Axonal Outgrowth and Dendritic Plasticity in the Cortical Peri-Infarct Area After Experimental Stroke

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Background and Purpose—Axonal remodeling is critical to brain repair after stroke. The present study investigated axonal outgrowth after stroke and the signaling pathways mediating axonal outgrowth in cortical neurons.

Methods—Using a rodent model of middle cerebral artery occlusion, we examined high-molecular weight neurofilament (NFH) immunoreactive axons and myelin basic protein-positive oligodendrocytes in the peri-infarct area. In vitro, using cultured cortical neurons in a microfluidic chamber challenged by oxygen-glucose deprivation (OGD), we investigated mechanisms selectively regulating axonal outgrowth after OGD.

Results—NFH⁺ axons and MBP⁺ oligodendrocytes substantially increased in the peri-infarct area during stroke recovery, concomitantly with an increase in dendrites and spines identified by Golgi-Cox staining. In vitro, cortical neurons subjected to OGD exhibited significant increases in axonal outgrowth and in phosphorylated NFH protein levels, concurrently with downregulation of phosphatase tensin homolog deleted on chromosome 10, activation of Akt, and inactivation of glycogen synthase kinase-3β in regenerated axons. Blockage of phosphoinositide 3-kinase with pharmacological inhibitors suppressed Akt activation and attenuated phosphorylation of glycogen synthase kinase-3β, which resulted in suppression of phosphorylated NFH and axonal outgrowth after OGD; whereas GSK-3 inhibitors augmented axonal regeneration and elevated phosphorylated NFH levels after OGD.

Conclusions—Stroke induces axonal outgrowth and myelination in rodent ischemic brain during stroke recovery, and the phosphoinositide 3-kinase/Akt/glycogen synthase kinase-3β signaling pathway mediates axonal regeneration of cortical neurons after OGD. (Stroke. 2012;43:2221-2228.)

Key Words: stroke ■ axonal outgrowth ■ high molecular weight neurofilament ■ GSK-3β ■ Akt

Brain injury including stroke induces limited axonal regrowth. Emerging data indicate that axonal remodeling is a critical aspect of brain repair and contributes to spontaneous improvements of neurological deficits after stroke.¹² Neurofilament (NF), a neuron-specific intermediate filament, is the most abundant architectural cytoskeletal element in axons and dendrites.³ Neurofilaments are composed of 3 different subunits, light (68kDa), medium (150 kDa), and heavy (NFH, 200 kDa).³ The activity of NF depends on its state of phosphorylation.⁴ Phosphorylated NFH (pNFH) participates in axonal growth and regulates synaptic function.⁵,⁶ Several articles have suggested that aberrant perikaryal accumulation hyperphosphorylated NFH in neurodegenerative disorders.⁷,⁸ Nonphosphorylated NFH (npNFH) is also described as being more abundant in regenerating axons of injured nerves, whereas npNFH indicates damaged and demyelinated axons in multiple sclerosis.⁹,¹⁰ Until recently, only a few studies have examined changes in pNFH after stroke. Stroke induces pNFH in perikarya of injured neurons in human brain.¹¹ In the rodent, cortical infarct results in reduction of pNFH within the peri-infarct region.¹² Mechanisms that regulate axonal regrowth and phosphorylation of NFH after stroke have not been fully investigated.

In the present study, we analyzed the profiles of axonal outgrowth and myelination in rat ischemic brains. We also investigated axonal outgrowth and the signaling pathways that mediate axonal outgrowth in cultured cortical neurons.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Focal Cerebral Ischemia

Adult male Wistar rats (~350 g, Charles River) were subjected to permanent right middle cerebral artery occlusion (MCAO) by

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advancing a 4 to 0 surgical nylon suture with an expanded tip.\textsuperscript{2,13} Rats were euthanized at 7, 28, or 56 days after MCAO. Peri-infarct area in cerebral cortex of the rat was defined on Hematoxylin and Eosin-stained coronal sections (8\textmu m) as the area encompassed by a 300 \textmu m distance from the infarction.\textsuperscript{14}

**Primary Cortical Neurons**

Cortical neurons were harvested from embryonic day-17 Wistar rats (Charles River), according to a published protocol. To separate axons from neuronal soma, a microfluidic chamber (Standard Neuron Device, Xona Microfluidics) was employed.\textsuperscript{15} Oxygen-glucose deprivation (OGD) was performed for 3 hours. See Supplemental Methods.

