Noggin Protects Against Ischemic Brain Injury in Rodents

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Background and Purpose—Bone morphogenetic proteins and their receptors are expressed in adult brains, and their expression levels increase after cerebral ischemia. The brain also expresses an inhibitor of bone morphogenetic protein signaling, noggin, but the role of noggin in ischemic disease outcome has not been studied.

Methods—We used transgenic mice overexpressing noggin to assess whether inhibition of bone morphogenetic protein signaling affects ischemic injury responses after permanent middle cerebral artery occlusion.

Results—Transgenic mice overexpressing noggin mice had significantly smaller infarct volumes and lower motor deficits compared to wild-type mice. CD11b+ and IBA1+ microglia along with oligodendroglial progenitors were significantly increased in transgenic mice overexpressing noggin mice at 14 days after permanent middle cerebral artery occlusion.

Conclusions—These results provide genetic evidence that overexpression of noggin reduces ischemic brain injury after permanent middle cerebral artery occlusion via enhanced activation of microglia and oligodendrocyte differentiation. (Stroke. 2010;41:357-362.)

Key Words: bone morphogenetic protein signaling; microglia; noggin; oligodendrocytes; stroke

Stroke is one of the leading causes of death and disability worldwide, and identification of new therapeutic agents is a major health imperative. Several studies of bone morphogenetic proteins (BMP) in ischemic rodent brains have suggested that this family of factors may play an important role in ischemic disease outcome. BMP belong to the transforming growth factor-β superfamily and play important roles in cell specification, neuronal migration, survival, and dendritic development. BMP act by binding to heterodimeric serine–threonine kinase receptors on the cell surface formed by BMP receptor I and BMP receptor II, which are widely expressed in the adult brain.1 BMP family members are also expressed in the adult brain, with differing distributions for various family members. BMP-5 and BMP-6 are expressed in hippocampus and neocortex, BMP-5 is expressed in cerebellum, BMP-5 and BMP-7 are expressed in striatum, BMP-6 and BMP-7 are expressed in meninges and choroid plexus, and BMP-4 is expressed in cortex, hippocampus, and cerebellum.1–3 Endogenous BMP-7 as well as BMP receptor II are upregulated after transient ischemia in rodents, whereas BMP-2/4 expression decreases in ischemic astrocytes in vitro.4,5 Administration of BMP-6 or BMP-7 in the brain before transient ischemia and reperfusion reduces infarct area and neurological deficit but has no effect on stroke volume when administered after ischemia.5–7 Pretreatment of rodents with retinoic acid has beneficial effects on stroke outcome that is prevented by inhibition of BMP signaling.8 However, BMP-2/4 are the major family members in cortex and hippocampus, and BMP signaling in the adult brain inhibits both neurogenesis and oligodendrogenesis.9 This suggested that inhibition of BMP signaling after cerebral ischemia might have an overall beneficial effect. To address this possibility, we analyzed the effects of stroke in transgenic mice overexpressing the BMP inhibitor, noggin, in the nervous system.

Noggin is an extracellular BMP antagonist that binds BMP-2/4 with high affinity and thus interferes with binding to receptors.10 In adult brains, noggin is expressed in the olfactory bulb, piriform cortex, hippocampus, and cerebellum, and overexpression of noggin results in an increase in oligodendrocytes in the cortex and neural stem cells in the proliferative regions of the brain.3,11,12 Our data indicate that noggin provides protection from ischemic disease by increasing the proliferation and activation of microglia and by enhanced repair of white matter.

Materials and Methods

Animals

The generation of the neuron-specific enolase (NSE)-noggin transgenic mice has been described previously.3,12 NSE-noggin mice were generated in FEV1 background and backcrossed to C57BL/6 mice for at least 5 generations. All the mice were housed in a facility with a 12-hour light/dark cycle and allowed access to food and water ad libitum. Experiments were conducted according to protocols approved by Institutional Animal Care and Use Committee and

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Northwestern Center for Comparative Medicine. The mice were generated in J.A.K.’s lab.

Focal Cerebral Ischemia

Four- to 5-month-old male mice were used for this study. After anesthesia (Avertin, 240 mg/kg, intraperitoneally), cortical brain ischemia was induced in accordance with the method described elsewhere. Briefly, an incision was made in the midline on the neck. The right common carotid artery was dissected and ligated with a 4-0 silk suture. After this wound was closed, an incision was made between the right ear and eye. The skull was exposed and a craniotomy was performed along the length of the middle cerebral artery (MCA). The MCA was coagulated with a cautzer (World Precision Instrument) distal to the striatal branch under a microscope. In mice undergoing sham surgery, the MCA was exposed but not cauterized. After irrigating and closing the wound, the mice recovered from anesthesia on a heating blanket. They were given a 1 mL bolus of lactated Ringer solution mixed with Buprenex 2 mg/kg after surgery and then daily for 2 days.

