Ischemia-induced neural stem/progenitor cells express pyramidal cell markers
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Introduction
Stroke is the major cause of death and invalidity in the western world. Although medical science has made immense progress in treating the consequences of stroke, an efficacious therapy to restore lost neural cells is yet to be conceived. A proposed therapeutic approach is a supply of neural stem cells (NSCs) to replace the lost cells in ischemic brain lesions. NSCs have been found in adult brain aside from the developing brain. Enhancing this endogenous neurogenesis might provide another strategy for treating neural loss in the poststroke brain. Accumulating evidence shows that adult neurogenesis also takes place outside conventional neurogenic sites such as the subventricular zone of the lateral ventricle [1] and the subgranular zone of the hippocampus [2], notably in the cerebral cortex [3,4], white matter [5], and spinal cord [6]. Stimulation of endogenous adult NSCs could contribute to brain function recovery, providing a less invasive stroke treatment.

Recently, we found that injury/ischemia-induced neural stem/progenitor cells (iNSPCs) developed in the poststroke cortex in adult mice [3]. These cells are capable of self-renewal and differentiation into electrophysiologically functional neurons, astrocytes, and myelin-producing oligodendrocytes. We also showed that iNSPCs migrated into the peri-infarct area of the poststroke brain [7], suggesting that iNSPCs contribute to restorative mechanisms of the poststroke brain. Determination of whether iNSPCs can differentiate into projecting pyramidal neurons is important for achieving functional recovery after stroke, as the projecting neurons directly contribute to the neural circuit that connects physically separated brain regions.

In this study, we show that ischemia-induced regenerative neurons express the pyramidal cell markers Emx1 and CamKIIz (Ca2+/calmodulin-dependent kinase IIz), as well as the nonpyramidal cell marker glutamic acid decarboxylase (GAD), suggesting iNSPC differentiation into projecting neurons in the poststroke brain.

Methods
All procedures were carried out under the auspices of the Animal Care Committee of Hyogo College of Medicine and were in accordance with the criteria outlined in the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the National Academy of Science.

Induction of permanent ischemia
Six-week-old male CB-17 (CB-17/Icr-+ /+ Jcl) and SCID (CB-17/Icr-seid/scid Jcl) mice (Clea Japan, Tokyo, Japan) were subjected to cerebral ischemia. Ligation of the middle cerebral artery causes highly specific and reproducible infarction of the ipsilateral cerebral cortex, as previously described [3]. In brief, the left middle cerebral artery was electrocauterized and disconnected just distal to crossing the olfactory tract (distal M1 portion) under halothane anesthesia (2%). The animals were allowed to rest for 7 days during which powdered chow and sterilized water were provided ad libitum.
Cell proliferation assessment

Previously, we demonstrated that immunodeficiency reduces neural stem/progenitor cell apoptosis and enhances neurogenesis in the cerebral cortex after stroke [8]. In this study, to determine cell proliferation in vivo, 7-week-old male SCID mice were injected with 5-bromo-2-deoxyuridine (BrdU) to visualize regenerating neurons. BrdU (50 mg/kg; Sigma-Aldrich, St. Louis, Missouri, USA) was administered from day 3 after stroke, three times per week, until the animals were killed on day 21 after stroke. Thereafter, the mice were strongly anesthetized using sodium pentobarbital (50 mg/kg) and perfused transcardially with 4% paraformaldehyde. Coronal brain sections (16 μm) were cut on a cryostat, stained with antibodies for BrdU (1:100; Abcam, Cambridge, UK) and Emx1 (1:200; Abcam), and visualized using Alexa-conjugated secondary antibodies (1:500; Alexa Fluor 488 or 555; Invitrogen, Carlsbad, California, USA). Images of stained sections were assessed using a fluorescent microscope (Olympus, Tokyo, Japan).

Culturing of neurospheres into pyramidal cells

On day 7 after stroke, infarcted tissue was extracted from the poststroke cerebral cortex of CB-17 mice as described before [3]. The ischemic tissue was dissociated into a single-cell solution. Cells were further cultured into neurosphere-like cell clusters in Dulbecco’s modified Eagle’s medium/F12 medium containing endothelial growth factor (20 nm/ml; Protech, Rocky Hill, New Jersey, USA), basic fibroblast growth factor (20 ng/ml; Protech), N2 (1%; Invitrogen), and Y27623 (10 nM; Wako Pure Chemical Industries Ltd, Osaka, Japan). After incubation for 21 days, neurospheres were incubated on poly-l-lysine-coated (0.05%) glass cover slips in neurobasal medium (Invitrogen) containing retinoic acid (0.06 μg/ml; Sigma-Aldrich) and B-27 supplement (0.4%; Sigma-Aldrich). Cell clusters were differentiated for 7 days before being processed further for immunocytochemistry or PCR.

