Remodeling of the Axon Initial Segment After Focal Cortical and White Matter Stroke

Jason D. Hinman, MD, PhD; Matthew N. Rasband, PhD; S. Thomas Carmichael, MD, PhD

Background and Purpose—Recovery from stroke requires neuroplasticity within surviving adjacent cortex. The axon initial segment (AIS) is the site of action potential initiation and a focal point for tuning of neuronal excitability. Remodeling of the AIS may be important to neuroplasticity after stroke.

Methods—Focal cortical stroke in forelimb motor cortex was induced by photothrombosis and compared with sham controls. White matter stroke was produced through stereotactic injection of a vasoconstrictor together with biotinylated dextran amine to retrogradely label injured cortical neurons. AIS length, morphology and number were measured using immunofluorescence and confocal microscopy 2 weeks after stroke.

Results—Within the peri-infarct cortex and after white matter stroke, AIS length decreases. This shortening is accompanied by altered AIS morphology. In peri-infarct cortex, the decrease in AIS length after stroke occurs from the distal end of the AIS, resulting in a Na 1.6 γ-aminobutyric acid type A receptor-α2 subunit staining at axoaxonic synapses along the AIS is significantly decreased. In addition, a significant increase in small, immature initial segments is present in layers 2/3 of peri-infarct cortex, reflecting maturation of axonal sprouting and new initial segments from surviving neurons.

Conclusions—Stroke alters the compartmental morphology of surviving adjacent neurons in peri-infarct cortex and in neurons whose distal axons are injured by white matter stroke. With a key role in modulation of neuronal excitability, these changes at the AIS may contribute to altered neuronal excitability after injury and prove crucial to increasing neuroplasticity in surviving tissue affected by stroke. (Stroke. 2013;44:182-189.)

Key Words: axon initial segment ■ plasticity ■ stroke ■ white matter

Stroke is a devastating neurological illness with limited functional recovery. The adjacent surviving cortex plays a pivotal role in the limited neuroplasticity and recovery seen after stroke. Regulation of neuronal excitability is an important feature of recovery after stroke. Tonic γ-aminobutyric acid (GABA) inhibition is increased in peri-infarct cortex, increasing overall basal inhibition and lowering neuronal excitability. Pharmacological blockade of tonic GABA signaling improves functional recovery after stroke. Stimulation of glutamate signaling through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor promotes neuronal excitability in peri-infarct cortex, stimulating local brain-derived neurotrophic factor production and enhancing recovery. Thus, stroke alters peri-infarct neuronal excitability, and these alterations play a role in the recovery of function.

The axon initial segment (AIS) regulates neuronal excitability by serving as a gatekeeper for action potential initiation. The AIS regulates the threshold for action potential initiation through voltage-gated sodium (Na+) channel clustering, neurotransmission, and axoaxonic synaptic connections. Recently, the molecular makeup of the AIS has been clarified and appears dependent on ankyrinG (ankG). β-IV spectrin follows ankG to the initial segment and serves to anchor the Na+ channel-ankG complex to the axonal cytoskeleton, creating a clustered zone of high-density depolarizing membrane ion channels. Initiation and propagation of the action potential is dependent on depolarization of a defined length of axon referred to as the spike trigger zone, thus the length of Na+ channel clustering at the distal AIS determines the trigger zone and participates in fine tuning of neuronal excitability.

As a gatekeeper of action potential initiation, the AIS is uniquely positioned to play an important role in brain plasticity after injury. Disruption of the presynaptic input of the avian brain stem auditory system lengthens the AIS and lowers the threshold for excitation by increasing the distribution of Na+ channel, whereas direct optogenetic stimulation of hippocampal neurons in culture alters AIS position (relative to the soma) over time. Mutation of α-II spectrin, similar to a microdeletion observed in a cohort of patients with early-onset West syndrome, alters clustering of the Na+ channel-ankG

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complex and increases the threshold required for action potential firing.14 In a rat model of temporal lobe epilepsy, pyramidal cells from the entorhinal cortex display increased action potential firing correlating with increases in NaV1.6 staining at the AIS.15 In the amyloid beta protein precursor/ presenilin-1 transgenic mouse model of Alzheimer disease, GABAergic axoaxonic synapses present along the AIS are lost from cells in proximity to amyloid plaques, suggesting an explanation for the altered neuronal excitability of cells adjacent to plaques;16 Thus, alterations in AIS molecular organization are relevant to a variety of neurological injuries.

