Intracerebral Infusion of Glial Cell Line–Derived Neurotrophic Factor Promotes Striatal Neurogenesis After Stroke in Adult Rats

Tohru Kobayashi, MD, PhD; Henrik Ahlenius, MSc; Pär Thored, MSc; Rina Kobayashi, MD, PhD; Zaal Kokaia, PhD; Olle Lindvall, MD, PhD

Background and Purpose—Stroke triggers increased progenitor proliferation in the subventricular zone (SVZ) and the generation of medium spiny neurons in the damaged striatum of rodents. We explored whether intrastriatal infusion of glial cell line–derived neurotrophic factor (GDNF) promotes neurogenesis after stroke.

Methods—Adult rats were subjected to 2-hour middle cerebral artery occlusion (MCAO). GDNF was infused into the ischemic striatum either during the first week after MCAO, with the animals being killed directly thereafter, or during the third and fourth weeks, with the rats being killed 1 week later. New cells were labeled with 5′-bromo-2′-deoxyuridine (BrdU) on day 7 or during the second week, respectively. Neurogenesis was assessed immunocytochemically with antibodies against BrdU and neuronal, glial, or progenitor markers. GDNF receptor expression was analyzed in SVZ tissue and neurospheres by reverse transcription–polymerase chain reaction and immunocytochemistry.

Results—GDNF infusion increased cell proliferation in the ipsilateral SVZ and the recruitment of new neuroblasts into the striatum after MCAO and improved survival of new mature neurons. The GDNF receptor GFRα1 was upregulated in the SVZ 1 week after MCAO and was coexpressed with markers of dividing progenitor cells.

Conclusions—Intrastriatal infusion of GDNF in the postischemic period promotes several steps of striatal neurogenesis after stroke, partly through direct action on SVZ progenitors. Because delivery of GDNF has biological effects in the human brain, our data suggest that administration of this factor may promote neuroregenerative responses in stroke patients. (Stroke. 2006;37:2361-2367.)

Key Words: cerebral ischemia ▪ corpus striatum ▪ middle cerebral artery occlusion ▪ nerve growth factors ▪ stem cells

In the adult mammalian brain, neural stem/progenitor cells are localized along the lateral ventricle in the subventricular zone (SVZ).1 Stroke induced by middle cerebral artery occlusion (MCAO) triggers increased cell proliferation and neuroblast formation in the SVZ.2-4 The neuroblasts migrate toward the injured striatum, where they mature and express markers characteristic of striatal medium spiny neurons.2-3 Striatal neurogenesis continues for several months after MCAO.5

Glial cell line–derived neurotrophic factor (GDNF) acts through the extracellular glycosylphosphatidylinositol (GPI)-linked receptor, GFRα1, and the transmembrane tyrosine kinase, c-Ret, or via a c-Ret–independent mechanism.6 After MCAO in rats, GDNF receptor expression is upregulated in penumbral areas,7 and short-term neuronal survival is improved by GDNF delivery.8 In contrast, long-term, viral vector–mediated high levels of GDNF can exacerbate neuronal loss.9

Several findings suggest that GDNF may affect striatal neurogenesis after stroke. First, GFRα1 mRNA levels are upregulated in the ipsilateral SVZ after MCAO.7 Second, overexpression of GDNF in neural progenitors induces genes involved in migration and differentiation.10 Third, intrastriatal GDNF infusion increases cell proliferation in the substantia nigra and hippocampal neurogenesis in the intact brain.11

In this experiment, we infused GDNF into the rat striatum after 2-hour MCAO to clarify whether GDNF delivery during the first week after MCAO affects cell proliferation in the SVZ and recruitment of neuroblasts into the damaged striatum. We also explored whether GDNF infusion, after the new cells had been generated, influences their maturation, survival, and migration.

