Functional Integration of Newly Generated Neurons Into Striatum After Cerebral Ischemia in the Adult Rat Brain

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Background and Purpose—Ischemic injury can induce neurogenesis in the striatum. Those newborn neurons can express glutamic acid decarboxylase and choline acetyltransferase, markers of GABAergic and cholinergic neurons, respectively. The present study investigated whether these GABAergic and cholinergic new neurons could differentiate into functional cells.

Methods—Retrovirus containing the EGFP gene was used to label dividing cells in striatal slices prepared from adult rat brains after middle cerebral artery occlusion. EGFP-targeted immunostaining and immunoelectron microscopy were performed to detect whether newborn neurons could anatomically form neuronal polarity and synapses with pre-existent neurons. Patch clamp recording on acute striatal slices of brains at 6 to 8 weeks after middle cerebral artery occlusion was used to determine whether the newborn neurons could display functional electrophysiological properties.

Results—EGFP-expressing (EGFP⁺) signals could be detected mainly in the cell body in the first 2 weeks. From the fourth to thirteenth weeks after their birth, EGFP⁺ neurons gradually formed neuronal polarity and showed a time-dependent increase in dendrite length and branch formation. EGFP⁺ cells were copositive for NeuN and glutamic acid decarboxylase (EGFP⁺-NeuN⁺-GAD₆₇⁺), MAP-2, and choline acetyltransferase (EGFP⁺-MAP-2⁺-ChAT⁺). They also expressed phosphorylated synapsin I (EGFP⁺-p-SYN⁺) and showed typical synaptic structures comprising dendrites and spines. Both GABAergic and cholinergic newborn neurons could fire action potentials and received excitatory and inhibitory synaptic inputs because they displayed spontaneous postsynaptic currents in picrotoxin- and CNQX-inhibited manners.

Conclusion—Ischemia-induced newly formed striatal GABAergic and cholinergic neurons could become functionally integrated into neural networks in the brain of adult rats after stroke. (Stroke. 2008;39:000-000.)

Key Words: functional neurogenesis | neural network | nonneurogenic regions | striatum | stroke

Stroke is a common neurological disease, and it is becoming a significant cause of disability around the world. Ischemic stroke induced by middle cerebral artery occlusion (MCAO), a major type of stroke seen in the clinic, causes neuronal damage mainly in the striatum and cortex. Decreasing neuronal death and enhancing brain repair are therapeutic strategies to facilitate neuroprotection against cerebral ischemic injury. It has been demonstrated that neurogenesis exists in the constitutive neurogenic regions, including the subventricular zone (SVZ) and subgranular zone (SGZ) throughout adult life. Newly generated (newborn) hippocampal neurons can develop into mature neurons displaying functional inhibitory and excitatory neurotransmission in the normal adult brain. Moreover, ischemic or traumatic injury can induce neurogenesis in the nonneurogenic regions, especially in the striatum, a vulnerable region to ischemic injury caused by MCAO. These remarkable observations suggest the brain has the potential to repair itself, which is relevant to therapies for various pathological conditions such as ischemic brain injury. For brain repair, it is important to have a certain amount of newborn neurons to replace injured neurons. Besides, we have to know whether these new neurons in the nonneurogenic regions can differentiate into regionally appropriate functional cells. Therefore, in the past years, several studies demonstrated that injury-induced neurogenesis in the nonneurogenic regions of the adult mammalian brain could be enhanced by various growth factors, antiapoptotic protein, and electroacupuncture. Besides, newborn neurons can express regional-appropriate neurotransmitter-synthesizing enzymes such as glutamic acid decarboxylase (GAD₆₇) in the hippocampus, choline acetyltransferase (ChAT), and DARPP-32, a marker of medium-
sized spiny neurons, in the striatum. However, these results cannot determine whether these newborn neurons are functional in fixed brain tissues.

In general, a functional neuron should have specific morphological characteristics and defined electrophysiological activities. Therefore, in this study, we performed EGFP-targeted immunostaining and immunoelectron microscopy to detect whether newborn striatal neurons could anatomically form neuronal polarity and synapses with pre-existent neurons in ischemia-injured regions of the adult rat brain. Additionally, we conducted patch clamp recording on acute striatal slices of brains at 6 to 8 weeks after MCAO to determine whether the newborn neurons could display functional properties. Our results demonstrated that ischemia-induced striatal newborn neurons could differentiate into functional GABAergic and cholinergic neurons that could become integrated into the neuronal network.

