Chronic Treatment With Minocycline Preserves Adult New Neurons and Reduces Functional Impairment After Focal Cerebral Ischemia

Zhengyan Liu, MD; Yang Fan, MS; Seok Joon Won, PhD; Melanie Neumann, PhD; Dezhi Hu, MD; Liangfu Zhou, MD; Philip R. Weinstein, MD; Jiaing Liu, PhD

Background and Purpose—Evidence suggests that activated microglia are detrimental to the survival of new hippocampal neurons, whereas blocking inflammation has been shown to restore hippocampal neurogenesis after cranial irradiation and seizure. The aim of this current study is to determine the effect of minocycline on neurogenesis and functional recovery after cerebral focal ischemia.

Methods—Four days after temporary middle cerebral artery occlusion, minocycline was administered intraperitoneally for 4 weeks. BrdU was given on days 4 to 7 after middle cerebral artery occlusion to track cell proliferation. The number of remaining new neurons and activated microglia were quantified in the dentate gyrus. Infarct volume was measured to assess the treatment effect of minocycline. Motor and cognitive functions were evaluated 6 weeks after middle cerebral artery occlusion.

Results—Minocycline delivered 4 days after middle cerebral artery occlusion for 4 weeks did not result in reduction in infarct size but significantly decreased the number of activated microglia in the dentate gyrus. Minocycline also significantly increased the number of newborn neurons that coexpressing BrdU and NeuN without significantly affecting progenitor cell proliferation in the dentate gyrus. Lastly, minocycline significantly improved motor coordination on the rotor rod, reduced the preferential use of the unaffected limb during exploration, reduced the frequency of footfalls in the affected limb when traversing on a horizontal ladder, and improved spatial learning and memory in the water maze test.

Conclusions—Minocycline reduces functional impairment caused by cerebral focal ischemia. The improved function is associated with enhanced neurogenesis and reduced microglia activation in the dentate gyrus and possibly improved neural environment after chronic treatment with minocycline. (Stroke. 2007;38:146-152.)

Key Words: dentate gyrus ■ hippocampus ■ inflammation ■ memory ■ motor function

The hippocampal formation is a cortical area involved in learning and memory. Increased proliferation of progenitor cells in the subgranular zone and neurogenesis in the dentate gyrus (DG) was often observed after a number of experimental models of brain injury, including cerebral ischemia, hypoglycemia, seizure, and traumatic brain injury.1 Neuronal cell death at the local network is not always associated with induced DG neurogenesis, suggesting that mitotic signals for progenitor cells might be transmitted synaptically or through humoral factors. Although many new neurons were produced immediately after brain injury in the DG, only a small fraction of them survived longer than 1 month of time.2,3 Promoting the survival of new progeny has become one of the most important priorities in enhancing endogenous neurogenesis after brain injury.

The role of neuroinflammation in regulating neurogenesis is beginning to emerge. T lymphocytes and microglia are required to maintain neurogenesis and memory function in the normal brain,4 whereas inflammation after brain insult impairs neurogenesis.5,6 Antiinflammatory agents have been shown to be effective in restoring neurogenesis after cranial irradiation, epilepsy, and ischemic stroke.6–8 Although the role of induced hippocampal neurogenesis in structural repair is unclear after the types of brain injuries that do not directly damage the DG, it is likely that the hippocampus might play an important role in mediating functional outcome by engaging in dialogue with other brain structures, including the cerebral cortex. Inhibiting DG neurogenesis after cerebral ischemia was associated with impaired functional recovery,9 whereas enhancing hippocampal neurogenesis was related to

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improved function. The goal of this study is to determine whether minocycline, a tetracycline derivative with anti-inflammatory action, enhances hippocampal neurogenesis and reduces functional impairment after experimental stroke.

Materials and Methods

Focal Cerebral Ischemia

This study was conducted in accordance with the animal care guidelines issued by the National Institutes of Health and by the Institutional Animal Care and Use Committee. Transient focal cerebral ischemia was induced in male Sprague-Dawley rats (2.5 months of age; Charles River, CA) by intraluminal suture middle cerebral artery occlusion method under isoflurane/O2/N2O (1.5/30/68.5%). Briefly, a 3-0 naked monofilament nylon suture, rounded at the tip, was introduced into the internal carotid artery through the external carotid artery stump and advanced intracranially for 18 to 19 mm. The suture was in place for 60 minutes and then withdrawn to restore cerebral blood flow. Core temperature was maintained at 37 ± 0.5°C with a heating blanket and rectal thermistor servoloop.