Materials and Methods

Animals and Experimental Design

Male Wistar rats (280 to 320 g; B&K Universal, Sollentuna, Sweden) were housed under a 12-hour:12-hour light/dark cycle with...
ad libitum access to food and water. Experimental procedures followed the guidelines established by the Malmö-Lund Ethics Committee for the use and care of laboratory animals. In the first experiment, (supplemental Figure I, available online at http://stroke.ahajournals.org), 20 rats were implanted with infusion cannulas (Alzet brain infusion kit, Durect) connected to subcutaneously placed osmotic minipumps (Alzet model 1002D, Durect), into the right striatum just before induction of the 2-hour MCAO. Coordinates were as follows: 1 mm rostral and 2.5 mm lateral to bregma, 5 mm ventral to dura, and toothbars at −3.3 mm. Recombinant human GDNF (1.5 μg/μL, Amgen) or phosphate-buffered saline (PBS) was infused (0.5 μL/h) for 7 days. 5′-Bromo-2′-deoxyuridine (BrdU, 50 mg/kg; Sigma) was injected intraperitoneally 3 times at 2-hour intervals on day 7, and the animals were humanely killed 2 hours thereafter. In the second experiment, BrdU was given 3 times daily for 1 week starting 6 days after the 2-hour MCAO. GDNF (n = 10) or vehicle (n = 10) was infused intrastratally via osmotic minipumps (Alzet, model 2002, Durect) from day 13 to day 26, after which the pumps were removed. The animals were humanely killed 1 week later.

**Middle Cerebral Artery Occlusion**

Transient MCAO was induced by the intraluminal filament techni-que. After being fasted overnight, rats were anesthetized with

**Immunohistochemistry**

After transcardial perfusion with ice-cold, 4% phosphate-buffered paraformaldehyde (PFA), brains were postfixed in PFA for 24 hours and sectioned coronally at 30 μm on dry ice with use of a microtome. Fluorescence double staining was used for visualization of BrdU and double cortin (Dcx), S100β, or neuronal nuclei (NeuN). In brief, free-floating sections were denatured in 1 mol/L HCl at 65°C for 30 minutes, followed by a 20-minute incubation at room temperature. After being blocked for 1 hour in potassium PBS (KPBS) containing 0.25% Triton X-100 (t-KPBS) and 5% of the appropriate normal sera, sections were incubated with rat anti-BrdU (1:100, Oxford Biotechnology) and goat anti-Dcx (1:400, Santa Cruz Biotechnology), mouse anti-NeuN (1:100, Chemicon) for (36 hours at 4°C), or mouse anti-S100β (1:5000, Sigma) (overnight at 4°C) in blocking solution. Sections were then incubated for 2 hours with Cy3-conjugated donkey anti-rat secondary antibody (1:200, Jackson ImmunoResearch Laboratories), together with either biotinylated horse anti-goat or horse anti-mouse secondary antibody (1:200, Vector Laboratories) in blocking solution at room temperature. Finally, sections were incubated with Alexa 488–conjugated streptavidin (1:200, Molecular Probes) in t-KPBS for 2 hours at room temperature. For double staining with BrdU and the oligodendrocyte marker APC, sequential staining was used. After incubation with mouse anti-APC antibody (1:200, Calbiochem) overnight at room temperature, APC antigen was visualized with Alexa 488–conjugated streptavidin, and sections were fixed with 4% PFA. Sections were then denatured with HCl, and BrdU was visualized as described earlier.

For GDNF, Ki67, and NeuN single labeling, diaminobenzidine staining was used. After quenching the reaction with 3% H₂O₂ and 10% methanol in KPBS followed by blocking, sections were incubated with anti-NeuN overnight at 4°C, monoclonal mouse anti-Ki67 antibody (1:50, Novoceastra Laboratories) for 36 hours at 4°C, or polyclonal goat anti-GDNF antibody (1:2000, R&D Systems) overnight at room temperature. Sections were then incubated with biotinylated secondary antibody (horse anti-mouse, 1:200, for NeuN and Ki67; horse anti-goat 1:200 for GDNF). After incubation with avidin-biotin-peroxidase complex (Elite ABC kit, Vector Laboratories), sections were peroxidase-catalyzed with diaminobenzidine and 3% H₂O₂. The specificity of staining was confirmed by omitting the primary antibody.