**Materials and Methods**

**Reagents and Animals**

Picrotoxin, 4-aminopyridine, Lucifer Yellow, dianinobenzenzidine, D(-)-2-amino-5-phosphonopantoic acid, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and rabbit polyclonal antibody against Ser tolerate SYN were purchased from Sigma (St Louis, Mo). 1, 1-di-Octadecyl-rhodamine, 6-di-(4-sulfophenyl)-3', 3', 3'-tetramethylindocarbocyanine (DiI) was from Molecular Probes, Inc (Eugene, Ore), and 5-bromodeoxyuridine (Brdu), from Roche Applied Science (Mannheim, Germany). Mouse monoclonal antibodies against Brdu, ChAT, and synaptophysin and (BrdU), from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Antimouse IgG-rhodamine, antirabbit IgG-rhodamine, antimouse IgG-Cy5, and ABC-alkaline phosphatase kit, and Vector Blue were obtained from Vector Laboratories, Inc (Burlingame, Calif).

**Preparation of Fresh Striatal Slices**

Adult male Sprague-Dawley rats (220 to 250 g) were from Shanghai Experimental Animal Center, Chinese Academy of Sciences. All experiments were approved by the Shanghai Medical Experimental Animal Administrative Committee. All efforts were made to minimize animal suffering and reduce the number of animals used.

**Packaging of the Recombinant Retroviruses**

Two hundred ninety-three T cells were cotransfected with a retroviral vector, pMSCV-EGFP, and a helper plasmid, pCL-Eco, by using the calcium phosphate-mediated transfection procedure. Three days after transfection, cell supernatants containing retrovirus were harvested and centrifuged at 2000 rpm at 4°C for 2 hours. Then, the pellet was resuspended in DMEM and stored at −70°C.

**Animal Experimental Protocol**

For marking ependymal cells lining the wall of the lateral ventricle, rats were stereotaxically injected with EGFP gene-bearing retrovirus (2×10^6 infectious units/5 μL; n=26), 2 μL of 0.2% DiI in DMSO (n=22), or both (n=4) into the lateral ventricle contralateral (stereo coordinates: A, −0.8 mm from bregma [anteroposterior]; L, 1.4 mm lateral; H, 3.6 mm below the dura mater) to the ischemia side 24 hours before MCAO. At day 6 after retrovirus and/or DiI injection, the animals were anesthetized with 10% chloride hydrate (360 mg/kg, intraperitoneally); and their arterial blood samples were then collected to measure pO2, pCO2, and pH with an AVL 990 Blood Gas Analyzer (AVL Co, Graz, Austria). A heating pad and lamp were used to maintain brain temperature. Rats within normal range of physiological variables were subjected to a 30-minutes MCAO, as previously described. After recovery from anesthesia, the rats were returned to their cages and provided ad libitum access to food and water.

Among the 22 rats receiving DiI 24 hours before MCAO and BrdU on the next 14 days after MCAO, rats were killed during 6 to 8 weeks of reperfusion for patch clamp recording.

**Morphological Analysis**

**Fluorescence Immunolabeling and Confocal Laser Scanning Microscopy**

At different survival time-points indicated in Figure 1, each rat was euthanized and their brains were removed for coronal sections at a thickness of 30 μm at the bregma level from 1.60 mm to −4.80 mm. Immunofluorescence staining was performed by using the following antibodies or antibody combinations: (1) NeuN antibody (1:1000); dopamine D2 receptor (long form) antibody (1:200); or NMDA receptor 2 antibody (1:200); (2) NeuN antibody (1:1000) and GAD67 antibody (1:100); (3) ChAT antibody (1:20) and MAP-2 antibody (1:200); (4) NeuN antibody (1:1000) and p-SYN antibody (1:500); (5) synaptophysin antibody (1:500) and MAP-2 antibody (1:200); or (6) BrdU antibody (1:100) and MAP-2 antibody (1:200). The sections were incubated with primary antibody at 4°C overnight followed with a 1:40 dilution of fluorescently conjugated (rhodamine, Cy5, or FITC) antimouse IgG and/or antirabbit IgG for 1 hour at 37°C. Negative controls received the same treatment omitting the primary antibodies and showed no specific staining. The signals were detected at excitation 650 nm and emission 670 nm (Cy5), 535 nm and 565 nm (rhodamine), 490 nm and 525 nm (EGFP, FITC), and 570 nm and 620 nm (DiI) by confocal laser scanning microscopy (TCS SP2, Leica, Germany).

