Modulation of Fate Determinants Olig2 and Pax6 in Resident Glia Evokes Spiking Neuroblasts in a Model of Mild Brain Ischemia

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Background and Purpose—Although in vitro studies suggest that non-neurogenic regions of the adult central nervous system potentially contain multipotent parenchymal progenitors, neurons are clearly not replaced in most brain regions after injury. Here, in a well-established model of mild transient brain ischemia, we explored Olig2 antagonism and Pax6 overexpression as potential avenues to redirect endogenous progenitors proliferating in situ toward a neuronal fate.

Methods—Retroviral vectors containing either Pax6 or a strong activator form of the repressor Olig2 (Olig2VP16), ie, a functionally dominant negative form of Olig2, were stereotaxically injected into the lateral striatum at 48 hours after 30 minutes middle cerebral artery occlusion (MCAo)/reperfusion.

Results—Retroviral modulation of fate determinants resulted in a significant number of infected cells differentiating into Doublecortin (DCX)-expressing immature neurons that were not observed after injection of a control virus. Whole-cell patch-clamp recordings in acute brain slices showed that the percentage of virus-infected cells with Na⁺ currents was increased by inhibition of the repressor function of Olig2 and by overexpression of Pax6. Furthermore, on retroviral transduction of fate determinants, we detected newly generated cells within the ischemic lesion that were capable of generating single action potentials and that received synaptic input.

Conclusions—Taken together, these data show that resident glia in the striatum can be reprogrammed toward functional neuronal differentiation following brain injury. (Stroke. 2010;41:2944-2949.)

Key Words: neurogenesis ■ ischemia ■ stroke ■ regeneration ■ NG2

The transcription factors Olig2 and Pax6 play largely opposing roles in determining progenitor cell fate in neurosphere culture, as well as in adult neurogenesis in vivo.¹–⁴ Following injury, an increase in the number of Olig2⁺ cells has been observed as a pan-lesion phenomenon throughout the brain.⁵–⁷ Repression of proneurogenic factors, such as Pax6, serves as a key mechanism underlying glial fate restriction by Olig2; using retroviral gene transfer, both Olig2 antagonism and overexpression of Pax6 have previously been shown to induce doublecortin (DCX)-expressing neuroblasts in a cortical stab wound lesion.⁶ This prompted us to examine the histopathologic and functional effects of retroviral gene transduction of neuronal fate determinants in the context of stroke.

Materials and Methods

Animals

All procedures were approved by an official committee. Nestin-GFP mice and GFAP-eGFP mice have been described in detail previously.⁸–¹⁰ 129Sv mice aged 10 to 12 weeks and weighing 20 to 25 g were used for retroviral injections.

Surgical Procedures

Mice were anesthetized with 1.5% isoflurane and maintained in 1% isoflurane in 69% N₂O and 30% O₂ using a vaporizer. Ischemia was induced by 30 minutes filamentous middle cerebral artery occlusion (MCAo)/reperfusion as described in detail previously.⁹ For retroviral injections, mice were mounted onto a stereotaxic head holder (David Kopf Instruments) in the flat-skull position. Approximately 1 mm anterior and 1.5 mm lateral to bregma, a 1-μL, 30–gauge, gas-tight syringe (Hamilton) was inserted to a depth of 4 mm and retracted to a depth of 3 mm from the dural surface, and 1 μL of retroviral suspension was injected.

Histological Procedures and Imaging

Brains were perfusion-fixed with 4% paraformaldehyde and cut into 40-μm sections. Antibodies were diluted in Tris-buffered saline containing 0.1% Triton X-100 and 3% donkey serum. Primary antibodies were goat antidoublecortin (Santa Cruz Biotecnologies) 1:200, polyclonal guinea-pig anti-GFAP (AdvancedInmunoChemical) 1:1000, polyclonal rabbit anti-NG2 (Chemicon) 1:100, mouse anti-NeuN (Chemicon) 1:100 rabbit anti-NG2 (Chemicon) 1:100, polyclonal rabbit anti-Sox2 (Chemicon) 1:500, and rabbit anti-Olig2 (DF308), FITC-, RhodX- or Cy5-conjugated secondary antibodies (Jackson Immuno-
Figure 1. Olig2 expression in glia reacting to brain ischemia. A, Two populations of GFP<sup>+</sup> cells (green) in normal striatum of GFAP-eGFP mice. Red: Olig2. B, GFAP-eGFP<sup>+</sup> cells (green) are induced at 4 days after MCAo. GFAP protein: blue, nestin: red. C and D, Following MCAo, GFAP-eGFP cells assume reactive morphologies with frequent nestin (red in C) and NG2 (red in D) coexpression. E, Olig2 expression (red) in GFAP-eGFP<sup>+</sup> cells in ischemic striatum at 4 days after MCAo. Note characteristic “rod-shaped” hypertrophic morphologies of GFAP-eGFP cells. F, Olig2 expression in nestin-GFP<sup>+</sup> cells at 4 days after MCAo. As we have previously reported, nestin-GFP<sup>+</sup> cells proliferate in situ in the ischemic lesion, show NG2 immunoreactivity and adopt a complex electrophysiological phenotype. Scale bar (in F) 30 μm in A, 235 μm in B, 37 μm in C, 27 μm in D, 29 μm in E, 38 μm in F.