Accelerated Rotorod Test

To analyze balance and coordination, all mice were trained and familiarized on the Rotorod treadmill (Med Associates) 5 times per day for 12 days before induction of infarct. Mice were placed on the rod rotating at 4 rpm between dividers. The accelerated speed was started and allowed to run until each mouse fell and disrupted the infrared beam located at the bottom of the Rotorod. The Rotorod test was performed at an accelerating speed of between 4 and 40 rpm for 20 minutes, with at least 30 minutes rest between runs for each mouse. Data from NSE-noggin mice were compared with those from age-matched wild-type mice. Statistical comparison between the 2 groups of mice was performed using Student t test.

Triphenyltetrazolium Chloride Staining

Mice were euthanized 14 days after MCA occlusion and the brains were sectioned coronally using a mouse brain matrix. Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma). The border between infarcted and noninfarcted tissue was marked by using NIH ImageJ. The area of infarction was measured using Scion software, and infarct volume was calculated blindly with a previously described semiautomated method that corrects for edema.

Immunohistochemistry

Mice were anesthetized and their brains were processed for immunohistochemistry as described previously. Primary antibodies used were: rat monoclonal antibodies to detect CD11b (1:500; ABD Serotec) and platelet-derived growth factor receptor-α (1:200; BD Biosciences); rabbit polyclonal antibodies to detect IBA1 (1:150; Wako, Japan) and NG2 (1:200; Chemicon); mouse monoclonal antibodies to detect CNPase (1:200; Covance); myelin basic protein (1:500; Covance); glial fibrillary acidic protein (1:100; Sigma); and Hu (1:150; Abcam) and guinea pig polyclonal antibody to detect doublecortin (1:500; Abcam) immunoreactivity. Images were taken using Zeiss LSM 510 meta confocal microscope, and cell counts were performed with NIH Image J software.

Results

Transgenic Overexpression of Noggin Reduces Infarct Volume

NSE-noggin transgenic mice overexpress noggin under the NSE promoter, which is specifically active in terminally differentiated neurons in the brain. NSE expression begins as early as embryonic day 12.5 and continues in adult rodents. We produced focal cerebral ischemia in adult NSE-noggin and wild-type mice by permanent MCA occlusion (pMCAO). We euthanized the mice 14 days after pMCAO or sham surgery and delineated the infarct area by triphenyltetrazolium chloride staining (Figure 1A–C). The NSE-noggin mice showed a significant reduction in the infarct volume as compared to wild-type mice (P=0.006; n=3; Figure 1D).

Overexpression of Noggin Protects Motor Function

To assess motor function, we used the accelerated Rotorod test and trained the mice before pMCAO until their performance reached a plateau. After pMCAO, we observed that NSE-noggin mice did not show any motor deficit as compared to levels before infarct. This was in contrast to wild-type mice, which showed significant motor deficit 24 hours after MCAO (Figure 1E). The NSE-noggin mice showed a significant reduction in the infarct volume as compared to wild-type mice (P=0.006; n=3; Figure 1D).

Noggin Increases the Numbers of Activated Microglia in the Peri-Infarct Area

Tolerance to ischemic brain injury can be induced by preconditioning with lipopolysaccharide, which is known to activate microglia, and administration of exogenous microglia...
during MCAO also ameliorates ischemic damage and reduces infarct volume.\textsuperscript{22,23} Therefore, we asked whether the beneficial effects of noggin reflected an alteration in the microglial response. At 14 days after pMCAO, we observed that NSE-noggin mice showed significantly higher numbers of CD11b\textsuperscript{+} microglia in the contralateral cortex \((P=0.05; t\text{ test}; n=3\) wild-type, and 8 NSE-noggin mice). The ischemic boundary zone of NSE-noggin mice also showed higher numbers of CD11b\textsuperscript{+} microglia compared to wild-type mice \((P=0.05; t\text{ test}; n=3\) wild-type, and 8 NSE-noggin mice). These results suggest that overexpression of noggin leads to proliferation of microglia in both hemispheres, along with an enhancement of microglial activation in the ipsilateral hemisphere after ischemic injury.