**Morphometric Analysis**

The total number of SVZ cells was estimated by stereological counting with the optical fractionator method. Four sections, 60 μm apart, starting at 1.7 mm rostral to bregma, were sampled for each brain. Analysis was performed with Computer Assisted Stereological Toolbox (CAST) software (Olympus). Section thickness was measured at multiple places in the SVZ with use of the microruler attached to the microscope stage. The number of cells in the SVZ was estimated by dividing the number of counted cells by the sampling fraction. The cell number in the striatum was counted on an epifluorescence microscope in 4 sections per brain, except for BrdU/APC staining, for which single APC+ and BrdU+/APC+ cells in the undamaged striatum were evaluated in 3 sections. BrdU/NeuN, BrdU/Dcx, BrdU/S100β, and BrdU/APC double labeling was validated with a confocal laser scanning microscope (Leica). Areas of the SVZ and remaining striatum were measured with use of the stereological system in NeuN-stained sections.

**Neurosphere Cultures**

Neurosphere cultures were initiated as described. In brief, adult Wistar rats were deeply anesthetized and humanely killed by decapitation. The ependymal layer and SVZ of the lateral wall of the lateral ventricle were dissected and enzymatically dissociated. Cells were grown in neurobasal-A medium supplemented with L-glutamine (2 mmol/L), B27-A, penicillin/streptomycin (100 U/mL and 100 mg/mL, respectively; Gibco), heparin (2 μg/mL, Sigma), epidermal growth factor (20 ng/mL), and fibroblast growth factor (10 ng/mL, R&D Systems).

Clonally derived neurospheres kept 5 to 7 days in vitro were attached to poly-L-lysine–coated chamber slides overnight and then fixed with 4% PFA for 15 minutes, washed, and blocked in t-KPBS containing 5% appropriate serum. Slides were stained with primary antibodies to GFRA (1:1000, a gift from Carlos Ibanez; Karolinska Inst; Stockholm, Sweden), GFRA2 (1:100, R&D Systems), c-Ret (1:1000, Amgen), Sox2 (1:100, R&D Systems), nestin (1:100, Chemicon), βIII-tubulin (1:300, Sigma), vimentin (1:50, Sigma), and Ki67 (1:200) at 4°C overnight. Primary antibodies were visualized with Cy3-conjugated donkey anti-rabbit secondary antibody or biotinylated horse anti-goat and goat-anti mouse secondary antibodies with Alexa 488–conjugated streptavidin. Cell nuclei were counterstained with Hoechst stain (Molecular Probes).

**Reverse Transcription—Polymerase Chain Reaction**

Brains from intact and 2-hour MCAO animals were cut into 1-mm coronal sections and transferred immediately to RNA later (Ambion). The SVZ was dissected and collected in RNA later. Total RNA was isolated with RNeaques for polymerase chain reaction (PCR; Ambion) and treated with DNase. For each condition, 0.5 μg RNA was reverse-transcribed (RT) into cDNA with the use of Superscript II and oligo-dT primers (Invitrogen) and used for PCR. cDNA concentrations were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase. Primers and PCR conditions have already been described. To control for genomic DNA contamination, PCR was also performed on non—reverse-transcribed samples. PCR products were analyzed on 1.5% agarose gels containing ethidium bromide and photographed in a UV transilluminator.

**Statistical Analysis**

Unless otherwise stated, values are mean ± SEM. Comparisons were performed with Student paired t test for side differences or an unpaired t test for differences between treatment groups. Differences were considered significant at P < 0.05.
Results

GDNF Stimulates Cell Proliferation in the SVZ and Recruitment of Striatal Neuroblasts After Stroke

