to the pPV and SGL in the intact brain (Figures 3G and 4I). However, ischemia increased their numbers in both the pPV and hippocampal parenchyma (Figures 3H and 4J), and growth factors further augmented their responses (Figure 4K).

Origins of Progenitors Responsible for Regenerating Hippocampal Neurons

The above results demonstrated that endogenous progenitors are abundant in the periventricular region near the hippocampus. To examine whether these cells are involved in the regenerative event, we labeled them in situ by two methods. First, the fluorescent dye 1,19-dioctadecyl-6,69-di(4-sulfophenyl)-3,3,39,39-tetramethylindocarbocyanine (DiI) was injected into the lateral ventricle at DAI2 in ischemic animals. DiI-labeled cells

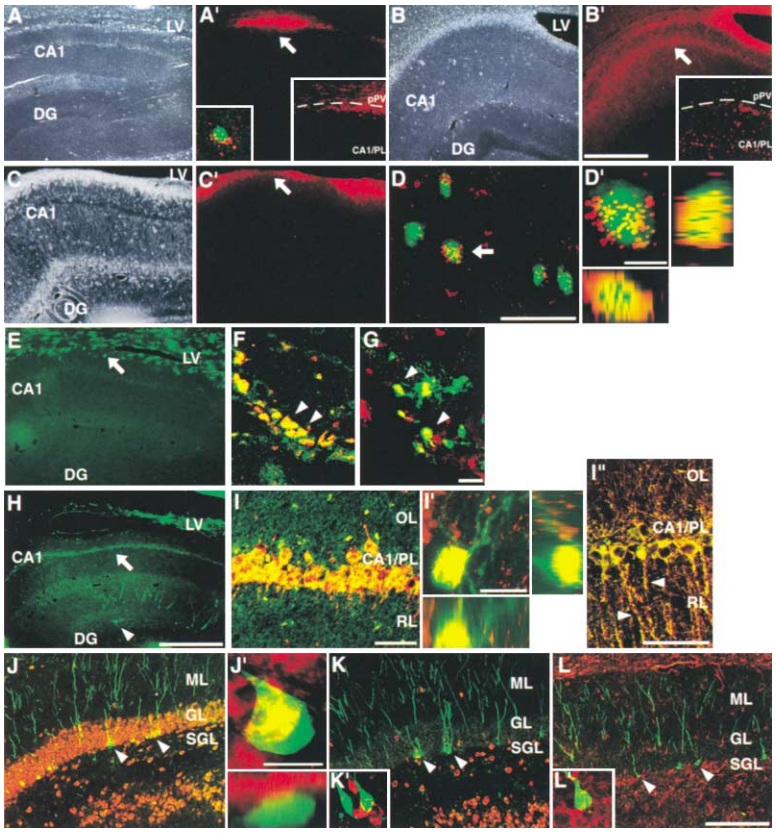

Figure 5. Migration of Periventricular Cells into the Hippocampus

(A–C′) Distribution patterns of DiI-labeled periventricular cells (red) near the hippocampus in ischemic animals at DAI4 (A′) and DAI28 (B′). (A), (B), and (C) are bright-field pictures of the sections shown in (A′), (B′), and (C′), respectively. The right insets in (A′) and (B′) are higher-magnification views of the areas indicated by arrows in (A′) and (B′). The left small inset in (A′) indicates Pax6$^+$ (green)/DiI$^+$ (red) cells in the CA1 pyramidal layer at DAI7. In the intact animals, DiI$^+$ cells remain around the ventricle 4 weeks after injection (C′).

(D) and (D′) show NeuN$^+$/DiI$^+$ cells in the CA1 pyramidal layer at DAI28.

(E–I) Genetic labeling of periventricular cells with GFP-expressing retroviruses. GFP-labeled progenitors in growth factor-treated animals (E, arrow) express Pax6 (F) and Mash1 (G) (arrowheads) at DAI7. At DAI28, however, many GFP$^+$ cells are found in the CA1 pyramidal layer (H, arrow) and become Hu$^+$ neurons (I and I′). MAP2$^+$ dendrites (indicated by arrowheads) are also GFP$^+$, and thus labeled yellow in (I″).

(J–L) GFP$^+$ cells in the DG at DAI28 (H, arrowheads). They were negative for NeuN (J and J′), Hu (K and K′), and GFAP (L and L′) (red). Note the green and red signals in separate cells in (J′), (K′), and (L′). ML indicates molecular layer of the DG. Scale bars for (A)–(C′), (E), and (H) (shown in B′ and H) are 500 μm; for (D), (I), and (I″) are 50 μm; for (D′), (I′), and (J′) are 10 μm; and for (J), (K), and (L) (shown in L) are 200 μm.

were confined to the pPV 2 days after injection (Figures 5A and 5A′, arrow). At DAI28, however, many DiI$^+$ cells became detectable in the hippocampus, particularly in the degenerated CA1 (Figures 5B and 5B′, arrow), where 88% of NeuN$^+$ cells were labeled with DiI (Figures 5D and 5D′; 126 cells in total 144 NeuN$^+$ cells examined, n = 2 animals, 4 hemispheres). Few DiI$^+$ cells could be detected in the CA1 when intact animals were labeled with the same method (Figures 5C and 5C′).