Minocycline Administration and BrdU Labeling

After middle cerebral artery occlusion (MCAO) and sham operation, rats in each group were randomly assigned to two subgroups and a given new animal identification number to conceal treatment plan. Decoding of the identity of each subject took place before statistical analysis to ensure blinded assessment. Minocycline (Sigma) dissolved in 0.1 mol/L phosphate-buffered saline, pH 7.4, or vehicle was administered intraperitoneally at 50 mg/kg once daily beginning 4 days after reperfusion for 1 week followed by 25 mg/kg daily for the remaining 3 weeks as the main paradigm to assess neurogenesis, microglia activation, and function. Another cohort of MCAO rats was treated with minocycline or vehicle beginning 6 hours after reperfusion using the same dose and duration to assess infarct size. To investigate the phenotype and survival of newborn cells, BrdU (50 mg/kg; Sigma) was injected intraperitoneally twice daily on days 4 to 7 after reperfusion to track divided cells. To detect activated microglia at the early stage of treatment, a third group of rats received a 1-week minocycline treatment with daily 50-mg/kg bolus injections of minocycline or vehicle 4 days after reperfusion.

Tissue Preparation, Immunohistochemistry, and Immunofluorescence

Forty-micron thick coronal free-floating sections were collected serially after transcardiac perfusion described previously. Immunohistochemistry and double immunofluorescence was performed according to the previous method. Fluorescence signals were detected by using the Zeiss LSM 510 confocal image system (Zeiss) with a sequential scanning model for Alexa 488 and 594. Stacks of images (1024×1024 pixels) from consecutive slices of 0.67 μm in thickness were obtained by averaging four scans per slice and processed with Adobe Photoshop (Adobe System).

Cell Counting and Infarct Measurement

The number of BrdU-labeled cells was determined in every 12th coronal in the hippocampus using the optical fractionator probe. Coronal frames (15×15×20 μm) were placed at the intersection of a 100×100-μm matrix randomly superimposed on the region of interest by the program. Cells were counted using an X63 oil objective. The CE (Gundersen) was between 0.07 and 0.14. As a result of the small number of ED1-immunoreactive cells in the granule cell layer and the hilus, they were counted in every 12th coronal hippocampal section manually and averaged. The phenotype of newborn cells was determined by confocal microscopy. The number of BrdU/NeuN, BrdU/NG2, and BrdU/GAP double-labeled cells was estimated by multiplying the percentages of colocalization (determined by confocal microscopy) to the total number of BrdU-labeled cells (determined by stereology). Infarct volume was indirectly measured by subtracting the volume of intact tissue in the ipsilateral hemisphere from that in the contralateral hemisphere on NeuN-stained serial sections (480 μm apart) after multiplying the perspective traced areas (using NIH ImageJ v1.3) by section interval thickness.

Behavioral Tests

Neurobehavioral performance was evaluated in a fourth group of rats (n = 6 to 7 per group) at 6 weeks after MCAO to avoid potential interaction between behavioral testing and neurogenesis. After minocycline or vehicle treatment, rats were acclimatized in the testing facility for 1 week before behavioral tests.

Forelimb Use Asymmetry Test

Forelimb use during explorative activity was analyzed in a 5-minute videotaped session as described. Each behavior was expressed as percentage in the independent use of either limb or the simultaneous use of both during “wall exploration” and “landing” after rearing.

Horizontal Ladder Test

Rats were videotaped while traversing a 30° ladder (60 cm in length with 3 cm between bars). The percentages of footfalls (slipping through the bars) with the affected or unaffected forelimb were averaged in three trials.

Rotor Rod

Rats were tested on an accelerating rotor-rod (San Diego Instruments) with a 1-minute adaptation before each trial with an accelerating rate at 5 rpm every 15 seconds and a final speed capped at 40 rpm. The latency to fall from the rod was averaged in three trials.

Water Maze

A 5-day paradigm was used as described. Rats were trained to locate first a visible platform (sessions 1 to 4) and then a hidden platform (sessions 5 to 10) in two daily sessions, each consisting of three 60-second trials with 10-minute intertrial intervals and a 2-hour intersession interval. Time to reach the platform (latency), path length, and swimming speed were recorded with an Ethovision video tracking system (Noldus Information Technology). After the last hidden platform session, a 1-hour break was given before a 60-second probe trial with the platform removed.

Statistical Analysis

Data were expressed as mean ± SEM. Water maze data were analyzed as repeated measures by “mixed model regression” using SAS proc MIXED. Other data were analyzed by one-way or two-way analysis of variance using Statview 4.01. Post hoc tests were used when appropriate. P values <0.05 were considered significant.