We infused GDNF into the striatum ipsilateral to the MCAO, starting just before the insult and continuing for 1 week thereafter. The mitosis marker BrdU was administered on day 7, with the last injection 2 hours before euthanization. GDNF protein diffused throughout the ipsilateral striatum in all rats and reached the SVZ, whereas there was no staining contralaterally (Figure 1A through 1C). In accordance with previous findings of increased cell proliferation 1 week after MCAO, we observed higher numbers of BrdU+ cells and cells immunoreactive for the cell cycle marker Ki67 in ipsilateral compared with the contralateral SVZ in the vehicle-treated group (Figure 1D through 1I, 1K, 1L). The SVZ volume was increased ipsilateral to the MCAO but was not influenced by GDNF treatment (Figure 1J). Also, the number of BrdU+ and Ki67+ cells on the contralateral side was unaffected (Figure 1K and 1L), whereas we observed further increases in cell numbers of 25% and 30%, respectively, evoked by GDNF in the ipsilateral SVZ (Figure 1F, 1I, 1K, and 1L). Taken together, our
We quantified the recruitment of new neurons by counting Dcx+/H11001 cells. This neuroblast marker is expressed for 2 to 3 weeks.15 GDNF markedly increased (by 86%) the number of Dcx+ cells in the damaged striatum but did not influence their distribution (Figure 2). The magnitude of neurogenesis is positively correlated to the extent of striatal injury.5,16 Because high levels of GDNF may aggravate neuronal death after MCAO,9 we explored whether increased cell proliferation and neuroblast numbers could have been caused by more extensive damage in the GDNF-treated rats. However, we found no significant group differences in the amount of damage (data not shown).

GDNF Increases Survival of Mature Striatal Neurons Generated After Stroke
We wanted to explore whether GDNF influences the fate of new neurons generated after 2-hour MCAO. Rats were given BrdU to label those cells generated during the second week after the insult. We then infused GDNF or vehicle into the striatum for 2 weeks and humanely killed the animals 1 week thereafter. Lesion size did not differ between groups (data not shown). GDNF infusion markedly increased (by 121%) the number of BrdU+ cells coexpressing the oligodendrocyte marker APC was unchanged after GDNF infusion (data not shown), whereas cells doubly labeled with BrdU and the astrocyte marker S100β (Figure 3A and 3I through 3K) were markedly fewer. How GDNF exerts this effect on astrocytes, which express GFRα1 in the stroke-damaged striatum,7 is unknown. Finally, we explored whether during the third and fourth week after stroke GDNF delivery also influences SVZ cell proliferation and striatal neuroblast recruitment. We obtained no supportive evidence for this concept, because 1 week after cessation of GDNF infusion, there were no group differences in the numbers of Ki67+ cells in the ipsilateral SVZ and of Dcx+ cells in the damaged striatum (data not shown).

Neural Stem/Progenitor Cells in the SVZ Express GDNF Receptors
To determine whether GDNF acts directly on SVZ cells, we analyzed the levels of GDNF receptors by RT-PCR. In intact animals, GFRα1 and GFRα2 were expressed at low and moderate levels, respectively (Figure 4A). At 1 week after 2-hour MCAO, GFRα1 expression was increased in the ipsilateral SVZ but unchanged contralaterally. This result extends our previous observations from in situ hybridization7 of elevated GFRα1 expression in the ipsilateral SVZ but unchanged contralaterally. This result extends our previous observations from in situ hybridization7 of elevated GFRα1 expression in the ipsilateral SVZ but unchanged contralaterally. This result extends our previous observations from in situ hybridization7 of elevated GFRα1 expression in the ipsilateral SVZ at 2 and 24 hours after 2-hour MCAO. GFRα2 levels were not affected by the insult. c-Ret was undetectable in the SVZ.

Most cells in primary neurospheres from the intact SVZ expressed both GFRα1 and GFRα2 (Figure 4B through 4D) but not c-Ret immunoreactivity. Ki67 and the stem/progenitor markers Sox2, nestin, and vimentin (data not shown), but not the early neuronal marker βIII-tubulin, were coexpressed with GFRα1 (Figure 4E through 4P). Thus, GFRα1 is mainly expressed on immature, dividing cells.

Figure 2. GDNF infusion increases recruitment of new striatal neuroblasts after stroke. Distribution of Dcx+ neuroblasts in the SVZ and dorsomedial striatum contralateral (A) and ipsilateral (C) to GDNF infusion and ipsilateral to vehicle infusion (B). Numbers of Dcx+ neuroblasts in the striatum (D), and their distribution (E) ipsilateral to infusion shown as a percentage of the total number of Dcx+ cells at different distances from the SVZ. GDNF or vehicle was delivered intrastriatally during the first week after 2-hour MCAO, and the animals were humanely killed on day 7. Values are mean±SEM. *P<0.05, n=9 for GDNF and vehicle groups. Scale bar=100 μm.
Discussion

This study shows that intrastriatal infusion of GDNF after stroke promotes striatal neurogenesis. Delivery of GDNF during the first week leads to enhancement of SVZ cell proliferation and neuroblast recruitment in the striatum. When administered for 2 weeks after the formation of striatal neuroblasts, GDNF increases the number of new mature neurons.