To selectively label proliferative progenitors, we next injected green fluorescent protein (GFP)-expressing recombinant retroviruses (Yamamoto et al., 2001a) into the ventricle of growth factor-treated animals. Two days after injection, GFP$^+$-infected cells accumulated around the ventricle (Figure 5E, arrow) and expressed Pax6 and Mash1 (Figures 5F and 5G, arrowheads). At DAI28, many GFP$^+$ cells were found in the CA1 (Figure 5H, arrow, and Figure 5I), and a large proportion (about 59%) of Hu$^+$ cells detected in this region were labeled with GFP (Figure 5I; 655 GFP$^+$ cells in total 1107 Hu$^+$ cells examined, n = 3 animals). MAP2$^+$ dendrites in the regenerated CA1 were derived from these GFP$^+$ cells (Figure 5I″, arrowheads). In turn, about 31% of GFP$^+$ cells became Hu$^+$ neurons (Figures 5I and 5I′; 655 Hu$^+$ cells in total 2084 GFP$^+$ cells examined, n = 3 animals). Some labeled cells were also scattered in other hippocampal regions and expressed neuronal markers (data not shown).

Interestingly, a few GFP$^+$ cells were also found in the DG at DAI28 (Figure 5H, arrowhead). They were aligned along the SGL, extending long radial processes toward the overlaying granule cell and molecular layers (Figure 5J, arrowheads). Few, if any, of these GFP$^+$ cells differentiated into NeuN$^+$ (Figures 5J and 5J′, arrowheads) or Hu$^+$ (Figures 5K and 5K′, arrowheads) neurons. They were also negative for GFAP (Figures 5L and 5L′, arrowheads), reminiscent of progenitors that normally reside in the SGL. We did not detect such cells, however, when intact animals were labeled with the same method. Thus, the migration of periventricular cells into the DG may be either specific following ischemia, or normally occurring at a very low frequency.

As described above, many BrdU$^+$ cells were detected at DAI7 in the pPV, but their number was decreased thereafter (Figure 4B). Concomitantly, TuJ1$^+$ and Hu$^+$ neurons once emerged around the ventricle around DAI14 (Figure 4B, and data not shown) and disappeared at DAI28 (Figure 4B). This timing appeared to coincide with the emergence of newborn CA1 neurons. Furthermore, some DiI$^+$ cells that migrated into the CA1 very early (during DAI7-10) expressed progenitor markers such as Pax6 (Figure 5A′, left inset). Thus, both newly generated neurons and undifferentiated progenitors may migrate into the hippocampus from the periventricular region. In addition, we detected Pax6$^+$, Emx2$^+$, and Mash1$^+$ cells in the CA1 at DAI2-4, the stage earlier than the timing when DiI- and GFP-labeled cells became detectable in this region (Figures 4J and 4K, and data not shown). These cells may correspond to neural pro-

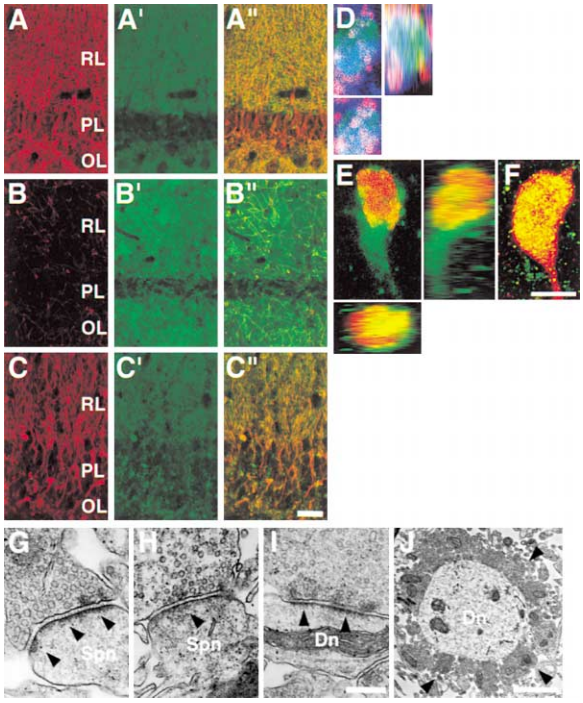

Figure 6. Integration of Newly Generated Neurons into the Brain Circuitry

(A–D) MAP2 (A–C, red) and synapsin I (A′–C′, green) staining of the CA1 in sham-operated control (A), untreated (B), and growth factor-treated (C) animals at DAI84. (A″)–(C″) are overlays of the images shown in (A)–(C) and (A′)–(C′). (D) shows triple-staining of a regenerated CA1 neuron with BrdU (green), MAP2 (blue), and synaptophysin (red).