Immunocytochemistry

Differentiated neurosphere-like cell clusters were subjected to immunocytochemistry using antibodies to Tuj1 (1:1000; Stemcell Technologies, Vancouver, Canada), MAP2 (1:1000; Convance, Berkeley, California, USA), CamKIIz (1:400; Abcam), and Emx1 (1:200; Abcam), as well as Alexa-conjugated (Alexa Fluor 488 or 555) secondary antibodies. Nuclei were stained with 4’,6-diamino-2-phenylindole (1:1000; Kirkegaard and Perry Laboratories, Gaithersburg, Maryland, USA).

Reverse transcriptase PCR

Total RNA was extracted from differentiated neurosphere-like cell clusters using an RNeasy Mini-plus kit (Qiagen, Valencia, California, USA), followed by treatment with DNase (Qiagen). To compare the pyramidal markers expressed in regenerated neurons with those in adult cortical neurons, RNA was extracted from the nonischemic cortex (contralateral cortex to infarction) of CB-17 mice. Total RNA (100 ng) was reverse transcribed under the following conditions: 15 s at 94°C, 30 s at 56°C, and 1 min at 68°C (40 cycles). Primer sequences used were: CamKIIz forward CCTACAGGAAGATGTGCGA CC and CamKIIz reverse TTGGGAGTGAGTCCGAC TA (amplicon size, 163); Emx1 forward primer GCAACC CCCTCACTCTTTCTTCT and Emx1 reverse primer GCA ACCCCCTCACTCTTTCTTCT (amplicon size, 380 bp); Otx1 forward primer Accttcagctgctcag and Otx1 reverse primer GCTCAGAGGAGGCTGCT (378 bp); and glyceraldehyde-3-phosphate dehydrogenase reverse TGG TATCGTGGAGGACTGATGAC and glyceraldehyde-3-phosphate dehydrogenase reverse ATGCCAGTGAAGCT TCCCGTTAGC (amplicon size, 180 bp).

Results

BrdU-positive cells express pyramidal cell markers in the poststroke cortex

We have shown previously that BrdU-positive regenerative neurons are present in the peri-infarct area of the poststroke cortex [9]. Immunohistochemistry for BrdU and the pyramidal cell marker Emx revealed that a number of BrdU-labeled cells were present throughout the ipsilateral cerebral hemisphere (Fig. 1a–c) on day 21 after stroke; however, they were never seen in the contralateral side. Some BrdU-labeled cells were identified simultaneously as being Emx1-positive neurons. These cells are frequently found in close association with the infarcted area (peri-infarct area) of the ipsilateral cortex (Fig. 1a, d–f) and in the ipsilateral striatum (Fig. 1c, g–i).

Injury-induced neural stem/progenitor cells differentiate into pyramidal-shaped neurons in vitro

To assess whether iNSPCs are capable of differentiating into pyramidal neurons in vitro, the nature of cells in neurosphere-like cell clusters from the ischemic core after incubation in culture was studied further as described previously [3,7,9]. Approximately 70% (68.2 ± 8.1%) of the clusters differentiated into adherent cells. Cell population analysis for differentiated sphere-like clusters showed that these cells predominantly differentiated into neuronal cells and oligodendrocytes rather than into astrocytes (Tuj1-positive neuronal cells, approximately 35%; GFAP-positive astrocytes, approximately 5%; and O4-positive oligodendrocytes, approximately 31%) similar to our previous study [3,7,9]. Approximately 6.7% (6.7 ± 3.1%) of the clusters differentiated into adherent cells characterized by a long apical dendrite-like structure in their processes (Fig. 1j), which is positive for the neuron markers Tuj1 and MAP2 (Fig. 1k).
Regenerated neurons and differentiated neural stem/progenitor cells. (a–i), pyramidal Emx1 and 5-bromo-2-deoxyuridine (BrdU)-positive cells in the poststroke brain. The postinfarct brain section obtained from mouse 21 days after stroke (a) was subjected to immunohistochemistry for Emx1 and BrdU. Emx1/BrdU double-labeled cells are distributed at the cerebral cortex (b, D–F) and the striatum (c, G–H) around the peri-infarct area. Higher magnification revealed that the nuclei of Emx1-positive cells were simultaneously labeled by BrdU [(D and G): Emx1; (E and H): BrdU; (F and I), merged], indicating that Emx1-positive neurons regenerated in the poststroke brain. (j–l): injury-induced neural stem/progenitor cells (iNSPCs) differentiate into pyramidal-shaped neurons in vitro. Cells in the neurosphere are processed to differentiate into neurons in culture. The adherent cells appeared pyramidal in shape with a long apical dendrite-like process under a light microscope (j). The pyramidal-shaped cells are Tuj1-positive (k and l) and MAP2-positive (m and n) neurons, which developed many dendritic spine-like structures in their processes [(k, m, and n): arrows; (l and n): higher magnification of (k and m), respectively]. These findings suggest that iNSPCs differentiate into pyramidal neurons in vitro. Scale bar: 200 μm (b and c), 50 μm (d–i, k, m), and 100 μm (j).
and l, Tuj1; m, n, MAP2). These neurons possess short dendritic spine-like structures (Fig. 1k–n, arrows; Fig. 1l and n are higher magnification of processes as indicated in Fig. 1k and m, respectively), suggesting that the iNSPCs differentiate into pyramidal-shaped neurons.