Here, we show that molecular remodeling of the AIS occurs after stroke within surviving peri-infarct cortex in focal cortical stroke and in cortical neurons injured by white matter stroke. We also demonstrate new AIS formation within peri-infarct cortex, suggesting that poststroke axonal sprouting may result in new functional axons. Together, these data suggest that plasticity in the length and morphology of the AIS may contribute to poststroke changes in neuronal excitability and provide a potential morphological neuronal target to enhance recovery from stroke.

**Methods**

**Photothrombotic Stroke Model**

Three separate cohorts (sham, n=12; stroke, n=15) of adult wild-type C57/B16 male mice (Jackson Laboratory) 2 to 4 months of age were subjected to either a sham or photothrombotic stroke over the left forelimb motor cortex according to previously published methods.1 For studies of specific Layer 5 cortical neurons, male Thy-1-eYFP-H homozygous transgenic mice17 at 2 to 4 months of age were used (sham, n=4; stroke, n=7). Animal maintenance and surgical procedures were consistent with UCLA Animal Research Council Guidelines. In all animal cohorts, tissue processing and analyses were performed 14 days after stroke.

**White Matter Stroke Model**

Focal ischemic lesions with retrograde neuronal tracing were produced in 5 adult wild-type C57/B16 male mice (Jackson Laboratory) at 2 months of age through stereotactic pneumatic injection of 150 nL of a 54 mg/mL solution of N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-Nio, Calbiochem) mixed 1:1 with a 20% solution of 10000 MW biotinylated dextran amine (Life Technologies, Inc; final concentration of L-Nio 27 mg/mL and 10% biotinylated dextran amine), at each of 3 stereotactic coordinates using a previously described approach.18

**Tissue Processing and Immunofluorescence**

Mice with focal cortical or white matter stroke were processed for tissue section immunofluorescent visualization of various AIS markers and GABA receptor (GABAR)-α2 subunit19 (Supplemental Methods).

**Quantitation of Axon Initial Segment Morphology**

From each animal, three 40-μm cryostat sections from a 160-μm series were immunostained for ankG and β-IV spectrin. Stitched 0.5 mm×0.7 mm 100× confocal z-stacks corresponding to 7.5-μm optical sections were obtained from peri-infarct cortex or sham motor cortex (Nikon C2 laser scanning confocal microscope). The 200-μm peri-lesional area adjacent to the stroke was cropped, rotated, and a 100-μm² square grid overlayed. Five 100-μm² areas were selected at random for initial segment measurement from each section and independently for each immunolabel. AIS length was measured in pixels (NeuronJ:Fiji). All linear structures within each measured field in which the beginning and end could be determined were measured, excluding nodes of Ranvier. This included a fraction of (short-appearing) initial segments not completely within the actual or optical section, but this fraction did not differ between control and stroke.

Quantitation of NaV1.6 and ankG length was performed by measuring the pixel length staining for each immunolabel in 5- to 10-layer 5-cortical neurons in five, 100× fields/animal in motor (sham) and peri-infarct cortex as above. In yellow fluorescent protein (YFP)-positive cells, AIS length was measured by tracing the labeled AIS of YFP-positive cells using the multipoint line feature of NIS Elements software (Nikon). The peri-infarct region of 200-μm lateral to the stroke edge was imaged from 2 sections from a 120 μm series. AIS length in 4 to 15 YFP-positive cells/animal was measured compared with a corresponding region of motor cortex from sham animals.

AIS number was quantified using the same 0.5 mm×0.7 mm stitched confocal images used for length measurements. These images included the upper boundary of cortical layer 2 to the lower boundary of layer 6 and included the lateral edge of the stroke. The region of interest for AIS number was defined as the region between 100 and 200 μm lateral to the stroke edge. Within this region of interest, all β-IV spectrin-positive structures were counted that had a typical AIS appearance: >5 μm in length with a wide base and tapered end. AIS number was binned according to 100-μm squares.

For AIS length measurement after white matter stroke, three 50-μm sections from a 150-μm series in each of 5 animals were imaged using a 0.5 mm×0.5 mm stitched 100× confocal z-stack of the motor cortex overlying the stroke. Biotinylated dextran amine-positive cells were counted and their AIS length measured as above (NeuronJ:Fiji). Percent change in AIS length was calculated by measuring AIS length from unlabeled cells within the same region.