(E and F) Retrograde labeling of $BrdU^+$ (E) and $NeuN^+$ (F) cells (red) with FluoroGold (green).

(G–J) Ultrastructures of the synapses in the regenerated CA1 of growth factor-treated animals at DAI84. Typical spine synapses with thick PSDs (G, arrowheads), spine synapses with thinner PSDs (H, arrowheads), and shaft synapses formed onto dendritic shafts (I) are shown. Multiple synapses often surround single dendrites (J, arrowheads). Abbreviations: Spn, postsynaptic spine; Dn, dendrite. The scale bar for (A)–(C) (shown in C″) is 20 μm; for (D)–(F) (shown in F) is 10 μm; for (G)–(I) (shown in I) is 100 nm; and for (J) is 1 μm.

genitors resident within the hippocampal parenchyma. Taking these results together, progenitors present in both the periventricular region and parenchyma may participate in regenerating new neurons after ischemia.

Regenerated Neurons Participate in the Brain Circuitry

We next examined the degree of maturation and connectivity of regenerated CA1 neurons. In the oriens and radiatum layers of the intact CA1, staining for microtubule-associated protein 2 (MAP2) detected abundant dendrites that contacted with multiple synapsin I^+ presynaptic fibers (Figures 6A, 6A′, and 6A″). Ischemic insults eliminated these dendrites almost completely, leaving presynaptic components largely undisturbed (Figures 6B, 6B′, and 6B″). In contrast, dendritic structures were restored, albeit at a reduced density, in growth factor-treated animals at DAI84 (Figures 6C, 6C′, and 6C″). Triple staining with BrdU, MAP2, and synaptophysin suggested that newly generated neurons received synaptic inputs (Figure 6D).

Electron microscopic examination revealed unambiguous synaptic structures (Figures 6G–6J). Asymmetric synapses onto dendritic spines, the major excitatory synapses in the intact CA1, were detected in growth factor-treated animals (Figure 6G). However, this type of synapse was not predominant, and instead spine synapses with a thinner postsynaptic density (PSD) (Figure 6H) and shaft synapses with a dense PSD (Figure 6I) were the major types. Multiple presynaptic components surrounding a single dendrite were frequently observed in the regenerated CA1 (Figure 6J). These characteristics are reminiscent of immature pyramidal neurons (Zhang and Benson, 2000) and may reflect the rearrangement of both pre- and postsynapses during regeneration.

We further asked whether regenerated neurons reconstruct the intrahippocampal connection between the CA1 and subiculum. The fluorescent tracer FluoroGold (FG) was injected into the subiculum at DAI49 in growth factor-treated animals, and retrograde labeling of CA1 neurons was examined at DAI56. The FG labeling was detected in about 40% of $NeuN^+$ cells in the CA1 pyramidal layer, 2 mm anterior to the injection site (Figure 6F; 83 FG^+ cells in total 196 $NeuN^+$ cells examined, $n = 2$ animals, 3 hemispheres), and many of these cells were $BrdU^+$ (Figure 6E). The above results collectively demonstrated that neurons newly generated after ischemia are integrated into the existing neural circuitry.

Synaptic Response of Regenerated Hippocampal Neurons

Next we examined the electrophysiological properties of synapses formed onto regenerated neurons. Stimulation of Schaffer collaterals elicited field excitatory postsynaptic potentials (fEPSPs) in slices prepared from sham-operated control animals (Figure 7A), but such a response was markedly attenuated in ischemic animals at DAI90-120 (Figures 7B and 7D). We observed, however, a significant increase in fEPSP amplitudes in growth factor-treated animals (Figures 7C and 7D, $p < 0.05$ in unpaired t test), albeit smaller than those in the control animals ($p < 0.01$). These fEPSPs were blocked by the AMPA/kainate-type glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Figure 7C).

As described above, the density of $MAP2^+$ dendrites and synapses restored in the growth factor-treated animals was lower than in the control. Thus, to compare the synaptic activities more directly, the relationship between the levels of presynaptic volley and fEPSP amplitude was examined, in which the former reflects the strength of presynaptic inputs, whereas the latter indicates the postsynaptic response to a given level of presynaptic stimulation (Figure 7E). Consistent with the anatomical observations, the maximum level of the presynaptic fiber volley was lower in the growth factor-treated animals (about 0.25 mV) than in the control animals (about 0.35 mV). Under these conditions, however, the slope of the input-output curve in the growth factor-treated group ($n = 29$, $r = 0.526$) was not statistically different from that in the control group ($n = 28$, $r = 0.777$; $p = 0.1074$ in comparison of two correlation coef-