Noggin Increases the Number of Oligodendroglial Progenitors in the Ischemic Boundary Zone

Transgenic overexpression of BMP-2/4 increases astrogliosis and decreases oligodendrogenesis, which are both reversed by noggin.\textsuperscript{12} Because the cortical white matter is susceptible to ischemic injury, we examined whether overexpression of noggin resulted in alteration of oligodendroglial cells.\textsuperscript{24} The ischemic boundary zone of NSE-noggin mice contained cells expressing platelet-derived growth factor receptor-\(\alpha\), the earliest marker of committed oligodendroglial progenitor cells, whereas these cells were not observed in wild-type mice (Figure 4A–D). There were also fewer CNPase\textsuperscript{+} and myelin basic protein-positive oligodendrocytes in the peri-infarct area of NSE-noggin mice as compared to wild-type mice (Figure 4E–H). This suggests that overexpression of noggin produces more oligodendroglial progenitor cells in response to ischemic injury. To assess whether the increase in oligodendroglial progenitor cells along with microglial activation is associated with enhanced neurogenesis, we counted the number of doublecortin-positive neuroblasts and Hu\textsuperscript{+} neurons. There were no significant differences in the numbers of doublecortin-positive (Figure 5A, B) or Hu\textsuperscript{+} (Figure 5C, D) cells in the ischemic boundary zone of NSE-noggin mice compared to wild-type mice. Finally, we examined if noggin affects the formation of glial scar after pMCAO. NSE-noggin mice did not show any difference in number of NG2\textsuperscript{+} cells, which produce chondroitin sulfate proteoglycans as compared to wild-type ischemic boundary zone (Figure 5A, B). We also compared the glial fibrillary acidic protein-positive...
area surrounding the infarct and did not observe any significant difference between the transgenic and wild-type mice (Figure 6C–F). These results indicate that overexpression of noggin specifically enhances the repair of white matter injury after ischemia.

**Discussion**

We have shown that overexpression of noggin in the brain provides protection or enhanced recovery from ischemic injury after pMCAO via enhanced activation of microglia and oligodendrogenesis. It is possible that noggin also activates other protective mechanisms. In human patients, activation of microglia increases after 72 hours after stroke and inflammation persists in both hemispheres up to 30 days. Further, Iba1 expression in activated microglia increases during the first 3 days after infarct and remains elevated in chronic stages. After pMCAO in rodents, activated resident microglia, along with hematogenous macrophages infiltrating the parenchyma, continue to increase in numbers up to day 14, when the debris removal reaches its peak. The role of microglia in stroke is controversial and different studies have given contradicting results. Most studies suggesting that microglia have neurotoxic effects have been performed in transient MCAO with a reperfusion stroke model. Thus, null mutation of CD11b reduces stroke volume after transient MCAO after 24 hours, indicating that microglia are neurotoxic at that stage. However, at 72 hours after transient MCAO, selective ablation of CD11b expressing microglia results in an increase in infarct volume, along with neuronal apoptosis, suggesting that microglia are neuroprotective. These divergent findings suggest that the role of microglia changes at distinct temporal phases after MCAO.

Our studies of the microglial response 14 days after ischemia in a permanent model suggest that microglia have beneficial effects on neurological function and infarct volume (Figures 1, 2). Our results also support other studies demonstrating that administration of exogenous microglia protect against neurodegeneration. The mechanism of neuroprotection by microglia has been attributed in some studies to the production of insulin-like growth factor-1 by activated microglia. Insulin-like growth factor-1 also promotes the differentiation of adult neural stem cells into oligodendro-
cytes by inhibiting BMP-2/4 signaling, and addition of noggin has a synergistic effect with insulin-like growth factor-1 on production of oligodendrocytes. In our study, we found the presence of oligodendrogial progenitor cells in the peri-infarct area of NSE-noggin mice (Figure 4), consistent with the idea that noggin and insulin-like growth factor-1 produced by activated microglia act synergistically to repair the white matter injury after stroke.

Whereas administration of BMP-6 and BMP-7 have been shown to improve neurological function and reduce stroke volume in transient MCAO models, our results show that more global inhibition of BMP signaling with noggin is beneficial in a pMCAO model. In addition to differences in the stroke model and timing of analysis, this could be explained by the fact that noggin binds BMP-2/4 more avidly than BMP-6 and BMP-7; thus, our results are probably attributable to the predominant inhibition of BMP-2/4 effects. BMP-2 and BMP-7 can exert opposite effects during development, and our findings may reflect the same divergence in the function of different BMP family members in adults.

Conclusion

In our study, overexpression of noggin protected the mice from neurological deficit 1 day after pMCAO, suggesting that noggin is neuroprotective. Noggin was present at the time of the stroke, so further studies must be performed to assess the therapeutic potential of administering exogenous noggin after ischemia. The incidence of stroke depends on genetic and environmental risk factors in different human populations; for example, certain Mediterranean populations have a significantly lower incidence. Further genetic studies may reveal whether noggin polymorphisms confer protection from stroke in human populations.

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Disclosures

None.

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