For length measurements in both focal cortical and white matter stroke, statistical significance was determined using Welch t test assuming unequal variance with a P<0.05. For AIS number analysis, a Bonferroni correction was applied for multiple comparisons and established statistical significance at P<0.007 (Microsoft Excel).

**Results**

**Peri-Infarct Changes in Axon Initial Segment Length**

Two weeks after photothrombotic stroke in the motor cortex, mice were euthanized and double-immunolabeled for ankG and β-IV spectrin, 2 key cytoskeletal scaffolds that position NaV channels to the AIS. In the peri-infarct cortex immediately lateral to the stroke (within 200 μm), AISs showed altered morphology and length compared with sham (Figure 1A and B). Focal cortical stroke produces near-complete cell death in the stroke core and results in the loss of all AIS labeling within the area of focal ischemia (Figure 1B), consistent with previous reports.11 In sham controls, AISs labeled for ankG (left panel) or β-IV spectrin (right panel) have a regular, linear morphology and project inferiorly (Figure 1C). Within peri-infarct cortex, AISs with nonlinear projection patterns were common (Figure 1D). The length of the initial segment is proposed as a key mechanism for the regulation of excitability.20 Therefore, we measured ankG, β-IV spectrin, and NaV1.6-positive AIS length within peri-infarct cortex compared with sham controls. Two weeks after stroke, ankG and β-IV spectrin AIS length decreases by 14.0% (±0.99%) and 15.4% (±1.04%), respectively, corresponding to a ≈3-μm absolute change in length (P<0.0001; Supplemental Table 1). Immunolabeling and quantification of the ankG to NaV1.6 length ratio shows that this ratio is unchanged from
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sham (76%) to stroke (77%; \(P=0.37, \text{NS; Supplemental Figure 1}\)). Thus, stroke reduces the length of the AIS by altering its distal end, thereby decreasing the overall number of \(\text{Na}_{1.6}\) channels at this region.

The previous data show that the AIS shortens in peri-infarct cortex in cells distributed throughout all cortical layers. However, we reasoned that subcortical projecting layer 5 cortical neurons in peri-infarct sensorimotor cortex might play a disproportionate role in poststroke plasticity and recovery given their role in motor function. Therefore, we utilized the Thy-1-eYFP-H transgenic mouse line to assess AIS length and morphology, specifically in this cell population, within peri-infarct cortex compared with sham control 2 weeks after stroke. This analysis demonstrates that YFP-positive layer 5 neurons show a similar alteration in AIS morphology as observed in other cortical layers (Figure 2). In peri-infarct cortex, the AIS of YFP-positive cells often showed a blunt distal end (arrows, Figure 2B and 2C) whereas in sham control, the AIS tapers off in the distal end (Figure 2A). Using \(\beta\)-IV spectrin immunostaining (red), quantification of AIS length in this specific cell population demonstrates a similar 14.2% (3.71 \(\mu\)m) decrease in peri-infarct cortex compared with sham (\(P<0.00001; \)Supplemental Table 1).

GABAR-\(\alpha\)2 Axoaxonic Synapses in Peri-Infarct Cortex

In cortical pyramidal neurons, chandelier cells form axoaxonic synaptic connections along the AIS. These synapses are exclusively GABAergic and specifically involve the \(\alpha\)-2 subunit of the GABA receptor. These axoaxonic synapses function at the AIS to further control the fidelity of neural circuitry. Thus, to demonstrate that poststroke plasticity at the AIS includes changes in its circuitry in addition to changes in its structure, we labeled GABAR-\(\alpha\)2–positive synaptical boutons in both sham and peri-infarct tissue 2 weeks after stroke. Within peri-infarct cortex, GABAR-\(\alpha\)2–positive synaptic boutons along the AIS are significantly decreased compared with sham control (Figure 3A and B). The absolute number of GABAR-\(\alpha\)2–positive boutons per AIS in peri-infarct is decreased (sham, 9.13; stroke, 4.41; \(P<0.005\)). When controlled for
ankG-positive AIS length, the average length of initial segment between GABAR-α2–positive boutons increases modestly but significantly in peri-infarct tissue (Figure 3C). Notably, there was substantial variability in the number of axoaxonic synapses in cortical neurons in both sham and stroke conditions. Thus, stroke causes a decrease in GABA innervation of the AIS by synaptic pruning of GABAergic synapses.