Research Laboratories) were all used at a concentration of 1:250. Phenotypic analyses of GFP<sup>+</sup> cells were performed using a spectral confocal microscope (Leica TCS SP2). Appropriate gain and black-level settings were determined on control tissues stained with secondary antibodies alone.

**Electrophysiology**

Acute striatal slices (150 μm thick) for in situ recordings were prepared as reported previously. The perfusion chamber was continuously perfused with bicarbonate-buffered bath solution composed of (in mmol/L): 134 NaCl, 2.5 KCl, 1.3 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 1.25 K<sub>2</sub>HPO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose; this solution was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at a rate of 1 to 2 mL/min to a final pH of 7.4. Drugs were applied via the bath. To minimize indirect neuronal effects induced by kainate application, 0.5 μm tetrodotoxin and 0.1 mmol/L CdCl<sub>2</sub> was added to the bath solution to block voltage-gated sodium and calcium channels, respectively. Patch-clamp recordings were performed with an EPC-9/2 double patch-clamp amplifier in combination with TIDA software (HEKA) in voltage-clamp mode. Whole-cell recordings were obtained with pipettes filled with the standard pipette solution composed of (in mmol/L): 130 KCl, 2 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2 Na-ATP, 5 EGTA, and 10 HEPES. The pH was adjusted to 7.3 with KOH. To confirm intracellular access, Alexa Fluor 594 (10 μg/mL, Molecular Probes, Invitrogen) was filled in the pipettes. Pipettes were pulled from borosilicate capillaries (inner diameter, 0.87 mm; outer diameter, 1.5 mm; open resistance, 6 to 8 MΩ; Hilgenberg) using a P-2000 laser-based pipette puller (Sutter Instrument Co). Experiments were performed at room temperature (21°C–25°C).

The ischemic striatum was identified using standard transmission optics (Zeiss Axioskop; ×5 Zeiss objective, numeric aperture: 0.15). Images were recorded with a charge-coupled device (CCD) camera (VarioCam, PCO Computer Optics, Kelheim, Germany). GFP<sup>+</sup> cells were identified by fluorescence optics (excitation at 488 nm using a monochromator, Polychrome IV, Till Photonics). The emitted light was collected at 530±10 nm with a CCD camera Quanticam (b/w VGA, Phase; ×60 water-immersion Olympus objective, numeric aperture: 0.8). Alexa Fluor 594 fluorescence was detected at an excitation wavelength of 589 nm, and an emission at 616±4 nm.

**Statistics**

Values are presented as mean±SEM. Comparisons between groups were performed by means of one-way analysis of variance, with level of significance set at 0.05 and with two-tailed probability values.