Injury-induced neural stem/progenitor cell (iNSPC)-derived neurons express pyramidal cell markers in vitro. (a–i), The Tuj1-positive neurons with long processes, which are derived from iNSPCs, express Emx1 in the cell soma [(a), Tuj1; (b), Emx1; (c), merged, arrow]. In contrast, Tuj1-positive neurons with short processes are negative for Emx1 [(a and c), arrowhead; (d), Tuj1; (e), Emx1; (f), merged], some of which express GAD in the cell soma and in the processes [(g), Tuj1; (h), GAD; (i), merged, arrows]. (j–l), the neurosphere consisting of iNSPCs cultured in vitro contains cells expressing MAP2 and CamKIIα after differentiation [(j), CamKIIα; (k), MAP2; (l), merge, arrow]. The long and rather thick dendrite-like processes stained with MAP2 are developed in radial direction from the neurosphere, suggesting that it predominantly consists of pyramidal neuronal progenitors. Scale bar, 50 μm (a–i) and 200 μm (j–l). (m–o) RNA isolated from the cells differentiating in vitro was subjected to PCR for Emx1 (m), Otx1 (m), and CamKIIα (n). Control RNA was extracted from the intact, nonischemic cortex (contralateral cortex to infarction) of CB-17 mice. Cultured iNSPC-derived neurons express both Emx1 and CamKIIα, similar to cortical neurons, but they do not express Otx1. The relative expression of pyramidal cell markers in iNSPC-derived neurons is shown in (o).

Injury-induced neural stem/progenitor cell-derived neurons express both pyramidal and nonpyramidal markers

To assess whether iNSPCs are capable of differentiating into pyramidal neurons in vitro, immunocytochemistry
was performed for the pyramidal marker Emx1. The cells differentiating into TuJ-1-positive neurons were simultaneously positive for Emx1 (5–7% of cells; Fig. 2a–c, arrow). However, the majority of neurons positive for TuJ1 were not positive for Emx1 (Fig. 2d–f), suggesting that these differentiate into both pyramidal and nonpyramidal neurons. As γ-aminobutyric acid neurons are known as interneurons [10], its synthesizing enzyme GAD was used as a marker for nonpyramidal neurons. Approximately 23–26% of cultured cells are GAD positive (Fig. 2g–i). GAD staining was found primarily to be present in the axon terminal-like processes and in the cell bodies of cultured cells (Fig. 2h, arrows). Emx1 staining was found throughout the whole cell body, with less staining in nerve cell processes (Fig. 2b). Emx1-positive cells displayed pyramidal-like morphology, whereas TuJ1-positive but Emx1-negative neurons showed nonpyramidal morphology (Fig. 2a–c, arrowhead, Fig. 2d–f).

The iNSPCs differentiate into MAP2-positive neurons. Immunocytochemistry revealed that approximately 6% of MAP2-positive neurons were positive for CamKIIz. We could find differentiating neurosphere-like clusters developing a number of long apical dendrites expressing MAP2 and staining at the same time for CamKIIz in cell bodies (Fig. 2j–k, arrow). CamKIIz staining was found primarily to be present in the cell bodies of cultured cells (Fig. 2j and 1, arrow), whereas MAP2 staining could be found in the dendrite-like processes and in the cell soma (Fig. 2k and l). These clusters may consist of pyramidal neuron/progenitor cells.

**Injury-induced neural stem/progenitor cell-derived neurons express Emx1 and CamKIIz but not Otx1**

To confirm the persistent expression of pyramidal molecular markers, RNA was isolated from the cells differentiating in vitro and subjected to PCR for Emx1 [10], Otx1 [11], and CamKIIz [12] (Fig. 2m, Emx1 and Otx1, n, CamKIIz). The relative expression levels of neurons differentiated from iNSPCs were calculated (Fig. 2o). As a result, expression levels of Emx1 and CamKIIz were similar to those observed in the intact adult murine cortex (control), but expression levels of another pyramidal cell marker Otx1 were found to be significantly lower in iNSPC-derived cells compared with nonischemic control.