**Coculture of Neurons With Oligodendrocyte Cells**

Standard coculture system was employed according to published protocols. Briefly, mouse premature oligodendrocyte cells (N20.1, generously provided by Dr. Anthony Campagnoni, University of California at Los Angeles) were incubated in DMEM/F12 at 39°C for 9 days.\textsuperscript{15} See Supplemental Methods.

**Experimental Protocol**

To examine the effect of Akt or (glycogen synthase kinase-3\textbeta) GSK-3\textbeta on axonal outgrowth and pNFH expression after ischemia, neurons were treated with pharmacological phosphoinositide 3-kinase (PI3K) inhibitors, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one, 10, 20, and 40 \textmu mol/L; Calbiochem) and Wortmannin (2 \textmu mol/L; Calbiochem), or GSK-3 inhibitors, inhibitor I (TDZD-8, Thiadiazolidinone analog, 5 \textmu mol/L; Calbiochem), and inhibitor VII (\alpha-4-dibromocacetophenone, 1 \textmu mol/L; Calbiochem) for 96 hours after OGD.\textsuperscript{16–18}

**Immunohistochemistry and Immunocytochemistry**

Single and double immunofluorescent staining was performed on brain sections and cultured cells, as previously described.\textsuperscript{16} See Supplemental Methods.

**Golgi-Cox Staining**

Golgi-Cox staining was performed on brain tissue according to a protocol kindly provided by Dr Crystal L. MacLellan.\textsuperscript{19}
Image Acquisition and Quantification
For histological study, 3 coronal sections at 200 μm intervals/rat per staining were used. Three images of peri-infarct area in cerebral cortex per section were acquired using a 2-photon microscope (Zeiss LSM 510 NLO) under a 40× objective. Images were analyzed using the National Institutes of Health Image analysis program version 1.43, as described previously.20 See Supplemental Methods.

Western Blot
Western blots were performed according to published methods.16 See Supplemental Methods.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction
Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on an ABI 7000 PCR instrument (Applied Biosystems) using SYBR Green real-time PCR method.16 See Supplemental Methods.

Time-Lapse Microscopy
Axons cultured in microfluidic chamber were incubated in a stage top chamber with 5% CO₂ at 37°C, which was placed on the stage of a TE2000-U inverted microscope equipped with a motorized z-stage (Nikon). See Supplemental Methods.

Statistical Analysis
All statistical analyses were performed using the Statistical Package for the Social Sciences (version 15; SPSS Inc). Student t test and 1-way ANOVA with post hoc Bonferroni were used when comparing 2 groups and more than 2 groups, respectively. Values presented in this study are expressed as mean±standard error. A probability value <0.05 was considered significant.