**Results**

**Olig2 Expression in Glial Cells With Complex Membrane Properties**

First, we characterized Olig2 expression in our mild ischemia model using GFAP-eGFP transgenic mice. In uninjured striatum, two types of GFAP-eGFP<sup>+</sup> cells were morphologically distinguishable: astrocytes with high-fluorescence intensity display ramified processes and show passive membrane currents with no apparent time and voltage dependence. Cells with lower-fluorescence intensity have round somata and few processes with little, if any, branching, and display voltage-gated complex currents. In agreement with reports from other brain regions, about half of all brightly-fluorescent cells in normal striatum showed GFAP immunoreactivity (85 of 150 randomly selected cells; 3 animals), whereas weakly-fluorescent cells did not. In contrast, most of the weakly-fluorescent GFAP-eGFP<sup>+</sup> cells displayed colabeling with Olig2 (95 out of 100 cells analyzed; 3 animals), which was never detected in brightly-fluorescent cells (Figure 1A). The GFAP-eGFP cell population increased during the first week after 30 minutes MCAo both in the vicinity of the ischemic lesion and, to a lesser degree, within the lesion core (Figure 1B). GFAP-eGFP<sup>+</sup> cells predominantly displayed the complex electrophysiological phenotype early after insult. GFAP-eGFP<sup>+</sup> cells in...
ischemic striatum adopted a variety of hypertrophic morphologies (especially rod-shaped cells) with frequent nestin and NG2 coexpression (Figure 1 C, D). Consistent with their frequent NG2 immunoreactivity, 72% of GFAP-NG2 cells (150 cells; 3 animals) were also Olig2 immunoreactive at 4 days postevent (Figure 1E).

Reactive glia can also be visualized by nestin promoter-GFP transgenic mice. Nestin-GFP$^+$ cells were induced during the first week after MCAo and showed voltage-gated currents. Here, immunohistochemical analysis revealed Olig2 immunoreactivity in the majority (ie, 90% of 200 cells characterized in 3 animals) of nestin-GFP$^+$ cells 4 days postlesion (Figure 1F). Thus, most glial cells reacting to injury as monitored here upregulated transcription factor Olig2, prompting analysis of its functional relevance.

**Transduction of Pax6 or of a Dominant Negative Form of Olig2 Induces Early Neuronal Characteristics in Proliferating Resident Progenitors**

Forty-eight hours postevent, retroviruses (titers: $10^6$ to $10^7$) containing either only GFP (CMMP), the potent neurogenic factor Pax6 and GFP (Pax6-ires-GFP), or a fusion of Olig2 to the transactivator VP16 and GFP (Olig2VP16) were injected into the ischemic lateral striatum (day 3). The fusion protein of Olig2 with the activator VP16 antagonizes Olig2 function, as Olig2 acts normally as a repressor. Mice were euthanized on days 10 or 17 after MCAo, and the fate of GFP$^+$ cells was analyzed (Table 1, Figure 2). The majority of GFP$^+$ cells displayed NG2 immunoreactivity. Only a few GFP$^+$ microglia and even fewer astroglial cells were observed after viral transduction. Independent of treatment group, Sox2 expression was rare in GFP$^+$ cells. No DCX$^+$/GFP$^+$ cells were detected in the control condition. However, DCX$^+$ cells emerged both after Olig2VP16 and Pax6 transduction (Table 1, Figure 2).

Next, we analyzed the physiological properties of GFP$^+$ cells in acute slices (Table 2). We selected for cells with a stable membrane potential between $-50$ and $-85$ mV ($n=182$). The membrane was clamped from the holding potential of $-70$ mV to hyper- and depolarizing potentials ranging from $-170$ to $100$ mV ($50$ ms, $10$ mV increments). Most GFP$^+$ cells displayed complex membrane properties characterized by voltage-dependent, outwardly-rectifying K$^+$ currents similar to those previously described for cells termed “glial precursors,” NG2 glia, or complex glial cells (Figure 3). A subset of these complex cells displayed rapidly-activating and inactivating inward currents elicited with depolarization followed by outward currents. The threshold for the activation of this inward current was about $-40$ mV and the time to peak was about $1$ ms, indicating the presence of voltage-gated Na$^+$ channels (Figure 3B). The percentage of cells with Na$^+$ currents was increased after Olig2VP16 or Pax6 transduction.

Control-virus infected complex cells failed to generate action potentials on membrane depolarization in current-clamp mode. By contrast, action potentials could be elicited after overexpression of Pax6 and, albeit in fewer cells, also after transduction of Olig2VP16 (current injections for $200$ ms). Commonly, we detected a single action potential during the depolarizing pulse. However, in some cases, more than one action potential was observed. To record spontaneous postsynaptic currents, the membrane was clamped at $-70$ mV, and we analyzed $10$ records for $10$ seconds. Spontaneous current events were observed in two of $36$ cells after Pax6 transduction (Figure 4). In line with immunohistological
results, only relatively few microglia were transduced (Figure 5A). Even fewer GFP\(^+\) cells displayed passive membrane properties typical of classical astrocytes (Figure 5B).