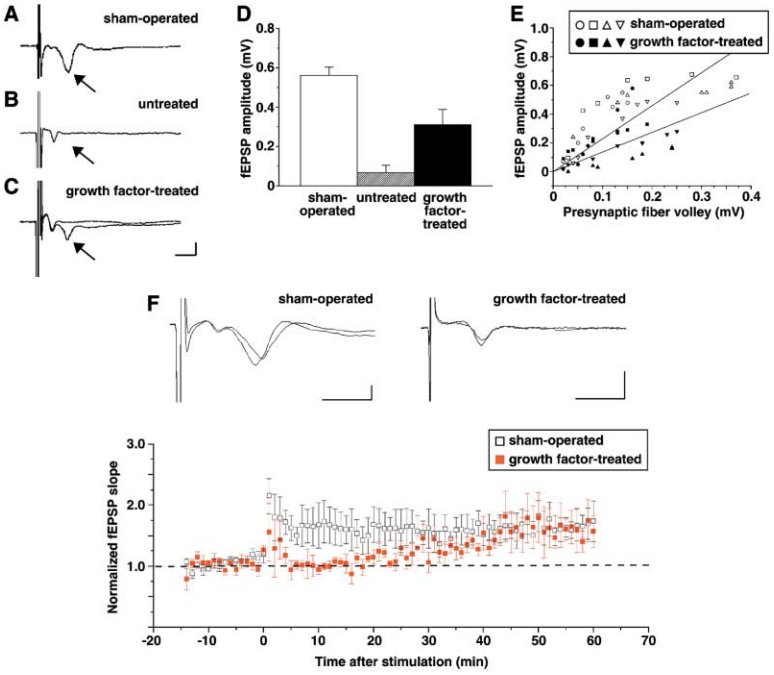

Figure 7. Electrophysiological Properties of Synapses Formed onto Regenerated Neurons

(A–C) Averaged traces of fEPSPs (arrows) evoked by presynaptic stimulation in sham-operated control (A), untreated (B), and growth factor-treated (C) animals. Each trace is the average of five fEPSPs, and stimulus artifacts are truncated. In (C), traces recorded with and without 50 μM CNQX are overlaid.

(D) Saturated amplitudes of fEPSPs (mean ± SD) in control (open bar, n = 4 slices/3 animals), untreated (hatched bar, n = 7 slices/3 animals), and growth factor-treated (filled bar, n = 4 slices/3 animals) animals.

(E) Relationships between the amplitude of fiber volleys and the corresponding amplitude of fEPSPs in sham-operated control (open symbols) and growth factor-treated (filled symbols) animals (n = 4 slices/3 animals for each group).

(F) Long-lasting synaptic potentiation evoked by a single tetanic stimulation (100 Hz, 1 s) in control (open squares, n = 4 slices/4 animals) and growth factor-treated (filled squares, n = 4 slices/3 animals) animals. Insets are superimposed traces of the averaged fEPSPs (5 traces) before (−5 to −1 min) and after (56 to 60 min) tetanus in a representative sham-operated (left) and growth factor-treated (right) animal. Calibration bars in (A)–(C) and (F) equal 0.3 mV and 5 msec.

ficients), although the former appeared to be slightly lower than the latter (Figure 7E). Thus, the basic properties of synaptic transmission were well restored in regenerated neurons.

In slices that showed typical postsynaptic responses, we further examined changes in fEPSP slopes following tetanic stimulation (Figure 7F). Clear potentiation, which is known as the long-term potentiation (LTP) (Malenka and Nicoll, 1993), was observed in the sham-operated control animals (67% ± 23% increase in normalized fEPSP slopes between 56–60 min after tetanus, n = 4 slices/4 animals). Importantly, significant potentiation could also be detected in the growth factor-treated animals. This effect was long-lasting, and the average of normalized fEPSP slopes was 58% ± 26% higher over the baseline 60 min after stimulation (n = 4 slices/3 animals). However, the synaptic potentiation once decayed to the baseline within 5 min, and thereafter gradually recovered to the extent comparable to that in the control slices at the time point of 40–60 min. Thus, the response appeared to lack a short-term component of the typical LTP. Nevertheless, these results clearly demonstrated that functional glutamatergic synapses are restored in the regenerated hippocampus.

Recovery of Brain Functions by Growth Factors

The preceding results demonstrated that endogenous progenitors are capable of regenerating functional hippocampal neurons. We also detected the generation of new neurons in other regions, including the cerebral cortex and striatum. Thus, many types of neurons in the forebrain were probably lost following ischemia, and at least a part of them could be replaced by newborn neurons. Taking these observations into account, we next sought to correlate the regeneration of new neurons and recovery of brain functions. Here we used the Morris water-maze task to examine hippocampus-involved learning and memory (Riedel et al., 1999). In this spatial learning task, rats learn to navigate to a platform submerged in a water pool by using extra-maze cues. To examine the role of newborn neurons, the same animal groups were tested at two distinct periods, i.e., before and after neurogenesis.