**Neurite Detection**

A total of 26 rats were used for this analysis. The numbers of rats per group were 6, 5, 5, and 5 in 1, 2, 4, 8, and 13 weeks after MCAO, respectively. Free-floating sections at a thickness of 50 μm made at bregma level from 0.4 mm to 1.0 mm of rat brains at the different times after MCAO indicated previously were incubated with primary antibody against EGFP (1:200) followed by secondary antibody for 1 hour at room temperature. EGFP signals were visualized with 0.05% dianinobenzidine. The signals were observed with a light microscope (×40 objective) and captured by a computer-assisted image system with a cooled CCD camera. The length and number of newborn neurons were measured assisted with Stereo Investigator Software (Micro-Bright Field, Inc, Williston, Vt). In this study, 5 sections of each rat were used for immunostaining and 6 views per each section were observed under a microscope assisted with 40 objectives. The total number of branches and length of newborn neurons was an average of 5 rats per group.

**Immunoelectron Microscopy**

Rats (n=4) at 8 weeks after MCAO with preinjection (ICV) of EGFP-engineered retrovirus were perfused with 4% PFA containing 0.25% glutaraldehyde. Coronal sections were cut at a thickness of 50 μm and subjected to EGFP immunostaining as described previously. Then, EGFP neurons were measured assisted with Stereo Investigator Software (Micro-Bright Field, Inc, Williston, Vt). In this study, 5 sections of each rat were used for immunostaining and 6 views per each section were observed under a microscope assisted with 40 objectives. The total number of branches and length of newborn neurons was an average of 5 rats per group.

**Observation of Synaptic Transmission**

**Preparation of Fresh Striatal Slices**

Rats at 6 to 8 weeks after MCAO were anesthetized with 10% chloral hydrate and decapitated. The brain was quickly removed and immersed in ice-cold artificial cerebrospinal fluid, constantly bubbled with 95% O2–5% CO2 containing (in mM, pH at 7.4) the following: 130 NaCl, 3 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaHPO4, 26 NaHCO3, and 10 glucose. Coronal striatal slices at 250 to 300 μm
thickness were cut by using a vibratome (VT1000 mol/L/E; Leica) and incubated at 22°C to 24°C for 1 hour. The slices were transferred into a recording chamber and superfused continuously with oxygenated artificial cerebrospinal fluid at 22°C to 24°C for patch clamp recording.

**Electrophysiological Recording**

Whole cell patch recording were performed as previously reported. The patch pipette (3 to 5 mol/LΩ resistance) was filled with solution containing (in mM, pH 7.25) the following: 135 KCl, 2 MgSO4·7H2O, 0.1 CaCl2, 1.1 EGTA, 2 K3ATP, 0.1 Na3GTP, 10 HEPES, and 2 Lucifer Yellow. Signals were amplified by an Axopatch 200B amplifier, digitized by Digidata 1200, and collected by pCLAMP6.0 software (Axon Instruments, Foster City, Calif). During the whole recording session, the series resistance (approximately 25 mol/LΩ) and membrane capacitance were not compensated. After rupture of the cell membrane, the resting membrane potential and membrane capacitance were measured immediately. Input resistance was calculated by measuring the steady-state passive current evoked by a hyperpolarizing pulse of 2-mV amplitude and 50-ms duration under a holding potential of −70 mV. To test the electrophysiological membrane properties of neurons being recorded, episodic currents, from hyperpolarizing 500 pA to depolarizing 150 pA at a step of 50 pA, were injected under the current clamp configuration. To test

**Figure 1.** Morphological development of newborn striatal neurons. Rats preinjected with retrovirus containing EGFP gene and euthanized at the indicated time points after MCAO (A). Photographs in A showed EGFP+ neurons in ipsilateral striatum of rat brains. Total neurite length of (B) and branch number (C) of newborn neurons increased time-dependently.

