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Electrophysiological neurodifferentiation of subventricular zone-derived precursor cells following stroke

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Abstract

Stroke in rodents is associated with increased neurogenesis and the migration of newborn neurons to sites of brain ischemia, where they may participate in repair and recovery. To determine if neurogenesis following stroke yields functional new neurons, we labeled neuronal precursors in the mouse subventricular zone with a lentivirus-green fluorescent protein vector, produced stroke by occluding the middle cerebral artery, and detected newborn neurons 8 weeks later by fluorescence microscopy. Patch-clamp studies on fluorescent neurons in the cortical region surrounding infarction showed tetrodotoxin-sensitive Na⁺ action potentials and spontaneous excitatory post-synaptic currents, suggesting that ischemia led to functional neurogenesis with synaptic integration. These findings support the hypothesis that enhancing endogenous neurogenesis after stroke might have therapeutic benefit.

Keywords

Neurogenesis; subventricular zone; ischemia; stroke; action potential; differentiation

Following stroke in rodents, neuronal precursor cells (NPCs) in the dentate gyrus and rostral subventricular zone (SVZ) proliferate at an accelerated rate, and SVZ-derived NPCs migrate to areas of ischemic injury, where they may contribute to postischemic repair and recovery [1,6,8,9,13,18]. A similar phenomenon appears to occur in human brain [10,12]. NPCs or their progeny may exert salutary effects that do not depend on their acquisition of uniquely neuronal properties, such as by secreting trophic factors that preserve or enhance the function of cells that survive ischemia [15]. However, the extent to which NPCs develop into fully mature, functional neurons after stroke is uncertain. Previous studies have shown that ischemia-induced adult neurogenesis results in the appearance of cells with morphological features [1,17] and gene [11] and protein [7,13,16,17] expression patterns consistent with a mature neuronal phenotype. In addition, ablation of NPCs is associated with worse outcome from stroke in rodents [14].

To determine if stroke-induced neurogenesis results in the production of new functional neurons, we sought evidence of Na⁺ action potentials and spontaneous post-synaptic currents

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in SVZ-derived neurons following stroke. Adequate measures were taken throughout to minimize pain or discomfort in animal subjects, and experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male CD1 mice (26–28g) were anesthetized with 1.2% isoflurane in 70% N₂O/30% O₂ and a third generation self-inactivating lentiviral vector expressing green fluorescent protein (LV-GFP) under the control of an human cytomegalovirus promoter (provided by Dr. Lisa Ellerby, Buck Institute for Age Research) was injected at a concentration of 8.0×10^8 IU/ml in 0.5 μ l into the left rostral SVZ (0.14 mm anterior to the bregma, 1.2 mm lateral to the midline and 2.3 mm deep from dura) (Figure 1A). One week later, mice were reanesthetized, and permanent middle cerebral artery occlusion (MCAO) was induced. The left temporoparietal region of the head was shaved, and a 1-cm incision was made between the left orbit and tragus. A 2-mm burr hole was made with a high-speed microdrill through the outer surface of the semitranslucent skull, just over the visually identified middle cerebral artery, at the level of the inferior cerebral vein. Saline was applied to the area throughout the procedure to prevent heat injury. The inner layer of the skull was removed with fine forceps, the dura and arachnoid were opened, and left MCAO was performed by electrocoagulation, without damaging the brain surface. Interruption of blood flow was confirmed under the microscope, the temporal muscle was repositioned, and the skin was closed. Rectal temperature was maintained at $37 \pm 0.2^\circ\text{C}$ with a heating blanket.

Eight weeks later, animals were anesthetized with chloral hydrate and decapitated. Brains were removed into ice-cold sucrose slicing solution containing 85 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 75 mM sucrose, 0.5 mM CaCl₂, 4 mM MgCl₂, 10 μ M D,L-aminophosphonovaleric acid (APV) and 100 μ M kynurenic acid (KA), and equilibrated with 95% O₂/5% CO₂. Transverse, 250- μ m brain slices were made through the cortical infarct and adjacent, penumbral tissue and incubated at 28°C for 30 min. Sucrose slicing solution was exchanged for artificial cerebrospinal fluid (ACSF) containing 125 mM NaCl, 2.4 mM KCl, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 25 mM glucose, 1 mM CaCl₂, 2 mM MgCl₂, 10 μ M APV and 100 μ M KA. Slices were transferred to a recording chamber and continuously perfused with ACSF containing 2 mM CaCl₂ and 1 mM MgCl₂.

Recordings were made using the whole cell patch-clamp technique. Electrodes were pulled from thick-walled borosilicate glass on a Flaming/Brown P-97 pipette puller (Sutter Instruments, Novato, CA), to resistances of 4–5 M Ω . Electrodes were filled with a solution containing 140 mM K-gluconate, 0.1 mM CaCl₂, 2 mM MgCl₂, 1 mM EGTA, 2 mM ATP•K₂, 0.1 mM GTP•Na₃ and 10 mM HEPES (pH 7.25 with KOH). For some experiments, 0.1% Lucifer yellow CH was added to the electrode solution. An Axopatch 200B amplifier was used to control pipette potentials or inject current, a Digidata 1322A interface to convert A/D values at 5–50 kHz, filtered at 2 kHz, and pClamp 9.2 software to control both the amplifier and the converter (Axon Instruments, Foster City, CA). Data were analyzed with pClamp 9.2. Action potentials were elicited by applying 100-ms depolarizing current pulses ranging from –0.06 to 0.16 nA.