The first block trials were performed during DAI7-11, at the period appropriate to detect impairment of brain functions caused by ischemia. When sham-operated control animals were trained twice a day for 5 consecutive days (sessions 1–10), they memorized the position of the hidden platform, thereby reaching it in a shorter latency during the training (n = 7 animals for Figure 8A and n = 6 for Figure 8B). In contrast, both untreated (n = 11 for Figure 8A and n = 8 for Figure 8B) and growth factor-treated (n = 9 for Figure 8A and n = 7 for Figure 8B) ischemic animals showed deficiencies during the initial 3 days (DAI7-9) (comparison by repeated ANOVA, $F[2,5] = 11.540$ in Figure 8A and 13.946 in Figure 8B). Post hoc analyses confirmed significantly longer escape latencies between sessions 1 and 6 in both animal groups than in the control ($p < 0.01$ in both Figures 8A and 8B). This initial impairment was not likely to be attributable to a defect in their swimming ability; the swimming velocities measured during the tasks were not significantly different among three animal groups ($F[2,9] = 0.888$ and $p = 0.431$ in Figure 8A, and $F[2,9] = 2.183$ and $p = 0.159$ in Figure 8B). The spontaneous locomotor activities measured in an open field were also indistinguishable among the groups (Figure 8C). These ischemic animals, however, were finally trained during the last 2 days (sessions 7–10). Thus, ischemic animals

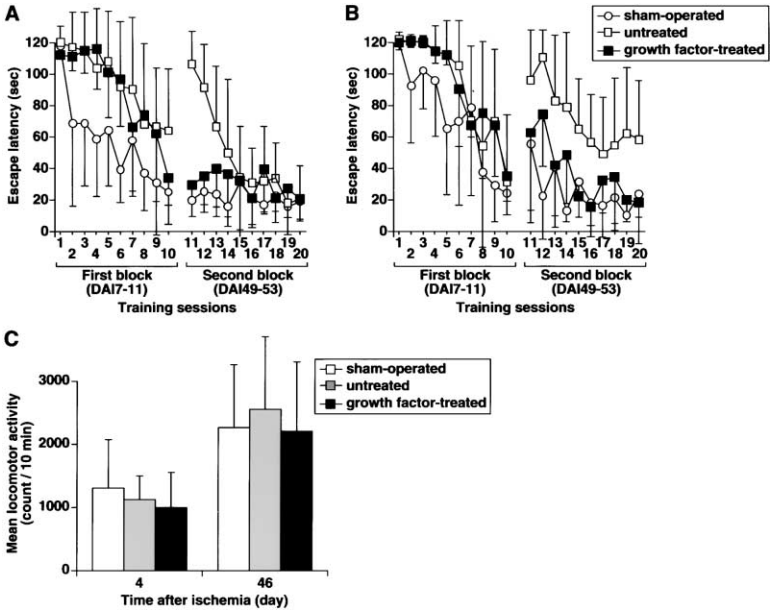

Figure 8. Performance in the Water-Maze Tasks

Two separate groups of rats (A and B) were subjected to two blocks of maze tasks at DAI7-11 (first block, sessions 1–10) and DAI49-53 (second block, sessions 11–20). Each animal was tested twice a day in both blocks. The position of the platform was either the same in the first and second blocks of task (A), or changed rightward at 90° between the first and second blocks (B). In both paradigms, the escape latencies (mean ± SD) are compared among sham-operated (open circles), untreated ischemic (open squares), and growth factor-treated ischemic (filled squares) animals. The spontaneous locomotor activities in an open field (C) are also measured at DAI4 and DAI46.

exhibited a partial impairment in the acquisition of new memory including both spatial memory and the search strategy. Importantly, FGF-2 and EGF showed no significant effect during this assay period, which is consistent with our histological observations.

To evaluate the contribution of regenerated neurons, the same animal groups were subjected to the second block trials between DAI49 and DAI53 (sessions 11–20). In the first paradigm, the platform was placed at the same position as in the first block trials (Figure 8A). The control animals ($n = 7$) retained the memory of spatial cues and the search strategy, and thus could reach it as fast as they did at the end of the first block. In contrast, the untreated ischemic animals ($n = 11$) exhibited severe impairment in this task. The growth factor treatment ($n = 9$) remarkably improved this long-term memory-dependent cognitive performance (between sessions 11 and 16, $F[2,5] = 15.794$, $p < 0.01$ comparing the untreated and treated groups).

Separate subgroups of trained animals were tested in a different paradigm, in which the platform was placed at a new position (Figure 8B). In this case, rats had to learn this new position, but could still utilize their memory of the search strategy. Under these conditions, the untreated ischemic animals ($n = 8$) again showed a severe deficiency compared to the control ($n = 6$). Although they were gradually trained and showed better performance, their escape latencies were still much longer than those of the control animals at the end of training. This difference might have reflected impairment of their ability to acquire new memory. In contrast, the growth factor-infused animals ($n = 7$) could reach the new platform position much faster than the untreated animals at all trial points ($F[2,9] = 8.547$, $p < 0.01$ comparing the two animal groups). Thus, the treatment also restored brain functions tested in this paradigm. We observed no significant difference in either the swimming velocities during the second block trials ($F[2,9] = 2.681$ and $p = 0.096$ in Figure 8A and $F[2,9] = 1.863$ and $p = 0.187$ in Figure 8B) or spontaneous locomotor activities (Figure 8C) among these animal groups.