Results
NFH Immunoreactivity in Experimental Stroke
Neurons express pNFH and npNFH in their axons and dendrites.5 We found that pNFH immunoreactive neuronal fibers were closely associated with myelin basic protein (MBP, a marker of myelinating oligodendrocytes) immunoreactive processes in the cortical layer of sham-operated rats (Supplemental Figure S1A); and approximately, 70% of pNFH fibers were associated with MBP in layer VI (Supplemental Figure S1A). This suggests that the majority of pNFH fibers are myelinated axons. The npNFH fibers were also adjacent to MBP processes in layer VI, although npNFH were expressed in neuronal soma and apical dendrites in layer II to V (Supplemental Figure S1B). The pNFH axons and npNFH axons, soma, and dendrites, substantially decreased in axonal and somal compartments in the peri-infarct area after stroke (Supplemental Figure S2A). In layer VI of rat cerebral cortex, MCAO resulted in a well-demarcated infarction in the territory supplied by the MCA during 7 to 56 days poststroke (Figure 1A). We measured pNFH immunoreactive fibers in the peri-infarct area (Figure 1A) and found a significant reduction of pNFH⁺ fibers of up to 63% at 7 days of MCAO in the peri-infarct area compared with homologous areas of sham-operated rats (Figure 1B, C). However, pNFH⁺ fibers in the peri-infarct area increased to 81% and 97% of intact levels at 28 and 56 days after MCAO, respectively (Figure 1B, C). Moreover, the number of NeuN⁺ cells in the area did not significantly change during this
period (Figure 1B). Interestingly, in the contralateral cortex, pNFH\(^+\) fibers substantially decreased to 68% at 7 days after MCAO compared with in sham-operated rats, and increased to 79% and 95% of intact levels at 28 and 56 days after MCAO, respectively (Supplemental Figure S2B). In addition, at 7 days of MCAO, pNFH\(^+\) fibers associated with MBP\(^+\) processes were reduced by 60% in the peri-infarct area compared with homologous areas of sham-operated rats (Figure 1B, D); whereas at 56 days after MCAO, pNFH\(^+\) fibers surrounded by MBP immunoreactivity increased to 98% of intact levels, which is equivalent to the levels of pNFH and MBP in sham-operated rats (Figure 1B, D). A comparable profile of npNFH immunoreactive fibers was also detected (Supplemental Figure S2C, S3A-C). These data suggest that myelinated axons are increased in the peri-infarct area during stroke recovery.

Axonal outgrowth is closely related to growth of dendrites and dendritic spines.\(^2^1\) At 7 days following MCAO, Golgi-Cox staining indicated that neurons showed a marked deterioration of basilar dendrites with connection to adjacent neurons, dramatic reduction of the number and diameter of dendrites, and reduction in spine number of apical dendrites compared with neurons in sham-operated rats (Figure 2A, B). Moreover, swollen dendritic spines increased (Figure 2B, Supplemental Figure S4A). However, number of spines in apical dendrites significantly increased at 56 days compared with 7 days after MCAO (Figure 2C, Supplemental Figure S4B). These dendrites increased in number and length and they connected to dendrites derived from adjacent neurons (Figure 2C, Supplemental Figure S4C, D), whereas only scattered swollen spines were present (Supplemental Figure S4A).

**pNFH in Cultured Cortical Neurons**

Aforementioned in vivo data suggest that regeneration of axons occurs in the peri-infarct area. To examine directly axonal regeneration and sprouting, we employed a microfluidic chamber, which separates axons from neuronal cell bodies and permits direct axonal outgrowth monitoring in cortical neurons.\(^1^5\) Cortical neurons cultured in a microfluidic chamber exhibited axonal morphology (Figure 3A). OGD for 3 hours did not significantly increase caspase-3 levels in cortical neurons (Supplemental Figure S5A), but induced damaged axons with beaded and vanishing appearance at 24 hours (Figure 3A, Supplemental Figure S5B). However, 96 hours after OGD, a large number of axons were regenerated (Figure 3A, B). RT-PCR and Western blot analysis showed that mRNA levels of *NEFH* and protein levels of pNFH substantially increased at 96 hours compared with 24 hours after OGD, as well as in control non-OGD neurons (Figure 3A, B, C).
3C, Supplemental Figure S5C). To examine whether these axons can be myelinated, we cocultured axons with differentiated N20.1 cells in the axonal compartment of the microfluidic chamber. Double immunostaining revealed that many pNFH/β/3-cyclic nucleotide 3'-phosphodiesterase/β oligodendrocyte processes at 96 hours after OGD (Figure 3D). Collectively, these data suggest that OGD induces axonal regeneration and sprouting, and newly generated axons can be myelinated by oligodendrocytes in vitro.