**Discussion**

This study has shown for the first time the presence of endogenously regenerating neurons that express several pyramidal cell markers in the poststroke cortex. Previously, we found that nestin-positive iNSPCs developed in the poststroke cortex of the adult murine brain. Such cells have the capacity for self-renewal and differentiation into three kinds of neural phenotypes, including neurons [3]. To achieve functional restoration of the poststroke cortex, lost brain cells need to be replaced by neurons and by astrocytes and myelin-producing oligodendrocytes. Moreover, the regenerated neurons should make synaptic connections with long-distance connecting neurons to form the neuronal circuit. We have already shown that iNSPC-derived neurons are electrophysiologically functional [3]; however, whether iNSPCs differentiated into pyramidal neurons was not shown before. As these long-distance spanning neurons are essential in maintaining cortical functionality, the regeneration of these particular brain cells is crucial for functional poststroke recovery.

The current immunohistochemical study showed that the pyramidal cell marker Emx1 was expressed in regenerating BrdU-labeled cells after cerebral infarction. Although Emx1 expression is reported to be especially high in the developing telencephalic cortex, the expression concurs with pyramidal cell distribution during their development and migration from the ventricular zone to the mature cortex in the adult cortex [10]. In contrast, Emx1 is not expressed in nonpyramidal cells, where alternatively Lhx6 fulfills a similar function [13]. Thus, involvement of Emx1 in both the developing brain and in cell development in the adult brain suggests that Emx1 functions as an initiator and preserver of the cortical neuron phenotype. The expression of this regulator by BrdU-labeled cells in the poststroke cortex and cultured neurons derived from cortical iNSPCs suggest that the adult poststroke cortex is capable of developing new pyramidal neurons to replace the neurons lost after cortical ischemia.

This study revealed that CaMKIIz, a multifunctional kinase, was also expressed in regenerating neurons differentiated from cortex-derived iNSPCs. The specific expression in cortical neurons allows CaMKIIz to be used as a marker for pyramidal cells [12,14]. Although we were unable to demonstrate CaMKIIz in the poststroke brain by using the current antibody, the expression of CaMKIIz by the regenerated neurons was confirmed both by in-vitro immunocytochemistry and by PCR. In such in-vitro conditions, we revealed CamKIIz immunoreactivity to be localized in the neural soma of cultured neurons. It was reported previously that CaMKIIz located in postsynaptic sites of mature neurons plays a crucial role in synaptic plasticity through postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors and N-methyl-D-aspartic acid receptors [12]. As CaMKIIz is activated by elevated intracellular Ca 2+, which causes calmodulin binding to CaMKIIz, the current finding may suggest that inactive CaMKIIz resides before calmodulin activation and transportation to the postsynaptic sites [15].

In this study, we performed PCR by using the RNA extracted from differentiating cultured neurons but not from the poststroke brain, which may contain the resident pyramidal neurons surviving after ischemic insult. PCR for Emx1 and CaMKIIz on mRNA showed that the expression levels of these genetic markers were...
found to be comparable to the expression range in the nonischemic adult cortex. However, expression levels of another pyramidal marker, Otx1, were found to be diminished in the same cell clusters. Otx1 is a transcription factor involved in telencephalon development and is especially highly expressed in layer-5 neurons that project to subcortical targets [16]. However, a previous report has shown that embryonic stem (ES)-derived neurons never express Otx1, although they appear to differentiate into fully functioning pyramidal neurons that integrate into the adult murine brain [11], suggesting that Otx1 is not essential in cortical neuronal development.

Stem cells have been regarded as a possible therapeutic tool for various pathologies. Both adult and ES cell-derived NSCs [17–20] are regarded as cell sources that can be used to restore crucial brain cells that were lost. A number of studies and the current study have shown that NSCs differentiate into a wide variety of neuronal subtypes and glia cells. However, the projecting pyramidal cortical neurons forming long-distance neuronal circuitry have been derived from these progenitor cells without much success, although a recent publication has demonstrated that ES cell-derived NSCs can differentiate into pyramidal cells that integrate into the adult poststroke cortex [11]. Although it would still be challenging to accomplish cortical neurogenesis after permanent ischemic stroke in vivo, we have shown that adequate support of iNSPCs in the in-vivo environment of the poststroke cortex (e.g., regulation of endothelial cells [7,9] and inflammatory cells [8]) could promote the proliferation of iNSPCs and enhance neurogenesis and cortical functional recovery.

Conclusion
This study showing that iNSPCs can differentiate into pyramidal neurons provides the possibility of cortical neurogenesis with functional recovery in the ischemic human brain through the regulation of iNSPCs, because our recent study has shown that iNSPCs are also present in the poststroke human cortex [21]. Further studies for their N-methyl-d-aspartic acid-induced glutamatergic response and morphological connectivity to distant regions are needed to confirm the regeneration of functional projecting neurons.

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Conflicts of interest
There are no conflicts of interest.

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