**Figure 2.** Maturation and migration of newborn striatal neurons. Rats injected with retrovirus (A–F) and Dil (G–I) were euthanized at 8 and 4 weeks of reperfusion, respectively. Their brains were sectioned for multiple immunostaining. EGFP−NeuN−GAD67− (A) and EGFP−MAP-2−ChAT− newborn neurons (B) were observed in the ischemic striatum. EGFP+ neurons expressed phosphorylated p-SYN (C), synaptophysin (D), dopamine D2 receptor (long form; E), and glutamate NMDA receptor 2 receptor (NR2, F). EGFP−Dil− newborn cells were seen in the SVZ (G). EGFP−NeuN−Dil− (H) and BrdU−MAP-2−Dil− newborn neurons (I) could be detectable in the ischemic striatum. Each multiple staining was repeated 4 times.
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ChAT

Figure 3. Ultrastructure of synapses between newborn neurons and the surrounding neurons in the ischemic striatum of rat brains. EGFP⁺ cell body (A) and neurite (D) are seen in the ischemic striatum. Photographs show dendron–axon (B, arrow), axon–axon synapse (C, arrow), synaptic glomerulus (F, arrow), and an autoysynapse (E, arrow). N, nucleus; black stars indicate diaminobenzidine particles.

whether recorded neurons received functional synaptic inputs, we recorded spontaneous postsynaptic currents (sPSCs) under the voltage patch clamp configuration with a holding potential of −70 mV. Picrotoxin (25 μmol/L) and CNQX (30 μmol/L) were bath-applied from reservoirs connected to the artificial cerebral spinal fluid flow line.

After electrophysiological recording, the slices were incubated with BrdU antibody at 4°C overnight and then with antimouse IgG-Cy5 for 30 minutes. Fluorescent signals were detected by confocal laser scanning microscopy at excitation 549 nm and emission 565 nm (DiI, red), 490 nm and 525 nm (Lucifer Yellow, green), and 650 nm and 670 nm (BrdU, blue). We produced digital reconstructions from a series of confocal images taken at 0.5-μm intervals and optical stacks of 10 to 12 images through the region of interest.

Data Analysis

All data were expressed as means±SEM and statistically analyzed by use of one-way analysis of variance with a post hoc least significant difference and Tukey test for multiple comparisons and unpaired Student t test for 2-group comparisons. P<0.05 was considered statistically significant.

Results

Morphological Integration of Newborn Neurons With Pre-existent Neurons in Adult Striatum After Transient Middle Cerebral Artery Occlusion

In our laboratory, we used the 30-minute MCAO model to induce focal cerebral ischemia and showed infarct areas at different times after MCAO demonstrated by the Cresyl Violet staining. The results indicated that the infarct area was involved in the lateral striatum and near cortex at the beginning and limited into the lateral striatum later after ischemic injury. Under this model, we demonstrated that newborn striatal neurons could further differentiate into GABAergic (BrdU⁺/GAD67⁺) and cholinergic (BrdU⁺/ChAT⁺) neurons at 4 weeks after MCAO (Supplemental Figure I and II, available online at http://stroke.ahajournals.org).

In this study, we injected an engineered retrovirus expressing EGFP into the lateral ventricle of rat brains 24 hours before MCAO to label the progeny of adult stem cells lining the ventricular wall. Such stem cells can give rise to new neurons and migrate into the ischemia-injured striatum. We observed the morphology of EGFP⁺ cells in the ipsilateral striatum at different times after MCAO. These EGFP⁺ cells included 2 distinct morphological types of neurons having time-dependent neurite outgrowth. In the first 2 weeks after ischemia, EGFP⁺ signals could be detected mainly in the cell body. From the fourth to thirteenth week after their birth, EGFP⁺ newborn neurons gradually formed neuronal polarity and showed a time-dependent increase in dendrite length and branch formation (Figure 1). In addition, we found that 51.5% of EGFP⁺ striatal cells contained MAP-2 (n=5) and 28.4% of them were BrdU⁺ (n=4) at 4 weeks after ischemia. Moreover, those newborn neurons could differentiate into GABAergic and cholinergic neurons, because EGFP⁺ cells were copositive for NeuN and GAD67 (EGFP⁺-NeuN⁻-GAD67⁻) or MAP-2 and ChAT (EGFP⁺-MAP-2⁻-ChAT⁺, Figure 2A–B). These newborn neurons could express proteins of dopamine receptors (EGFP⁺-D2L⁺), glutamate NR2 receptors (EGFP⁺-NR2⁺), nonphosphorylated and phosphorylated synaptic vesicule-associated proteins, synaptophysin, and synapsin-I (EGFP⁺-MAP-2⁻-SYP⁺ and EGFP⁺-NeuN⁺-p-SYN⁺), thus indicating the presence of active synaptic vesicles and formation of neurotransmitter receptors, key structures for establishing neurotransmission (Figure 2C–F).