Phosphorylation of GSK-3β Enhances pNFH and Axonal Growth

Several signaling pathways, including PI3K/Akt signaling, mediate growth of axonal and dendritic branches.22 Western blot analysis showed a significant increase in pAkt in neurons at 96 hours after OGD compared with neurons without OGD, which was coincident with elevation of pGSK-3β Ser9 (Figure 4A). These data suggest that the activation of PI3K/Akt phosphorylates GSK-3β at Ser9. To examine whether stroke induces pGSK-3β, we performed immunostaining on...
brain coronal sections. Double immunofluorescent staining revealed that pNFH processes in peri-infarct areas were pGSK-3β (Figure 4B), suggesting activation of inhibitory GSK-3β in vivo. To examine further the connection between PI3K/Akt activity and phosphorylation of GSK-3β, we treated neurons subjected to OGD with PI3K inhibitors, LY294002 and Wortmannin; both inhibitors significantly decreased pAkt and reduced pGSK-3β Ser9 (Figure 4C, Supplemental Figure S6B, C). To test whether increases in the levels of pGSK-3β that inactivate GSK-3β are functionally relevant to elevated pNFH, we blocked GSK-3β activity using 2 structurally unrelated non-ATP competitive GSK-3 inhibitors, inhibitor I and VII. Treatment of OGD-challenged neurons with both GSK-3 inhibitors further elevated pGSK-3β Ser9 and pNFH, whereas PI3K inhibitors substantially decreased pNFH protein levels (Figure 4C, Supplemental Figure S6A, C). In parallel, time-lapse microscopy revealed that inhibition of GSK-3β activation by GSK-3 inhibitor I increased axonal elongation (Figure 5A, C) and pNFH+ arborization (Figure 5B, D). In contrast, a PI3K inhibitor, LY294002, significantly suppressed axonal outgrowth (Figure 5A, C) and pNFH+ arborization (Figure 5B, D).

LY294002 (40 μmol/L) did not significantly induce cell death measured by TUNEL positive cells (Supplemental Table S1), and did not significantly increase the number of damaged axons compared with neurons without treatment after OGD (Supplemental Figure S6D). Consistent with concentration of 40 μmol/L, we found that LY294002 at 10 and 20 μmol/L significantly (p<0.01) blocked axonal elongation and arborization in OGD-challenged primary cortical neurons. A, representative time-lapse microscopic images of primary cortical neuronal culture in a microfluidic chamber, showing axonal outgrowth (lower images, arrowhead) from a starting point at 24 hours (upper images, black arrow) to ending point at 48 hours (lower images, red arrow) in OGD-challenged neurons without any treatment (left), with LY294002 treatment (middle), or with GSK-3 inhibitor I treatment (right). B, confocal tiling images of pNFH+ axons of primary cortical neurons cultured in microfluidic chambers at 96 hours after OGD without treatment (left), LY294002 treatment (middle), or GSK-3 inhibitor I (right). C, quantitative data of axonal elongation after treatment with PI3K inhibitors LY294002 (LY) and Wortmannin (W), or GSK-3 inhibitor I (I) and VII (VII) in OGD-challenged neurons. D, quantitative data of percentage of pNFH-positive axons that distended from the entrance of axonal compartments (B, dot lines) in at 96 after OGD without treatment, with treatment of LY294002 (LY) or GSK-3 inhibitor I (I). N=3/group. Values are mean±SE *p<0.05 versus the OGD without treatment group; **p<0.01 versus the OGD without treatment group. Scale bars: 50 μm in A;100 μm in B.
Inhibits Akt activity and mediates axonal outgrowth compared with the control group (n = 80 axons/group), compared with the control group (n = 70 axons/group).