**Peri-Infarct Changes in Axon Initial Segment Number**

While assessing AIS length and morphology differences in peri-infarct cortex, we observed an apparent increase in small, irregular appearing initial segments in upper cortical layers. Because of the randomized way AIS length was assessed in Figure 1, this increase is not responsible for the shorter AIS length observed throughout peri-infarct cortex; however, it was intriguing because axonal sprouting is known to occur within peri-infarct cortex. Therefore, we examined AIS number according to relative cortical depth within lateral peri-infarct cortex 2 weeks after stroke (Figure 4A and B). The number of β-IV spectrin–positive AIS increases significantly between 47% and 109% greater than sham animals at cortical depths consistent with layers 2/3 (Figure 4C). Small, irregular appearing initial segments (arrows; Figure 4B) were largely responsible for the increase in AIS number after stroke. Rarely, occasional neurons within peri-infarct cortex could be identified with supernumerary AIS (Figure 4D; Supplemental Figure 2), perhaps accounting for some of the increase in total AIS number. When present, these new initial segments typically arose from the most proximal portion of β-IV spectrin labeling and were oriented laterally rather than the typical inferiorly projecting orientation for cortical motor neurons.

**White Matter Stroke Alter AIS Morphology and Length**

To further support that AIS remodeling is part of poststroke plasticity, we utilized a mouse model of focal white matter
stroke combined with retrograde labeling. This method produces precise localization of stroke in the white matter underlying forelimb motor cortex (Figure 5A). Combined with retrograde labeling, this technique allows visualization of individual cortical neurons that have undergone distal ischemic anatomy because uninjured axons do not readily pick up the retrograde label (data not shown). As established in the previous study, only a minority (≈10%) of neurons within overlying motor cortex project through the focal ischemic lesion and pick up the tracer (red; Figure 5B), thus allowing unlabeled cells in the adjacent cortex to serve as internal controls because they lie adjacent to neurons that have injured axons. Co-immunolabeling for AIS markers allows identification and measurement of the AIS of individual cortical neurons injured by stroke (Figure 5C). Two weeks after white matter stroke, biotinylated dextran amine–positive cells (arrows; Figure 5C) have both altered morphology and length of their AIS (green) when compared with adjacent, unlabeled cells (arrowheads; Figure 5C) within the same region of motor cortex. Quantification of β-IV spectrin–positive AIS length in labeled versus neighboring unlabeled cells, demonstrated a 33.0% decrease in AIS length 2 weeks after stroke (P<0.0001; Supplemental Table 2). In labeled cells, decreases in AIS length were often accompanied by altered morphology, particularly a decrease in the caliber or width of the AIS both at its proximal base and distal end, as well as a relative decrease in the β-IV spectrin (arrows; left panel Figure 5C) and ankG (data not shown) immunoreactivity. Thus, white matter stroke produces remodeling of the AIS in neurons far from the stroke site. Figure 5D summarizes how cortical and white matter stroke alters the AIS in surviving neurons.

**Discussion**

Recovery from stroke depends in large part on plasticity within peri-infarct cortex. Understanding the regulation of surviving cortical networks after injury is essential to designing neural repair therapies. Modulation of neuronal excitability in peri-infarct cortex can benefit recovery from stroke. Poststroke changes in peri-infarct dendritic arborization are known to occur and thereby alter the morphology of surviving neurons after stroke. The AIS provides a structural platform for the regulation of neuronal excitability through dense localization of Na\(_{\text{v}}\) channels and integration of axoaxonic input. Here we examined AIS length and morphology in surviving neurons within peri-infarct cortex with structurally intact but stunned circuitry. Two weeks after stroke, cell death and peri-infarct inflammation are waning, whereas axonal sprouting, gene expression changes, and dendritic remodeling are robust, suggesting that poststroke neuroplasticity is at a first peak. Within peri-infarct cortex, neurons are characterized by a decrease in the length of the AIS, occurring from the distal end of the initial segment, and lose axoaxonic synapses along the length of the AIS. These changes potentially alter the spike trigger zone and may negatively impact peri-infarct excitability. We also show that the absolute number of AISs increases within peri-infarct cortex, perhaps reflecting maturation of poststroke axonal sprouting into functional axons. Finally, we demonstrate that AIS remodeling occurs in a model of white matter stroke, thus supporting the concept that this axonal microdomain is sensitive to neuronal injury and essential for neuroplasticity after varied types of stroke.