Consistent with the findings of others [5], GFP was detected in SVZ cells of neuronal lineage 1 week after injection of the vector, as demonstrated by the co-expression of GFP and doublecortin (DCX), a marker for NPCs (Figure 1B). GFP fluorescence was then used to detect SVZ-derived cells in the cortical ischemic penumbra 8 weeks after MCAO, and these cells were analyzed by patch-clamp electrophysiology (Figure 1C).

Two distinctive features of neurons are Na⁺ action potentials and spontaneous excitatory or inhibitory post-synaptic currents, both of which develop late in the course of SVZ-derived neuronal differentiation [2]. To determine if SVZ-derived (GFP-labeled) cells in the region surrounding cortical infarction exhibited these features, cell excitability was measured in the absence and presence of 0.5 μ M TTX. Action potentials were detected in the absence of TTX

(Figure 2A), but were abolished by TTX (Figure 2B), consistent with the presence of functional, TTX-sensitive Na⁺ channels. Also observed were spontaneous excitatory post-synaptic currents (Figure 2C), implying that SVZ-derived cells found in cerebral cortex post-stroke received synaptic inputs from other neurons and had, therefore, integrated into neuronal networks.

Although we considered it most likely that the GFP-positive cells from which we recorded had originated in the SVZ, where LV-GFP vector was injected, it is also conceivable that leakage of vector along the needle track used for injection caused direct labeling of cells in the cortex. To distinguish between these possibilities, brain sections through the injection site, the SVZ and the infarct were prepared and stained for GFP and DCX. Figure 3 shows that the injection site was remote from the site of infarction, and that whereas few GFP-labeled cells in the vicinity of the injection site expressed DCX, many such cells in the peri-infarct cortex stained for both markers. Not all DCX-positive cells co-expressed GFP, even in the SVZ, which is consistent with less than 100% transfection efficiency. Not all GFP-positive cells expressed DCX, which indicates that (a) some GFP-labeled cells in the SVZ went on to assume a non-neuronal (e.g., astroglial) phenotype, (b) some GFP-labeled cells of neuronal lineage had ceased to express DCX as they achieved a mature neuronal phenotype, or (c) both of the above.

The natural history of stroke in humans is characterized by the abrupt onset of neurological deficits followed over up to several months, in most patients, by gradual improvement [4]. The basis for this improvement is not fully understood, but may involve changes in brain plasticity that involve alterations in gene expression, neuronal excitability, axonal sprouting, synaptogenesis, somatotopic organization, and interneuronal connectivity [3]. It has also been proposed that enhanced neurogenesis in the adult SVZ may promote post-stroke recovery by partly compensating for neuronal loss. The current findings provide additional evidence for this possibility, by documenting functional maturation of at least some SVZ-derived neurons in rodents in the aftermath of stroke.

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Figure 1.

Labeling and post-ischemic detection of SVZ-derived neurons. **(A)** LV-GFP vector was injected into the SVZ, followed 1 week later by MCAO and 8 weeks after that by identification of and patch-clamp recording from SVZ-derived cells. **(B,a)** A section through the rostral SVZ shows the location of LV-GFP injection. **(B,b)** Double-label immunohistochemistry shows co-localization of GFP (top panel, green) and DCX (middle panel, red) in merged image (bottom panel, yellow). DAPI (blue) was used to stain nuclei. **(B,c)** Cell with patch electrode. **(B,d)** SVZ-derived cell in (B,c) identified by GFP fluorescence.

Figure 2.

Electrophysiological properties of post-ischemic SVZ-derived neurons. (A) Action potentials were elicited by applying 100-ms depolarizing current pulses ranging from -0.06 to 0.16 nA. (B) Blockade of action potentials by 0.5 μ M TTX. (C) Spontaneous excitatory post-synaptic currents were recorded at a holding potential of -70 mV.

Figure 3.

Anatomical and immunohistochemical relationships between postischemic GFP and DCX expression. The sites of LV-GFP injection and of cerebral infarction were distinct and non-contiguous (top left panel). High-magnification views showed little DCX expression in the vicinity of the needle track (top right panel), consistent with a paucity of locally labeled neurons, but numerous DCX-expressing cells adjacent to the cortical infarct (bottom left panel). Further magnification revealed co-localization of GFP and DCX immunoreactivity in some but not all cells in the peri-infarct cortex (three bottom right panels). GFP-positive/DCX-negative cells could be non-neuronal (e.g., astroglial) cells, or neurons that ceased to express DCX as they differentiated more fully.