Phosphatase tensin homolog deleted on chromosome 10 (PTEN) inhibits Akt activity and mediates axonal outgrowth. Cyclin-dependent kinase regulates axonal transport and phosphorylates NFH. Axons at 96 hours after OGD exhibited a substantial reduction of phosphorylated PTEN (Figure 4D), but levels of total and phosphorylated cyclin-dependent kinase did not significantly change compared with levels in the control group (data not shown); this suggests that downregulation of PTEN activates Akt, whereas cyclin-dependent kinase may not play an important role in OGD-elevated pNFH. Collectively, our data suggest that inactivation of GSK-3β by its phosphorylation at Ser9 enhances axonal outgrowth via the PI3/Akt pathway in OGD-challenged neurons.

**Discussion**

The present study demonstrates that in rodent ischemic brain, stroke substantially increased NFH+ axons in peri-infarct cortex and in homologous areas of the contralateral cortex during the recovery period, and increased NFH+ axons were closely associated with myelinating oligodendrocytes. Moreover, in vitro data show that inhibition of GSK-3β elevated pNFH levels and enhanced axonal outgrowth of the cortical neurons challenged by OGD via the PI3/Akt pathway. These data suggest that the PI3K/Akt/GSK-3β signaling pathway could be a potential therapeutic target for promoting axonal regrowth after stroke.

Stroke acutely causes a loss of pNFH+ axons in mammals. In human brains after stroke, pNFH accumulates in perikarya and dendrites. However, little information is available on the distribution of pNFH+ axons in ischemic brain during stroke recovery. Our experimental data revealed that acute stroke induced loss in NFH+ axons. More importantly, we found gradual, but substantial, increases in NFH+ axons in the peri-infarct cortex, and many of these axons were surrounded by MBP+ processes during stroke recovery; this suggests that stroke induces axonal regrowth and newly generated axons are myelinated. Likewise, in the contralateral cortex, we detected a substantial reduction and subsequent increase of pNFH+ axons in acute stroke and recovery from stroke, respectively. This finding is consistent with recently published studies showing an increase in axonal outgrowth in the contralateral cortex during stroke recovery in the rodent, which was facilitated by treatment with inosine or bone marrow stromal cells.

A major challenge for studying axonal outgrowth of cultured cortical neurons is the need to isolate axons from other cellular debris, which impedes investigation of mechanisms underlying axonal outgrowth. We employed a recently developed microfluidic platform that isolates axons from dendrites of cortical neurons. We demonstrated that cortical neurons injured by OGD have elevated pNFH levels and regenerate axons, which was associated with increases in phosphorylation of GSK-3β. Moreover, inhibition of GSK-3β with pharmacological inhibitors increased phosphorylation of GSK-3β and pNFH, and it promoted axonal regeneration and arborization. Phosphorylation of GSK-3β inactivates GSK-3β. GSK-3β acts as a negative regulator of axon formation. Phosphorylated GSK-3β has been implicated in the phosphorylation of NFH, given that GSK-3β has affinity to the NFH side arm. Thus, our data for the first time indicate that in cortical neurons, pGSK-3β mediates pNFH expression and axonal regeneration after OGD. However, in addition to neurons, GSK-3β plays important roles in brain parenchymal cells, including in neural stem cells. Administration of GSK-3β inhibitors will not selectively block neuronal GSK-3β activity in vivo. Investigation of the role of GSK-3β in axonal outgrowth after stroke by use of a conditional GSK-3β mouse (GSK-3βflox/lox) to create mice with neuron-specific GSK-3β deficiency is warranted.

PI3K activates Akt. On activation, Akt phosphorylates diverse substrates, including GSK-3β. PI3K inhibits PI3K/AKT signaling. Activation of Akt and subsequent phosphorylation of GSK-3β increases axonal outgrowth of dorsal root ganglial neurons. The present study demonstrates that elevation of pNFH in cortical axons was associated with downregulation of PTEN and activation of Akt, whereas inhibition of PI3K suppressed Akt activation and downregulated phosphorylation of GSK-3β. Therefore, our data indicate that reduction of PTEN activates PI3K/Akt signaling, which in turn inhibits GSK-3β by its phosphorylation, leading to axonal outgrowth of cortical neurons after OGD. A caveat is that these in vitro findings cannot be directly applied to in vivo observation. However, our in vivo immunohistochemistry data suggest the activation of inhibitory GSK-3β in pNFH fibers in the peri-infarct area. Others have demonstrated that deletion of PTEN enhances regeneration of adult corticospinal tract in mouse model of spinal cord injury. Therefore, inhibition of GSK-3β activity in axons has a potential therapeutic effect to enhance axonal generation in the adult injured brain after stroke.