**Discussion**

The adult brain displays a narrow capacity for self repair. In vitro studies suggest that non-neurogenic regions may harbor multipotent parenchymal progenitors.\(^1^4-^1^6\) However, neurons are clearly not replaced in most brain regions after injury. We have previously demonstrated that reactive glia characterized by nestin-GFP, NG2, or GFAP-eGFP expression become proliferative and express voltage-gated currents in ischemic striatum.\(^9,^1^1\) Here, we show that in parallel, these cells also express Olig2, a key antineurogenic transcription factor implicated in proliferation and glial scar formation.\(^6,^1^7,^1^8\) Moreover, antagonizing the repressor function of Olig2 or overexpressing Pax6 by intraparenchymal injection of retroviral vectors permitted a small but significant number of infected cells to adopt an immature neuronal phenotype characterized by DCX expression. Similarly, the percentage of GFP\(^+\) cells with Na\(^+\) currents was increased by modulation of fate determinants. Furthermore, we were able to elicit single action potentials in GFP\(^+\) cells after overexpression of Pax6 and, albeit in fewer cells, also by interfering with Olig2 function. Finally, spontaneous postsynaptic currents were detectable in a small subset of transduced cells after overexpression of Pax6.

Retroviruses were injected at 48 hours after 30 minutes MCAo into the ischemic lateral striatum. In line with an earlier study, we did not detect DCX expression in the progeny of infected cells under control conditions.\(^1^9\) At both time points investigated, the majority of GFP\(^+\) progenitors expressed chondroitin sulfate proteoglycan NG2, which is also expressed by nestin-GFP\(^+\) cells after MCAo.\(^9\) These data are in line with recent findings showing that even after injury, cells labeled by Olig2::CreERT\(^2^M\) mediated recombination generate mostly NG2\(^+\) glia, few astrocytes, and no cells colabeling with neuroblast or neuronal antigens.\(^2^9\)

**Table 2. Electrophysiologic Results**

<table>
<thead>
<tr>
<th>Day</th>
<th>CMMP</th>
<th>Olig2</th>
<th>PAX6</th>
<th>CMMP</th>
<th>Olig2</th>
<th>PAX6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportions of all transduced cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n(^*) (number of animals)</td>
<td>16 (5)</td>
<td>38 (7)</td>
<td>39 (6)</td>
<td>18 (6)</td>
<td>38 (5)</td>
<td>33 (6)</td>
</tr>
<tr>
<td>Microglia</td>
<td>31%</td>
<td>5%</td>
<td>0%</td>
<td>28%</td>
<td>10%</td>
<td>0%</td>
</tr>
<tr>
<td>Passive astrocytes</td>
<td>0%</td>
<td>29%</td>
<td>0%</td>
<td>5%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>Complex cells without Na(^+) current</td>
<td>50%</td>
<td>26%</td>
<td>23%</td>
<td>28%</td>
<td>24%</td>
<td>15%</td>
</tr>
<tr>
<td>Complex cells with Na(^+) current</td>
<td>19%</td>
<td>40%</td>
<td>77%</td>
<td>39%</td>
<td>63%</td>
<td>82%</td>
</tr>
<tr>
<td>Characteristics of complex cells with Na(^+) current</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. Na(^+) current Mean±SEM (pA)</td>
<td>168±51</td>
<td>226±38</td>
<td>200±26</td>
<td>125±41</td>
<td>157±22</td>
<td>318±55(^\dagger)</td>
</tr>
<tr>
<td>Membrane Potential Mean±SEM (mV)</td>
<td>−59±2</td>
<td>−55±2</td>
<td>−66±1(^\dagger)</td>
<td>−56±3</td>
<td>−54±2</td>
<td>−66±1(^\dagger)</td>
</tr>
<tr>
<td>Membrane Resistance Mean±SEM (MΩ)</td>
<td>167±23</td>
<td>231±89</td>
<td>1927±235(^\dagger)</td>
<td>393±181</td>
<td>215±36</td>
<td>1983±284(^\dagger)</td>
</tr>
</tbody>
</table>

**Neuronal properties of all transduced cells**

| Elicited single AP (n) | 0% (11) | 3% (36) | 15% (39) | 0% (13) | 3% (33) | 25% (32) |
| Spontaneous activity (n) | 0% (10) | 0% (30) | 5% (36) | 0% (13) | 0% (33) | 0% (31) |

\(^*\)n=Number of GFP\(^+\) cells analyzed.