Then, we analyzed the ultrastructure of EGFP⁺ neurons by electron microscopy and found typical synaptic structures comprising dendrites, spines, and cell bodies of surrounding neurons. The neurons were presynaptic or postsynaptic and appeared to form symmetrical or asymmetrical synapses (Figure 3). These results provide the first morphological evidence that newborn striatal neurons could form synapses with pre-existent neurons in the ischemia-injured region of an adult mammalian brain.

Electrophysiological Evidence of Integration of Newborn Neurons Into Neuronal Networks in Adult Striatum After Transient Middle Cerebral Artery Occlusion

Next, we asked if these new neurons could become functionally integrated into striatal neural networks. As indicated previously (Figure 2C), we observed that EGFP⁺ cells were copositive for NeuN and p-SYN, indicating that newborn neurons possess activated synaptic vesicles. To determine whether newborn neurons were electrically active and whether morphologically defined synapses were functional, we further used the whole cell patch clamp technique to record electrophysiological activities of newborn neurons in freshly prepared striatal slices from adult rat brains. As described before, stroke-induced newborn striatal neurons migrate primarily from the ependymal cells of the ventricle wall and from progenitors of the SVZ, both of which can be labeled by the intraventricular injection of DiI or retrovirus-expressed EGFP. Therefore, besides using retrovirus, we also injected DiI (ICV) to label the ependymal cells lining the lateral ventricular wall of ischemic rats in the present study. By multiple fluorescence immunostaining, we found that striatal DiI-labeled cells were positive for EGFP (63% DiI⁺ cells) or for BrdU (37% DiI⁺ cells). These DiI⁺-EGFP⁺ or DiI⁺-BrdU⁺ cells could further positively stain with NeuN
Either BrdU or retrovirus could be used to label dividing cells. However, the half-life time of BrdU after systemic injection was approximately 2 hours. Theoretically, we could speculate the age of the newborn neurons according to the latest time of BrdU injection. In our study, we needed to detect the electric activity of newborn neurons at the time ranging from 6 to 8 weeks postischemia, because by this time, the newborn neurons had established neuronal polarity (Figure 1) and formed synaptic connections with pre-existent neurons (Figure 3). Therefore, we next recorded the electric activity of DiI-labeled cells in freshly prepared striatal slices of ischemically injured brains at 6 to 8 weeks of rats with BrdU injection. To determine if the neurons recorded were newly generated, we marked them with Lucifer Yellow dye after recording and then assayed them for BrdU immunofluorescence staining combined with confocal scanning after patch clamp recording. Then, we divided the recorded cells into DiI^-BrdU^- and DiI^-BrdU^+ groups.

Twenty-two DiI-labeled large neurons from 16 rats were selected for electrophysiological recording in acute striatal slices. We grouped the recorded cells into BrdU^-DiI^- (newborn, n=4) and BrdU^-DiI^+ cells (n=18) based on the results from BrdU immunostaining. Electrophysiological properties of different groups are summarized in the Table, which indicated that each parameter of newborn cells was similar to that of pre-existent neurons (BrdU^-DiI^+, n=9) under comparable recording conditions. In the example shown in Figure 4, a recorded large neuron was labeled with DiI (red), marked with Lucifer Yellow (green), and positively stained with BrdU (blue), indicating that the recorded neuron was newborn and had migrated from the ependymal cells of the ventricle. Electrophysiological recording showed that depolarizing currents triggered repetitive spiking, and negative current pulses produced initial hyperpolarization followed by depolarizing "sags" in the membrane potential, which are typical properties of cholinergic neurons (Figure 4E). To assess whether this cell had the capacity to receive synaptic inputs, we recorded sPSCs (Figure 4F). Under the voltage patch clamp configuration, neurons displayed sPSCs of small amplitude and low frequency (approximately 0.1 Hz/s). Therefore, we applied 4-aminopyridine, a potassium channel blocker, to enhance the activity of sPSCs. 4-Aminopyridine (100 μmol/L) increased the frequency and amplitude of sPSCs, consistent with a previous report. In the presence of 4-aminopyridine, both the frequency and amplitude of sPSCs were partially reduced by application of picrotoxin, a GABA receptor antagonist, and abolished by...
further application of CNQX, an antagonist of non-NMDA receptors (Figure 4F–G).