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**Disclosures**

None.

**References**

6. Sanchez I, Hassinger L, Sihag RK, Cleveland DW, Mohan P, Nixon RA. Local control of neurofilament accumulation during radial growth of...
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SUPPLEMENTAL MATERIAL

Title: Axonal outgrowth and dendritic plasticity in the cortical peri-infarct area after experimental stroke

Abbreviated title: Axonal outgrowth in cortical peri-infarct area

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Supplemental Methods

Primary cortical neurons
Briefly, the dissociated cerebral cortical cells were cultured in neurobasal medium with 2% B27 which includes serum albumin, corticosterone, insulin, and progesterone (Gibco, Grand Island, NY). To kill astrocytes, uridine and 5-fluorodeoxyuridine were added for 2 days. At 14 days in vitro, media was changed to Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks balance salt solution, and neurons were subjected to oxygen-glucose deprivation (OGD) for 3 h. To separate axons from neuronal soma, a microfluidic chamber (Xona Microfluidics) was employed. The cells were cultured in the same medium as indicated above. Medium was changed daily. At 7 days in vitro, neurons were challenged by OGD for 3 h, morphologically analyzed at 24 h and 96 h after OGD using time-lapse microscope, and then prepared for immunocytochemistry.

Co-culture of neurons with oligodendrocyte cells.
N20.1 cells were differentiated into mature oligodendrocytes. Concurrently, neurons cultured in the microfluidic chamber were subjected to OGD for 3 h. Immediately after OGD, harvested differentiated N20.1 cells were placed at a density of \(3 \times 10^4\) cells/chamber in the axonal compartment of microfluidic chambers with the neurobasal medium for 96 h.

Immunohisto and immunocytochemistry.
The following primary antibodies were used in the present study: mouse anti-SMI31 (a marker of phosphorylated NFH, 1:1500; Covance, Emeryville, CA); anti-SMI32 (a marker of nonphosphorylated NFH, 1:1000; Covance); rabbit anti-phosphorylated glycogen synthase kinase-3β (pGSK-3β, 1:15; Cell Signaling, Technology Beverly, MA); rabbit anti-myelin basic protein (MBP, 1:400; Dako); chicken anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, 1:200; Aves, Tigard, OR). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (1:10,000, Vector Laboratories, Burlingame, CA).

Image acquisition and quantification.
Briefly, pNFH\(^+\) areas were binarized and digitally level-adjusted with intensity threshold setting at pixel value 22 as black pixel and 255 as white pixel, so that the black pixels represent pNFH immunoreactive areas. The pNFH\(^+\)-MBP\(^+\) areas were split into green- and red-channel images, and the image showing common positive areas in both images was obtained and adjusted as above. The area of black pixels was divided by the total area in each image to estimate the profile of axonal outgrowth after stroke. In Golgi-Cox staining, 15 pyramidal neurons in peri-infarct
areas per rat were selected. In vitro, for axonal elongation, ten axons having growth cone were randomly selected at 24 and 48h after OGD and an axonal length was then measured (length at 48 h minus length at 24 h of the selected axon).