Functioning as the integrative site of neuronal excitability, the AIS is a finite cellular compartment that plays a key role in neuronal plasticity. Several studies suggest the primary mechanism for AIS regulation of cellular excitability is through control of its length. For example, deafferentation of the chick brain stem auditory nucleus resulted in an increased AIS length with a greater distribution of Na\(_{\text{v}}\) channels and a lower threshold for excitation. In a mouse model of Angelman syndrome, the AIS lengthened in CA1 and CA3 areas by similar percentages as seen here. In this model, the action potential amplitude and rate of rise of the action potential increased, whereas the threshold potential was lower, correlating with increased expression of AIS proteins ankG and Na\(_{\text{v}}\)1.6. Of these, ankG appears to be the driver of AIS formation and maintenance. In its absence, axonal compartments revert to dendritic molecular makeup. In direct neuronal ischemia, proteolysis of ankG is mediated by calpain-dependent proteolysis and partially reversed by calpain-inhibition. Calpain activity increases in peri-infarct cortex, therefore AIS remodeling in peri-infarct cortex may be dependent on calpain activation.

Although the poststroke change in AIS length is small, alterations within this critical cell compartment are likely to have an impact on cellular excitability. The observed decrease
in the length of Na\textsubscript{v}1.6 staining at the AIS in peri-infarct cortex suggests that fewer Na\textsubscript{v} channels are present at this location. Stroke directly alters the gene expression of sodium channels in sprouting neurons adjacent to the infarct\textsuperscript{28} and in human peri-infarct tissue after intracerebral hemorrhage.\textsuperscript{32}

Electrophysiological techniques to measure the excitability effects of sodium channel changes at the AIS are difficult. Only recently have voltage-sensitive dyes and imaging techniques improved enough to allow observations of current at the AIS\textsuperscript{9,33} and these techniques still have resolution limits that preclude their application to this model system.\textsuperscript{9} However, localization of the action potential spike trigger zone to the distal half of the AIS,\textsuperscript{9} where we observed the greatest degree of change, suggests the peri-infarct decrease in AIS length from its distal end would decrease the ability of neurons to fire action potentials with the same fidelity as unaffected cells, thereby altering cortical circuitry poststroke.

Axoaxonic synapses present at the AIS introduce an additional regulatory element to the initiation site of action potentials. Chandelier cells that provide axoaxonic input are predominantly GABAergic interneurons whose role in cortical networks is thought to be inhibitory at the AIS.\textsuperscript{34} However, recent data have suggested that axoaxonic synapses may have depolarizing effects at rest despite their exclusive use of GABA.\textsuperscript{35–37} Loss of these synapses after stroke provides evidence that remodeling at the AIS is not limited to intrinsic pyramidal cell regulation of AIS length but includes synaptic remodeling. The actual electrophysiological meaning of GABAergic synapse loss at the poststroke AIS remains to be clarified as the field determines whether these are inhibitory or excitatory synapses. However, the data presented here support the idea that AIS remodeling changes not just the intrinsic excitability of peri-infarct pyramidal neurons but also their local circuitry.

In rodent models of stroke, axonal sprouting occurs in the peri-infarct region, resulting in reorganization of topographical maps of adjacent somatosensory cortex\textsuperscript{38–40} and stimulating a sprouting transcriptome that varies with age.\textsuperscript{28} The extent to which axonal sprouting occurs from the neuronal cell body or from more distal axon collaterals is unknown. Regardless, the functional maturation of these new axons requires the molecular clustering of Na\textsubscript{v} channels at either nodes of Ranvier or AIS to facilitate conduction. Here, we show that in upper layers of cortex adjacent to stroke, the absolute number of initial segments increases. The significance of this increase in AIS is not clear, but previous data have demonstrated that there is not significant neurogenesis within this region of peri-infarct cortex.\textsuperscript{51,42} Thus, we believe that the increased number of initial segments seen here reflects supernumerary initial segments arising from neurons in this region as a consequence of axonal sprouting, evidenced by the example provided in Figure 4. Axonal sprouting from the cell body has