Forty Dil-labeled, medium-sized neurons from 16 rats were also electrophysiologically examined. Among them, 8 were BrdU-positive, indicating that they were newly generated, and 32 were BrdU-negative. The Table summarizes the electrophysiological properties of these neurons, which data indicated that the values of the parameters assayed from the newborn neurons were similar to those of pre-existing neurons from the striatum of ischemia-operated animals. Figure 5, showing morphological and electrophysiological features of a recorded neuron, indicated that application of suprathreshold depolarizing current pulses elicited repetitive firing. However, administration of hyperpolarizing current pulses did not produce the “sag” phenomenon, making these cells different from the larger neurons described in the previous paragraph (Figure 4E). Under the voltage patch clamp configuration, this neuron exhibited relatively active sPSCs (6.2 Hz/s) compared with the large-sized neurons. The sPSCs were partially decreased by treatment with picrotoxin and abolished by further addition of CNQX (Figure 5F–G).

Overall, morphological and electrophysiological findings suggest that newborn large- and medium-sized neurons were functional cholinergic and GABAergic neurons, respectively. They could receive both inhibitory (GABA-mediated) and excitatory (glutamate-mediated) synaptic inputs.

### Table. Comparison of Electrophysiological Parameters of BrdU+/Dil− and BrdU−/Dil+ Large- and Medium-Sized Neurons in Striatal Slices of the Adult Brain 6 to 8 Weeks After MCAO∗

<table>
<thead>
<tr>
<th>Electrophysiological Property</th>
<th>Large-Sized Neurons</th>
<th>Medium-Sized Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BrdU+/Dil−</td>
<td>BrdU−/Dil+</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>229.75±37.41</td>
<td>179.61±17.67</td>
</tr>
<tr>
<td>Membrane capacitance, pF</td>
<td>32.5±3.48</td>
<td>31.72±0.99</td>
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<tr>
<td>Resting potential, mV</td>
<td>−58.75±1.72</td>
<td>−57.94±1.33</td>
</tr>
<tr>
<td>Threshold, mV</td>
<td>−47.0±1.41</td>
<td>−46.56±1.41</td>
</tr>
<tr>
<td>Spiking frequency, Hz</td>
<td>13.17±2.62</td>
<td>13.88±0.93</td>
</tr>
<tr>
<td>Spontaneous activity, Hz</td>
<td>0.08±0.04</td>
<td>0.11±0.02</td>
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<tr>
<td></td>
<td>136.13±23.08</td>
<td>113.88±9.33</td>
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<td>14.38±0.35</td>
<td>13.91±0.32</td>
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<tr>
<td></td>
<td>−72.13±2.33</td>
<td>−74.78±1.04</td>
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<td>−41.13±2.11</td>
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<td>20.20±4.13</td>
<td>15.05±1.02</td>
</tr>
<tr>
<td></td>
<td>4.79±0.84</td>
<td>5.20±0.38</td>
</tr>
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</table>

∗Input resistance was calculated by measuring the steady-state passive current evoked with a hyperpolarizing pulse of 2-mV amplitude and 5-ms duration under the voltage clamp mode from a holding potential of −70 mV. Spike threshold is the voltage difference between the resting membrane potential and spike onset. Spike frequency is the inverse of the interspike interval between the first 2 spikes of the train in response to 60 pA and 150 pA depolarizing current steps during recording large- and medium-sized neurons, respectively.