**Western Blot**

The following primary antibodies were used: mouse anti-SMI 31 (1:2000; Covance), rabbit anti-phosphorylated Akt (Ser 473, 1:1000; Cell Signaling), rabbit anti-Akt (1:3000; Cell Signaling), rabbit anti-phosphorylated phosphatase tensin homolog deleted on chromosome 10 (PTEN, Ser 380/Thr 382/383, 1:1000; Cell Signaling), rabbit anti-PTEN (1:3000; Cell Signaling), rabbit anti-phosphorylated GSK-3β (pGSK-3β, Ser 9, 1:2000; Cell Signaling), rabbit anti-GSK-3β (1:3000; Cell Signaling), rabbit anti-caspase-3 (1:3000; Cell Signaling), goat anti-phosphorylated cyclin-dependent kinase 5 (cdk5, Ser 159, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-cdk5 (1:3000; Upstate Biotechnology, Lake Placid, NY), and β-actin (1:10000; Abcam, Cambridge, MA). Normalization of results was ensured by running parallel gels and blotting for β-actin levels as an internal control.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction.**

Each sample was tested in triplicate, and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method. Primers to amplify the following transcripts are as follows: β-actin forward primer, 5′-CCATCATGAAGTGTGACGTTG, and reverse primer, 5′-CAATGATCTTGATCTTCATGGTG (150 bp); neurofilament, heavy polypeptide (NEFH) forward primer, 5′-CGAGAGGGACACCCAGAATA, and reverse primer, 5′-CCGCCATCTAAGTGCTT (118 bp).

**Time-lapse microscopy.**

A 10x objective with 1.5x zoom was used for acquiring images. Bright-field images were acquired at 30 ms exposure time (Universal Imaging, West Chester, PA).
Supplemental Reference


Supplemental Figure Legend

Figure S1. Association of NFH$^+$ neuronal fibers with MBP$^+$ fibers in cerebral cortex of the rat. Panels A is double immunofluorescent confocal images obtained from a representative sham-operated rat, showing pNFH (red) and MBP (green) positive fibers as well as merged images in 6 layers of the cerebral cortex. Panel B is double immunofluorescent confocal images obtained from a representative sham-operated rat, showing npNFH (red) and MBP (green) positive fibers as well as merged images in 6 layers of the cerebral cortex. Scale bars: 20 µm.

Figure S2. Morphological alteration of NFH$^+$ axons, dendrites, and soma after MCAO. Panels A is immunofluorescent confocal images of pNFH and npNFH positive fibers in axonal (layer VI), and somal (layer V) compartments obtained from a representative sham-operated rat (sham), and rat at 7 days after MCAO (stroke). Panel B is quantitative data of pNFH$^+$ fibers in the homologous area of contralateral cortex. Panel C is quantitative data of npNFH$^+$ fibers in the homologous area of contralateral cortex. N=4/group. Values are mean ± SE. *$P < 0.05$ vs sham group; **$P < 0.01$ vs sham group; ***$P < 0.001$ vs sham group; ##$P < 0.01$ vs the 7 day group; ###$P < 0.001$ vs the 7 day group; †††$P < 0.001$ vs the 28 day group.

Figure S3. Distribution of npNFH$^+$ and MBP$^+$ fibers in the peri-infarct cortical region of ischemic rat. Panel A is double immunofluorescent confocal images of representative rats at 7, 28 and 56 days after MCAO, showing npNFH$^+$ and MBP$^+$ fibers as well as merged images in the peri-infarct areas. Arrows and arrowheads indicate that npNFH$^+$ axons were not associated and closely associated, respectively, to MBP$^+$ processes in the peri-infarct areas. Panels B is quantitative data of npNFH$^+$ axons in the ipsilateral peri-infarct area. Panel C is quantitative data
of co-localization of npNFH$^+$ axons and MBP$^+$ processes in peri-infarct areas. N=4/group. Values are mean ± SE. **P < 0.01 vs sham group; ***P < 0.001 vs sham group; ###P < 0.001 vs the 7 day group; †††P < 0.001 vs the 28 day group. Scale bars: 20 µm.

Figure S4. Dendrites and dendritic spines after MCAO. Panels A-D are quantitative data of number of swollen spines in each neuron (A), number of spines (> 0.5 µm in diameter) /20 µm of apical dendrites (B), and diameter (C) and number (D) of dendrites in each neuron. IC = ischemic core. N=3/group. Values are mean ± SE. *P < 0.05 vs sham group; ***P < 0.001 vs sham group; #P < 0.05 vs the 7 day group; ##P < 0.01 vs the 7 day group; ###P < 0.001 vs the 7 day group; †††P < 0.001 vs the 28 day group.