In summary, ischemic animals showed severe deficiencies in spatial cognitive performance during both early (DAI7-11) and late (DAI49-53) periods after a transient ischemic episode. Postischemic administration of growth factors significantly ameliorated such deficiencies at the late but not early assay period, which could be correlated to its remote action to augment regeneration of new neurons.

Discussion

Here we have demonstrated a remarkable regenerative capacity of endogenous neural progenitors in the adult brain. Hippocampal CA1 pyramidal neurons undergo extensive degeneration following transient ischemia (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984). We have shown that intraventricular infusion of growth factors can recruit endogenous progenitors in situ, thereby inducing massive regeneration of pyramidal neurons after ischemia. We discuss the implication of our findings with reference to the regenerative capacity of the adult CNS and its potential therapeutic applications for ischemic injury and other neurological diseases.

Recruitment of a Regenerative Capacity of Adult Progenitors

Our results, together with those in recent studies, have demonstrated that adult neural progenitors proliferate in situ in response to various insults such as ischemia (Liu et al., 1998; Jin et al., 2001; Yoshimura et al., 2001), trauma (Gould and Tanapat, 1997; Johansson et al., 1999; Yoshimura et al., 2001; Yamamoto et al., 2001a), and neurodegeneration (Fallon et al., 2000; Magavi et al., 2000). However, their endogenous regenerative capacity appears to be very limited, being capable of replacing only a small fraction of neurons (Fallon et al.,

2000; Magavi et al., 2000) or unable to generate new neurons (Johansson et al., 1999; Yamamoto et al., 2001a, 2001b). This study showed that we can markedly augment such a latent regenerative potential of the adult CNS.

To this end, several manipulation conditions appear to be critical. Previous studies have shown that either FGF-2 or EGF alone stimulates neurogenesis only to a limited extent in vivo (Craig et al., 1996; Kuhn et al., 1997). On the other hand, both FGF-2 and EGF are necessary for the maximum proliferation of adult progenitors in vitro (Weiss et al., 1996). Therefore, we combined both factors here to effectively recruit endogenous progenitors. We also noticed that in the intact brain, exogenous factors stimulate progenitors in normally active neurogenic sites, but much less do so around the hippocampus (H.N. and M.N., unpublished observation). In contrast, ischemic insults can activate otherwise dormant progenitors in this region, albeit transiently. Thus, this transient activation period appears to be most appropriate to mobilize progenitors by exogenous factors.

Various neuroprotective agents such as FGF-2 and brain-derived neurotrophic factor have been tested for their ability to rescue ischemic cell death (Bethel et al., 1997; Kiprianova et al., 1999). However, irreversible degeneration is triggered in the initial 1–2 days after ischemia (Kirino and Sano, 1984; Colbourne et al., 1999b), and hence little success has so far been achieved beyond this critical period (Colbourne et al., 1999a, and references therein). We instead took advantage of these observations to distinguish between regeneration and neuroprotection. When administered to ischemic animals at DAI2 and thereafter, FGF-2 and EGF did not protect CA1 pyramidal neurons against cell death, yet they could selectively stimulate endogenous progenitors. Furthermore, our preliminary study has suggested that a long-term treatment with growth factors rather delays and/or inhibits differentiation of progenitors in situ. Thus, our growth factor-infusion paradigm was defined so as to induce substantial regeneration of new neurons after ischemia. In order to apply similar strategies to other disease models, we may have to consider many parameters, such as the time window and site of injection of growth factors and their doses, depending on their pathophysiology.

Control of Neurogenesis in the Adult Brain

It is noteworthy here that hippocampal CA1 pyramidal neurons, which have not been thought to be continuously replaced in the normal adult brain, can be regenerated following ischemia. Although adult neurogenesis has begun to be widely recognized during the last decade, there have still been lots of debates with regard to the sites of neurogenesis (Gould and Gross, 2002). Recent studies have demonstrated, however, that the production of new neurons occurs in many parts of the adult brain albeit at a low frequency (Rietze et al., 2000; Pencea et al., 2001, Gould and Gross, 2002). This study, together with other recent studies (Fallon et al., 2000; Magavi et al., 2000), has further demonstrated that adult neurogenesis can be markedly stimulated under certain pathological conditions. In line with these observations, recent studies have revealed the widespread occurrence of progenitors in the adult CNS (Weiss et al., 1996; Palmer et al., 1999; Johansson et al., 1999; Yamamoto et al., 2001a).