**Discussion**

The findings reported here demonstrate by molecular, structural, and functional criteria that ischemia-stimulated new-
born neurons could differentiate into GABAergic and cholinergic neurons that become functionally integrated into neural networks in the ischemia-injured striatum of the adult rat. Morphological analysis revealed that EGFP-positive cells could establish neuronal polarity with multiple neurites, form synapses with pre-existent neurons in the ischemic region, and express GAD67 or ChAT. Electrophysiological recording combined with histological confirmation in striatal slices indicated that both newborn GABAergic and cholinergic neurons could fire action potentials and display spontaneous postsynaptic currents in a picrotoxin- and CNQX-inhibited manner, indicating that these striatal newborn GABAergic and cholinergic neurons had acquired the capacity to fire action potentials and receive both excitatory and inhibitory synaptic inputs.

In the present experiment, we also observed new striatal GABAergic and cholinergic neurons as evidenced by multiple staining EGFP-NeuN-GAD67 and EGFP-MAP-2-ChAT neurons. If newborn neurons could replace ischemia-injured or lost neurons in the adult brain, they should be morphologically and functionally mature. Using retrovirus bearing the EGFP gene to label dividing cells, we found that EGFP+ new neurons morphologically established neuronal polarity with multiple neurites (Figure 1) and made synaptic connections with new neurons and pre-existing neurons in the injured brain regions (Figure 3). We also observed the presence of functional synaptic vesicles by immunostaining with phosphosynapsin I, a marker of vesicle activation. Therefore, we demonstrated that these new striatal neurons possessed the capacity for active synaptic vesicles and neurotransmitter release.

As noted earlier, mature neurons should morphologically form appropriate axons and dendrites as well as synaptic structures, which are key steps of neuronal differentiation; functionally fire action potentials; and, most importantly, communicate with other cells, which has been well documented by Kandel. Therefore, electrophysiological evidence combined with findings relevant to synaptic morphology should be evaluated to determine the possibility of “silent synapses” of newborn neurons. In the present study, we performed electrophysiological recording of newborn neurons in the striatal slices by using patch clamp techniques. To make sure newborn striatal neurons we recorded were at a similar age and had migrated from the ventricular wall, we made sure newborn striatal neurons we recorded at a functional age with multiple neurites (Figure 1) and made synaptic connections with new neurons and pre-existing neurons in the injured brain regions (Figure 3). We also observed the presence of functional synaptic vesicles by immunostaining with phosphosynapsin I, a marker of vesicle activation. Therefore, we demonstrated that these new striatal neurons possessed the capacity for active synaptic vesicles and neurotransmitter release.

As noted earlier, mature neurons should morphologically form appropriate axons and dendrites as well as synaptic structures, which are key steps of neuronal differentiation; functionally fire action potentials; and, most importantly, communicate with other cells, which has been well documented by Kandel. Therefore, electrophysiological evidence combined with findings relevant to synaptic morphology should be evaluated to determine the possibility of “silent synapses” of newborn neurons. In the present study, we performed electrophysiological recording of newborn neurons in the striatal slices by using patch clamp techniques. To make sure newborn striatal neurons we recorded were at a similar age and had migrated from the ventricular wall, we recorded electrophysiological parameters from DiI-positive newborn striatal neurons as pre-existent cells. Here, we provided evidence for the electrophysiological activity of newborn striatal neurons. As seen in the Table and in Figures 4 and 5, we recorded different parameters of electrophysiological activity in both medium-sized neurons and large-sized neurons. It should be noted that the parameters of newborn neurons did not significantly differ from those of the pre-existing ones. All of them could fire action potentials in response to synaptic activation and showed spontaneous postsynaptic currents that could be antagonized by GABA and glutamate receptor blockers, suggesting that these new striatal neurons were electrically active and capable of firing action potentials and of receiving excitatory and inhibitory synaptic inputs.