Results

Minocycline Enhances the Number of Hippocampal Newborn Neurons After Stroke

An increase in the remaining BrdU cells in the granule cell layer was observed in the minocycline-treated MCAO rats compared with vehicle treated MCAO rats (Figure 1A), although this increase was not statistically significant (two-way analysis of variance). Minocycline by itself did not alter the number of remaining divided cells or new neurons in the normal hippocampus. Transient focal cerebral ischemia also did not significantly affect the level of neuronal differentiation in the DG (sham: 60.1 ± 1.3%; MCAO: 66.4 ± 3.8%; P = 0.54). Similar to previous findings in the distal MCAO model, newly divided cells coexpressed DCX, TuJ1, NG2, NeuN, and glial fibrillary acid protein markers in the granule cell layer (Figure 1C). However, minocycline increased the ratio of remaining newborn cells adopting neuronal phenotype in the DG in MCAO (78.8 ± 1.8%, P<0.01) but not in...
sham-operated rats (61.7 ± 1.9%, \( P = 0.59 \)). Furthermore, minocycline also increased the number of newborn neurons in the ipsilateral granule cell layer in MCAO (\( P = 0.05 \)) but not in the sham-operated ones (\( P = 0.70 \)). C, Orthogonal reconstructions of confocal microscopic merged images with BrdU as green and cell marker as red as viewed in the x–z (top) and y–z (right) planes. The majority of newly divided cells assumed neuronal identity in the granule cell layer between 2 to 4 weeks after BrdU labeling in MCAO rats treated with minocycline. Scale bar, 10 \( \mu m \).

### Minocycline Reduces Microglia Activation

Minocycline treatment for 4 weeks starting 4 days after temporary MCAO did not lessen infarct volume (MCAO: 77.2 ± 11.9 \( mm^3 \); MCAO+minocycline: 76.9 ± 10.9 \( mm^3 \), \( P = 0.98 \)), suggesting that it no longer confers subacute neuroprotection in reducing ischemic core. In addition, the pattern and distribution of ischemic injury was similar between the minocycline- and vehicle-treated MCAO rats. However, significantly less activated microglia expressing the ED1 marker were observed in the granule cell layer and hilus of MCAO rats after a 4-week treatment plan (Figure 2B). At 1 week after MCAO, ED1-positive microglia were detected adjacent to newly divided cells in the subgranular zone (Figure 2C). Treatment of minocycline for only 1 week

### The Number of Remaining New Cells That Colabeled With a Phenotypic Marker in the Granule Cell Layer of Rats With MCAO After 4 Weeks of Minocycline or Vehicle Treatment

<table>
<thead>
<tr>
<th>Markers</th>
<th>MCAO</th>
<th>MCAO + Minocycline</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ipsilateral</td>
<td>Contralateral</td>
</tr>
<tr>
<td>NeuN/BrdU</td>
<td>6147.2 ± 850.9</td>
<td>4777.2 ± 525</td>
</tr>
<tr>
<td>NG2/BrdU</td>
<td>638.6 ± 253</td>
<td>562.9 ± 269.5</td>
</tr>
<tr>
<td>GFAP/BrdU</td>
<td>3588.9 ± 951</td>
<td>2857.5 ± 592</td>
</tr>
</tbody>
</table>

Significant difference between groups is indicated by asterisk (\( P < 0.05 \)).
Minocycline did not reduce the amount of activated microglia in the DG (Figure 2A). In contrast to the delayed paradigm, 4-week minocycline treatment reduced infarct volume by nearly 50% when delivered 6 hours after reperfusion (MCAO: 72.6±8.4 mm³, n=9; MCAO+minocycline: 34.5±12.3 mm³, n=6; P<0.05).

Minocycline Reduces Stroke-Induced Functional Impairment

To determine the effect of minocycline on motor function after experimental stroke, we conducted a battery of motor function tests, including the Schallert cylinder, foot fault, and rotor-rod tests, in the 4-week minocycline-treated and vehicle-treated rats after experimental stroke and sham surgery. Spontaneous paw use in the cylinder indicated that MCAO rats displayed asymmetry in favoring use of the ipsilateral (unaffected) limb during wall exploration and landing. There was also a reduction of simultaneous use of both limbs in the MCAO rats during landing (P<0.05). Minocycline reduced the limb use asymmetries during wall (P<0.005) and landing (P<0.001) motion after experimental stroke (Figure 3). Minocycline also significantly reduced the percentages of footfalls of the affected limb after MCAO while traversing a 30° angle horizontal ladder (P<0.05). Finally, minocycline restored the reduced fall latency after MCAO during the rotor-rod balancing test (P<0.0005).