Another notable feature is that adult progenitors can regenerate specific neuronal subtypes appropriate for the sites of damage. For instance, targeted degeneration of corticothalamic neurons in the neocortex and dopaminergic neurons innervating to the striatum has been shown to induce regeneration of the same cell types (Magavi et al., 2000; Fallon et al., 2000). Our data also demonstrated that progenitors near the hippocampus contribute to the genesis of hippocampal neurons. Thus, adult progenitors present in distinct regions may have distinct intrinsic properties. Alternatively, distinct environmental cues may instruct endogenous progenitors to differentiate into specific neuronal subtypes depending on their locations. Details of such a specificity and the underlying mechanisms still remain poorly understood.

The above evidence collectively suggests that the latent regenerative potential of the adult CNS may be much greater than previously thought. Another important question is how adult neurogenesis can be manipulated under damaged conditions. The availability of signaling factors in local environments may be limiting the behavior of endogenous progenitors. Exogenous growth factors increase the rate of neurogenesis in the normal brain (Craig et al., 1996; Kuhn et al., 1997). In these cases, however, the observed neurogenesis is largely restricted to the regions where production of new neurons normally occurs at a high frequency. Thus, a certain combination of exogenous factors and endogenous stimulatory signals may be necessary for inducing substantial regeneration of neurons at lesions with normally slow turnover rates. Furthermore, some inhibitory signals have also been implicated in tightly restricting neurogenesis in vivo (Yamamoto et al., 2001b). Thus, multiple stimulatory and inhibitory signals are probably involved in controlling the rate of neurogenesis, depending on the location and type of lesions. Elucidation of the molecular nature and mechanisms of the actions of these signals will be essential for further understanding of the regenerative capacity of the adult CNS.

Integration of Regenerated Neurons into the Neural Circuitry

Integration of regenerated neurons into the existing neural circuitry is a prerequisite for restoration of brain functions. We showed here that CA1 neurons regenerated after ischemia survived for a long period of time (at least 6 months), received synaptic inputs, and participated in reconstruction of the intrahippocampal connection. However, synapses of these neurons appeared to remain immature in morphology and exhibited altered electrophysiological properties even 3 months after ischemia. The maturation of regenerated CA1 neurons may therefore proceed over an extended period of time (van Praag et al., 2002).

The above evidence has demonstrated that regenerated hippocampal neurons are functional at least in certain aspects, and therefore suggests that they may contribute to the restoration of brain functions. In fact, postischemic growth factor treatment significantly amel-

iorated deficits in spatial cognitive performance in ischemic animals. Growth factors appeared to improve the ability of long-term retention of memory and also possibly had an effect on the acquisition of new memory. However, it remains to be further explored what aspects of brain functions were actually restored by the treatment.

It is noteworthy here that growth factors did not show any detectable effect on the cognitive performance of ischemic animals during the period of neuronal loss. Their effect was rather seen at much later time points. This remarkable remote action can most simply be explained by the idea that growth factors did not prevent ongoing neuronal degeneration during the period of their administration, but rather stimulated regeneration of new neurons that would occur in a relatively long period of time after the treatment. In fact, growth factors showed no significant neuroprotective effect on hippocampal neurons. However, it remains possible that mechanisms other than neurogenesis, such as promotion of regrowth of damaged nerve fibers, may also underlie the observed behavioral recovery. Thus, the direct causal relationship between the neuronal regeneration and improvement of cognitive functions must await further intensive studies. Nevertheless, our data strongly suggest that together with many other possible mechanisms, integration of newly generated neurons also likely contributes to the recovery of brain functions.

Cell Replacement Therapies by Using Endogenous Progenitors

Various strategies preventing neuronal cell death have so far been explored toward developing therapeutics for neurological disorders. Recent studies, however, have begun to imply the potential use of neural progenitors for transplantation therapies (Björklund and Lindvall, 2000). Our results may further provide an alternative, novel strategy for cell replacement therapies, which utilizes the latent regenerative capacity of endogenous progenitors. Such a strategy may be applicable for a wide range of diseases in which relatively broad areas and many types of neurons are affected simultaneously.

Various exogenous factors have been shown to regulate adult neurogenesis in vivo. Notably, subcutaneous administrations of FGF-2 and IGF-I have been shown to stimulate neurogenesis in the adult hippocampus (Wagner et al., 1999; Åberg et al., 2000). Furthermore, adult neural progenitors are abundant in the periventricular areas, and hence amenable to a direct access of viral and nonviral vehicles for gene delivery. Thus, adult neural progenitors may provide a novel target for drug and gene therapies for neurological diseases. Future development of a range of strategies to efficiently mobilize endogenous progenitors will facilitate significant structural and functional repair of the damaged adult CNS, which may hopefully be applicable in the future for curing various human diseases including stroke.