Figure S5. Caspase-3, number of damaged axons, and NEFH mRNA levels in primary cortical neuronal cultures. Panel A shows quantitative western blot data of caspase-3. N=5/group. Panel B is quantitative data of number of damaged axons before OGD, and at 24 and 96h after OGD. N=4/group. Panel C shows mRNA levels of NEFH measured by real-time RT-PCR. N=5/group. NEFH = neurofilament, heavy polypeptide. Values are mean ± SE. *P < 0.05 vs the control; **P < 0.01 vs the control; #P < 0.05 vs the 24 h group.

Figure S6. pNFH, pAKT, and pGSK-3β levels, and number of damaged axons in cortical neurons after OGD treated with PI3K and GSK-3 inhibitors. Panels A to C are quantitative western blot data of pNFH (A), pAkt (B), and pGSK-3β (C), as well as number of damaged axons (D) after OGD treated with PI3K inhibitors, LY294002 (40 µM, LY) and Wortmannin (2 µM, W), or GSK-3 inhibitor I (5 µM, I) or VII (1 µM, VII). N=4/group. Values are mean ± SE. *P < 0.05 vs the OGD without treatment group; **P < 0.01 vs the OGD without treatment group.
Supplemental Table 1: TUNEL positive cells in microfluidic chambers

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<th>Groups</th>
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</tr>
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<tbody>
<tr>
<td>Non-OGD (n=3)</td>
<td>10±1</td>
<td>NA</td>
</tr>
<tr>
<td>OGD (n=4)</td>
<td>12±9</td>
<td>0.819532</td>
</tr>
<tr>
<td>OGD+LY 10 µM (n=3)</td>
<td>10±4</td>
<td>1</td>
</tr>
<tr>
<td>OGD+LY 20 µM (n=3)</td>
<td>5±4</td>
<td>0.355348</td>
</tr>
<tr>
<td>OGD+LY 40 µM (n=5)</td>
<td>13±4</td>
<td>0.463496</td>
</tr>
</tbody>
</table>

N= 3 to 5 different chambers/group. LY= LY294002
**A** Axonal compartment (layer VI) vs. Somal compartment (Layer V)

Sham vs. pNFH vs. npNFH vs. pNFH vs. npNFH

**B** % of pNFH+ fibers (mean ± SE) in the contralateral cortex

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>7</th>
<th>28</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>[Bar]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCAO</td>
<td>[Bar]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C** % of npNFH+ fibers (mean ± SE) in the contralateral cortex

<table>
<thead>
<tr>
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<th>28</th>
<th>56</th>
</tr>
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<tr>
<td>Sham</td>
<td>[Bar]</td>
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<td></td>
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<tr>
<td>MCAO</td>
<td>[Bar]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
% of npNFH+ fibers (mean ± SE) in the peri-infarct area

**A** npNFH

**B** % of npNFH+ fibers (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>7</th>
<th>28</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAO (Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C** % of npNFH+MBP+ fibers (mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>7</th>
<th>28</th>
<th>56</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCAO (Days)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Number of swollen spines (mean ± SE)

A

sham 7 28 56

MCAO (Days)

Number of spines (mean ± SE)

B

sham 7 28 56

MCAO (Days)

Number of dendrites (mean ± SE)

C

sham 7 28 56

MCAO (Days)

D

sham 7 28 56

MCAO (Days)
A. Caspase-3

- Control
- 24 h
- 96 h

B. Number of damaged axons

- Control
- 24 h
- 96 h

C. NEFH mRNA

- Control
- 24 h
- 96 h

OGD (3 h)

**Casepase-3 (mean ± SE)**

**Number of damaged axons (mean ± SE)**

**NEFH mRNA (mean ± SE)**