To study the effect of minocycline on memory function after experimental stroke, we compared the spatial learning performances of minocycline-treated and untreated rats after experimental stroke in the water maze test. Both sham and stroke rats, independent of minocycline treatment, learned to locate a visible platform (Figure 4A). However, cerebral ischemia had an effect on water maze acquisition as demonstrated by prolonged latency to locate a hidden platform in stroke rats compared with three other groups (P<0.05). Minocycline reduced functional impairment in stroke rats (P<0.05) during hidden platform search without affecting the performance in sham rats (P=0.28). Neither stroke (P=0.62)
nor minocycline \((P=0.81)\) affected swim velocity. The probe trial showed that stroke rats had severe deficit in retaining spatial memory (Figure 4B), whereas minocycline-treated stroke rats showed an increased preference to search the target quadrant compared with any of the other quadrants \((P<0.0001)\).

**Discussion**

The principal finding of this article is that minocycline in a 4-week delayed treatment paradigm preserved stroke-induced hippocampal newborn neurons, decreased ED1-positive microglia in the DG, and reduced functional impairment. The effect of minocycline on microglia was not observed in a 1-week treatment plan, suggesting the longlasting nature of brain inflammation after ischemic stroke that might interfere with the restoration of the neural environment.

Minocycline confers neuroprotection in a number of experimental models of brain injury, including cerebral focal and global ischemia, if given within its therapeutic window.\(^{15,16}\) We found that when minocycline was delivered as early as 6 hours after reperfusion, it reduced infarct volume by nearly 50% and associated inflammation, confirming its neuroprotective effect in the acute phase of ischemic stroke. In addition to its acute neuroprotective effect in reducing ischemic core, we found that chronic treatment of minocycline also protected stroke-induced newborn neurons and improved neurologic function. The effect of minocycline in enhancing the number of newborn hippocampal neurons is likely related to its ability in promoting the survival of newly divided cells, resulting in an elevated ratio of progenies expressing late neuronal marker NeuN (Figure 1). The mech-
Consistent with previous results, we found hippocampus by ischemic brain injury is complex and not well understood. Minocycline, can potentially block the production of inflammatory cytokines or activation of the cell death pathway directly. This blocking effect might require a prolonged presence of minocycline, because short-term treatment of minocycline did not confer neuroprotection in a rat model of neonatal focal ischemia, nor did it affect the number of ED1-immunoreactive cells (Figure 2A). Although less pronounced compared with the ipsilateral hemisphere, the effect of minocycline on the reduction of microglia and increased trend of surviving newborn neurons in the contralateral hemisphere suggest an improved global neural environment, which might aid in functional recovery. In conclusion, our data suggest that chronic treatment of minocycline reduces stroke-induced functional deficits and enhances the number of surviving hippocampal newborn neurons. The benefit and mechanism of chronic minocycline treatment in improving neural environment warrants further investigation.

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Disclosures
None.

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4. Ziv Y, Ron N, Butovska O, Landa G, Sudai E, Greenberg N, Cohen H, Kipnis J, Schwartz M. Immune cells contribute to the maintenance of minocycline-enhanced survival of newborn cells is, in part, related to its antiinflammatory action, because activated microglia were detected adjacent to newborn cells in the subgranular zone (Figure 2C). In support of this notion, indomethacin was recently found to increase the amount of activated microglia were detected adjacent to newborn cells in restricted locations. Although the exact molecular mechanism of minocycline’s neuroprotective action is not completely understood, the inhibition of p38, a known target of minocycline, can potentially block the production of inflammatory cytokines or activation of the cell death pathway directly. This blocking effect might require a prolonged presence of minocycline, because short-term treatment of minocycline did not confer neuroprotection in a rat model of neonatal focal ischemia, nor did it affect the number of ED1-immunoreactive cells (Figure 2A). Although less pronounced compared with the ipsilateral hemisphere, the effect of minocycline on the reduction of microglia and increased trend of surviving newborn neurons in the contralateral hemisphere suggest an improved global neural environment, which might aid in functional recovery. In conclusion, our data suggest that chronic treatment of minocycline reduces stroke-induced functional deficits and enhances the number of surviving hippocampal newborn neurons. The benefit and mechanism of chronic minocycline treatment in improving neural environment warrants further investigation.

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