\(^\dagger\)P<0.05 compared with CMMP treatment.

\(^\dagger\)P<0.01 compared with CMMP treatment.

Figure 3. Most GFP\(^+\) cells show complex membrane properties. GFP fluorescence induced by Pax6 vector in cells in acute slices 17 days after MCAO (left). Membrane currents were recorded as described in the text (middle). Insets show beginning of depolarizing-voltage jumps in expanded time scale after correction for passive current components. On the right, the responses of these two cells to current injection (current clamp mode) are displayed. Note that in B as opposed to A, a Na\(^+\) current could be elicited by depolarizing voltage steps.
Under both treatment conditions, cells capable of eliciting depolarization-induced single action potentials emerged. Importantly, in our earlier stroke studies using nestin-GFP and GFAP-eGFP reporter mice as well as in control virus-infected cells investigated here, we never detected complex cells capable of producing action potentials.\(^9,11\) Furthermore, the subgroup of NG2\(^+\) cells that generated action potentials and received synaptic input as previously described in white matter showed a particularly high vulnerability to hypoxia/ischemia.\(^20\) Taken together, it therefore seems improbable that a similar cell type is both present in lateral striatum under baseline conditions and induced to divide after ischemia. Physiological parameters indicate that DCX\(^+\) cells mature toward functional neuronal differentiation. However, we acknowledge that there was no relevant change in physiological characteristics between days 10 and 17 after MCAo. Furthermore, we did not detect virus-infected cells expressing neuronal marker NeuN. Clearly, many factors may represent a major impediment to the generation of fully mature neurons after brain ischemia, possibly the most important of which is chronic neuroinflammation.\(^21\)

NG2\(^+\) O-2A progenitors are capable of firing single action potentials in vitro.\(^22\) NG2\(^+\) precursors in early postnatal mouse cortex have also been shown to generate a single immature action potential, possibly indicating that these cells are undergoing neuronal differentiation.\(^23\) Importantly, early postnatal NG2\(^+\) cells can be transformed into neurons under appropriate culture conditions.\(^24\) By contrast, NG2\(^+\) cells reacting to injury of the adult mammalian cortex do not give rise to multipotent neurospheres.\(^16\) The limited neurogenic response observed in a prior study\(^6\) and again here, may be caused by the fact that viral vectors predominantly target NG2\(^+\) progenitors. When early postnatal cortical astroglia are reprogrammed to adopt a neuronal fate, electrophysiological differentiation into a neuronal phenotype in vitro leads to repetitive spiking behavior after current injection; however they may also pass through a developmental stage characterized by firing single action potentials.\(^25,30\)

**Figure 4.** Single action potentials and spontaneous postsynaptic currents after transduction of Pax6. GFP\(^+\) cell recorded 10 days after MCAo is shown on the upper left panel (A). Membrane currents (B) and membrane potential (C) were recorded as described in text. D, sample current traces are displayed from the cell described in A, B, and C, which show spontaneous inward currents while the membrane was clamped at \(-70\) mV.

**Figure 5.** A subset of GFP\(^+\) cells displays membrane characteristics of activated microglia or classical astrocytes. A, Fluorescence image of GFP\(^+\) cell (17 days postevent) dialyzed with Alexa Fluor 594 during recording (left). Cell displays current pattern typical of activated microglia with delayed outward rectifying currents in addition to the inwardly rectifying currents with inactivation at hyperpolarizing potentials.\(^28\) B, Example of a cell expressing passive, voltage-independent currents characteristic of classical astrocytes 17 days after MCAo.
Pax6-transduced cells stand out in displaying significantly more hyperpolarized membrane potentials, higher peak Na⁺ currents, and higher input resistances as compared with the other groups (Table 2). At the 17-day time point, the percentage of Pax6-ires-GFP–transduced cells capable of producing an action potential reached 25%. Similarly, newly generated granule neurons in the adult hippocampus show high input resistances and, unlike mature granule neurons that generate trains of action potentials under current clamp, they predominantly fire single action potentials.²⁶⁻²⁷

In conclusion, our results provide additional proof-of-principle evidence that neurogenesis can be evoked in an injured, non-neurogenic region of adult mammalian central nervous system, such as in the spinal cord,³¹ the cerebral cortex,⁶ and the striatum (this work), and present for the first time functional physiological data on these regenerated neurons.

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Disclosures
None.

References


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