The GDNF-induced stimulation of cell proliferation in the SVZ was observed as elevated numbers of BrdU+/Ki67+ cells. After 4 weeks of intrastriatal GDNF infusion in intact rats, Chen et al.\textsuperscript{11} reported increased numbers of BrdU+ cells in the dentate gyrus and substantia nigra. Dempsey et al.\textsuperscript{17} administered GDNF intraventricularly during the first week after 1-hour MCAO with concomitant BrdU injections. Similar to Chen et al.,\textsuperscript{11} they observed increased numbers of BrdU+ cells in the dentate gyrus, but in contrast to our findings, no effect of GDNF delivery on cell proliferation in the SVZ. There are several possible explanations for this discrepancy. First, GDNF was administered by different.
routes, and whereas we demonstrate that GDNF had diffused throughout the striatum, including the SVZ, Dempsey et al.\textsuperscript{17} reported no data on GDNF distribution. Second, we gave BrdU pulses on day 7 to label newly formed SVZ cells at the peak of proliferation, whereas Dempsey and coworkers injected BrdU during the entire first week after stroke. Their number of BrdU\textsuperscript{+} cells reflects proliferation both before and during the stroke-triggered stimulation, as well as survival of proliferated cells. Finally, different species were tested, which could be relevant, because the spontaneously hypertensive rats used by Dempsey and coworkers have a higher basal progenitor cell proliferation compared with normotensive rats.\textsuperscript{18}

GDNF also stimulates proliferation of other stem/progenitor cells, notably retinal progenitor cells in vitro,\textsuperscript{19} and neural precursors from the embryonic gut.\textsuperscript{20} Our data indicate that this effect is mediated by receptors on the stem/progenitor cells themselves. GFR\textsubscript{α1} levels in the SVZ were elevated at 1 week after stroke, when GDNF stimulated SVZ cell proliferation in vivo. Moreover, GFR\textsubscript{α1} was coexpressed with markers of immature, dividing cells in SVZ-derived neurospheres. The lack of c-Ret expression in SVZ tissue and neurospheres indicates that GDNF mediates its effects on neurogenesis through c-Ret–independent mechanisms.\textsuperscript{21}

The numbers of striatal Dcx\textsuperscript{+} neuroblasts and mature BrdU\textsuperscript{+/NeuN\textsuperscript{+}} neurons were also increased by GDNF. In the first experiment, the higher numbers of Dcx\textsuperscript{+} cells could be secondary to the enhanced SVZ cell proliferation. Although Chen et al.\textsuperscript{11} reported that a high percentage of BrdU\textsuperscript{+} cells in the dentate gyrus after intrastriatal GDNF infusion were NeuN\textsuperscript{+}, data on changes in mature neuron numbers were not given. Dempsey et al.\textsuperscript{17} did not determine the phenotype of the BrdU\textsuperscript{+} cells after GDNF delivery. We obtained no evidence that GDNF influences the speed of neuronal maturation. The BrdU\textsuperscript{+} cells in the second experiment were formed up to 1 week before the start of the infusion, and it is inconceivable that a substantial portion of them had been driven from a glial to a neuronal lineage by the GDNF treatment. Most likely, GDNF promotes survival of new neurons in the postischemic phase.

Our findings raise the possibility that GDNF administration could have therapeutic value for stroke patients. Infusion of GDNF induced sprouting of dopaminergic neurons in a patient with Parkinson’s disease,\textsuperscript{22} but this mode of delivery is probably not ideal.\textsuperscript{23} Gene therapeutic strategies leading to local production of GDNF may be more suitable to promote neurogenesis after stroke.

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Disclosures

None.

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