Our present study also identified GABAergic and cholinergic neurons based on their distinct morphologies and electrophysiological properties. First, cholinergic neurons had a large soma (20 to 60 μm diameter), aspiny dendrites, and dense local axonal branches compared with medium-sized GABAergic neurons (10 to 20 μm diameter). Second, cholinergic neurons had a less negative resting membrane potential, a lower threshold for spike generation, and a lower frequency of sPSCs than GABAergic neurons. In particular, cholinergic neurons displayed prominent “sag” during hyperpolarizing current pulses, whereas GABAergic neurons did not. Injection of depolarizing currents induced action potentials in both newborn GABAergic and cholinergic neurons. However, in newborn cholinergic neurons, increasing the magnitude of depolarizing current induced repetitive action potentials and increased firing frequencies. Third, in voltage clamp recordings, spontaneous postsynaptic currents were detected frequently in newborn GABAergic neurons but rarely in newborn cholinergic neurons, which may be due to the relatively less negative resting membrane potential with higher membrane resistance of the latter. These electrophysiological characteristics clearly demonstrated that the large- and medium-sized neurons we recorded should be cholinergic and GABAergic neurons, respectively.

Taken together, we concluded that stroke-induced newborn GABAergic and cholinergic neurons could become integrated into striatal neural networks. These results provide very comprehensive and strong evidence that neuronal replacement strategies through adult endogenous neurogenesis may be of potential therapeutic value for stroke and other neurodegenerative disorders.

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**Disclosures**

None.

**References**


Figure 1. Double-labeled cells of BrdU and CRMP-4 (BrdU−CRMP−4, A), MAP-2 (BrdU−MAP−2, B), ChAT (BrdU−ChAT+, C), or GAD67 (BrdU−GAD67+, D) in the ipsilateral striatum of the rat brain at 4 weeks after MCAO. Animal experimental protocols were described in the “Materials and Methods.” Double immunohistochemical staining and cell counting were performed based on our previous reports by Wang12 and Zhang.14 The photographs demonstrate that BrdU−ChAT+ cholinergic neurons showed a larger soma with longer dendritic processes (C) and BrdU−GAD67+ GABAergic neurons showed triangular and small cell bodies with short dendrites (D). The numbers of double staining cells were counted at the different time as indicated in Figure 1E. The results showed that, in sham-operated rats, few cells were BrdU-positive, and those cells rarely costained with GAD67 (BrdU−GAD67+ cells: 1.80±1.24/section). No BrdU− cell showed ChAT-positive staining. However, BrdU−GAD67+ cells and BrdU−ChAT+ cells increased to 15.86±5.10/section and 7.43±1.54/section at 4 weeks after MCAO and declined to 12±3.09/section and 5.33±0.92/section at 8 weeks, respectively (P<0.05 versus sham-operated rats and P>0.05 versus 4-week reperfused rats, n=6 per group). These results suggest that newly generated neurons differentiate into GABAergic and cholinergic neurons and keep survival even at 8 weeks after their birth.

Figure II. Representative photographs of the ischemic infarct area in rat brains at different time after 30-minute MCAO. The operation of MCAO was performed as described in the “Materials and Methods.” The rats were euthanized at indicated times. Coronal sections were cut serially (30 mm thick at 420-mm intervals) from phosphate-buffered paraformaldehyde (PFA) -fixed brains and stained with cresyl violet (CV) to identify viable cells. Photographs were taken under microscope assisted with computer image analyzer (Leica Q570). A, Three areas: area i (white color region within gray curve) was an intact region in the striatum ipsilateral to MCAO; area ii (white color area between blue and green curves) was an infarct region, which will disappear mostly at 8 weeks after a 30-minute MCAO; area iii (white color area within green curve) was an infarct region, which still existed even at 13 weeks after 30-minute MCAO. B–H, The CV staining of a section from a normal rat brain (B) and ischemic brain at 3 days (C), 1 week (D), 2 weeks (E), 4 weeks (F), 8 weeks (G), and 13 weeks (H) after 30-minute MCAO. b–d, High-power magnification corresponding to the boxes in B–D, respectively. Scale bars=0.6 mm in B–H; 60 μm in b–d; 20 μm in b’, c’, d’. Photographs of CV staining indicated that the infarct area was involved in the lateral striatum and near the cortex at the beginning and limited into the lateral striatum later after ischemic injury, which was consistent with reports by another laboratory.26 Besides, the majority of newly generated striatal neurons were detected in the outer part of the damaged area in the striatum of the rat brain after MCAO, which was previously reported by Lindvall’s and our laboratory.14,27,28 Therefore, in the present study, we observed neurogenesis in the outer part of the damaged striatum as indicated in the gray color region of A.
Functional Integration of Newly Generated Neurons Into Striatum After Cerebral Ischemia in the Adult Rat Brain
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