Experimental Procedures

Animals and Surgery

Adult male Wistar rats (8–10 weeks of age and weighing 250–300 g) were subjected to transient forebrain ischemia by a method combining those described previously (Kirino, 1982; Pulsinelli et al., 1982; Smith et al., 1984). Details of the surgical procedure and animal care are available as Supplemental Data at http://www.cell.com/cgi/content/full/110/4/429/DC1.

Labeling and Manipulations of Endogenous Progenitors

Dividing progenitors were labeled by intraperitoneal injection of BrdU (50 mg/kg body weight, Sigma). To examine the kinetics of cell divisions, BrdU was administered every 2 hr for 48 hr prior to sacrifice of rats. The fate of labeled cells was examined by allowing the animals to survive for various periods of time after BrdU injection. Cells in the periventricular region were labeled by injecting either 20 μl of DiI (0.2% in dimethylsulfoxide, Molecular Probe) or 25 μl of a solution of high-titer recombinant retroviruses that express GFP (approximately 5×10^8 colony-forming unit/ml) (Yamamoto et al., 2001b) into the lateral ventricle.

To stimulate the proliferation of endogenous progenitors, a cocktail of FGF-2 and EGF (Roche) was infused bilaterally into the ventricle during DAI2-5 by osmotic minipumps (model 1003D, Alzet) at a flow rate of 1.0 μl/hr, resulting in a delivery of 1440 ng of each growth factor per day, per brain for 3 days. The vehicle solution was artificial cerebrospinal fluid (aCSF) containing 0.1 mg/ml rat serum albumin (Sigma). The growth factors did not cause detectable hypothermia during or after infusion. In some experiments, the antimitotic drug Ara-C (20 μg/μl in aCSF; Sigma) was combined with growth factors and infused between DAI0 and DAI7.

The above stereotactic injections were performed at the position of anteroposterior +0.0 mm and lateral +1.5 mm from the center of the bregma, and the cannula depth was 3.3 mm below the surface of the dura matter. Sham-operated control animals were treated identically, except that they received aCSF without growth factors, and carotid arteries were not occluded. The positions of the injection cannulae were identified after sampling the brain, and it was confirmed that no structural damage of the hippocampus was caused by the cannulae themselves.

Histology

Affinity-purified rabbit antibodies against Pax6, Emx2, and Mash1 were described previously (Yamamoto et al., 2001b; Suda et al., 2001). Double staining was visualized with Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Molecular Probe), and confocal images were obtained under Zeiss LSM-510 confocal microscopy.

The number of neurons was quantified by staining 10-μm-thick cryosections (for details, see Supplemental Data at http://www.cell.com/cgi/content/full/110/4/429/DC1). CA1 pyramidal neurons were defined as large cells of >10 μm diameter, with pale nuclei, discrete nucleoli, and basophilic cytoplasm. Their morphology was further examined by using 4-μm-thick paraffin sections. Neuronal connections of regenerated CA1 neurons were examined by injecting 0.2 μl of aCSF containing 3% FluoroGold (Fluorochrome) into the subiculum at the position of anteroposterior −5.3 mm and lateral +1.2 mm with cannula depth 3.3 mm below the surface of the dura matter. The morphology of neuronal cell body and synapses was examined in hippocampal slices of 300–400 μm thickness by transmission electron microscopy using JEOL JEM-1010 TEM at 80 kV.

Electrophysiology

Animals that survived various treatments for 3–4 months (22–27 weeks of age) were subjected to electrophysiological studies as described (Okabe et al., 1998). The Schaffer collateral pathway of hippocampal slices was stimulated by 0.2 ms constant-current pulses every 30 s or 1 min by using a bipolar electrode positioned in the CA1 radiatum layer, and fEPSPs evoked in the pyramidal layer were recorded with glass microelectrodes. At the beginning of each recording, the strength of presynaptic fiber stimulation was increased stepwise (50 μA each, ranging from 30 to 480 μA) until fEPSP amplitudes were saturated, and subsequently reduced to elicit a fEPSP with 50%–70% of the maximal amplitude. Baseline fEPSP slopes were recorded for 20–30 min before tetanic stimulation, and LTP was subsequently induced by a high-frequency stimulation (100 Hz, 1 s). Evoked fEPSPs were filtered 1 kHz, and digitized at 10 kHz. CNQX (50 μM) was added to the perfusion medium to block AMPA/kainate-type glutamate receptor-mediated response.

Behavioral Study

The spatial cognitive performance of rats was examined by using the Morris water maze as described (Yonemori et al., 1999). Rats were subjected to two blocks of maze tasks at 7–11 and 49–53 postoperative days, respectively, and the escape latency and mean swimming velocity to reach the hidden platform were compared among different animal groups. The position of the platform was kept unchanged in the first and second blocks of the task, or changed rightward at 90 degrees between the two blocks. The spontaneous locomotor activity of rats was measured for 60 min with 10 min intervals using activity box at DAI4 and DAI46 as described (Yonemori et al., 1999) and indicated as mean counts per 10 min.

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