Figure 5. Focal white matter stroke alters the axon initial segment (AIS). White matter stroke in the yellow fluorescent protein (YFP)-H transgenic mouse produces an area of focal axonal loss (*) in the white matter underlying motor cortex (A). Neurons with axons projecting through and injured by stroke (*) are retrogradely labeled with biotinylated dextran amine (BDA; red; B). High magnification of BDA-labeled cells (red) immunolabeled for β-IV spectrin (green) shows decreased AIS length and altered morphology (arrows; C) compared with neighboring unlabeled cells with intact AIS (arrowheads) 2 weeks after stroke. Schematic showing the 3 major effects cortical and white matter stroke have on the AIS (red; D). Scale bar in A and B=250 μm, C=5 μm.
been observed in models of spinal cord injury, in which these supernumerary axons from axotomized spinal motor neurons were shown to be cholinergic, GAP-43 positive,43 and form synapses.44 Here, we demonstrate evidence for supernumerary initial segments arising from the existing AIS in adult cortical neurons in vivo after stroke and show that these axons begin the molecular organization that begets function.

The loss of AIS integrity after white matter stroke heralds a new concept in our understanding of the widespread effect of ischemic lesions deep within the brain. The retrograde effect of white matter stroke on the neuronal cell body is poorly understood, but here we show that its ability to fire action potentials is likely jeopardized through remodeling of the AIS. In this disease model of white matter stroke, the neuronal cell body is protected from direct injury, and thus, only the effect of distal ischemic axotomy on the neuron is observed. These data expand on the observations by Schafer et al (2009) that the molecular organization that begets function.

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Stroke alters many cortical networks, simultaneously deafferenting both excitatory and inhibitory neurons, and producing stunned neuronal circuitry. Extrapolating from a comparatively simple sensory network, in which deafferentation increases the excitability of central auditory neurons through elongation of the AIS,12 to the complex multicellular network present in neocortex, is difficult. However, the observed decrease in AIS length in neurons surviving stroke may reflect a common injury response; a final common effect in the AIS.

Likewise, the effect of pharmacological manipulation of post-stroke excitability may further alter AIS morphology/length in ways that suggest this morphological compartment of the cell holds key molecular signals for neuroplasticity.

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Title: Remodeling of the axon initial segment after focal cortical and white matter stroke

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Supplemental Methods

Photothrombotic stroke model
Three separate cohorts (sham n=12, stroke = 15) of adult wild type C57/Bl6 male mice (Jackson Laboratory) 2-4 months of age were subjected to either a sham or photothrombotic stroke over the left forelimb motor cortex according to previously published methods 1. Sham animals were administered Rose Bengal without transcranial illumination. Mice were maintained for 2 weeks following the stroke. For studies of specific Layer 5 cortical neurons, male Thy-1-eYFP-H homozygous transgenic mice 2 at 2-4 months of age were used (sham n=4, stroke n=7). Animals were maintained and surgical procedures were consistent with UCLA Animal Research Council Guidelines.

White matter stroke model
Focal ischemic lesions with retrograde neuronal tracing were produced in five adult wild type C57/Bl6 male mice (Jackson Laboratory) at 2 months of age, through stereotactic pneumatic injection of 150 nL of a 54 mg/mL solution of N5-(1-iminoethyl)-L-ornithine, dihydrochloride (L-Nio, Calbiochem) mixed 1:1 with a 20% solution of 10,000 M.W. biotinylated dextran amine (BDA) (Life Technologies, Inc.) (final concentration of L-Nio 27 mg/mL and 10% BDA), at each of three stereotactic coordinates using a previously described approach 3. Mice were maintained for 2 weeks following the stroke, sacrificed as below, and tissue processed as indicated.

Tissue processing
Mice with focal cortical stroke were administered a lethal dose of isoflurane and then transcardially perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde in phosphate buffered saline, and post-fixed overnight. Thy-1-eYFP-H transgenic mice were processed identically as wild-type mice. For NaV immunofluorescence, a third cohort of mice was transcardially perfused with ice-cold 1% paraformaldehyde and post-fixed for 2h. A fourth cohort of mice was processed specifically for GABAR-α2 immunofluorescence (see below). Mice subjected to white matter stroke were perfused and fixed as above. Unless otherwise specified, brains were cryoprotected stepwise in 20% and 30% sucrose and frozen on dry ice. Floating cryostat sections were obtained and tissue sections were maintained in cryoprotectant until ready for immunostaining.

Immunofluorescence
Forty-micron sections were rinsed twice in phosphate buffered saline, pH 7.4 (PBS), placed in 10 mM sodium citrate buffer, pH 8.5 for 30 min at 80°C for antigen retrieval. Following 3 brief washes in PBS, sections were then blocked and permeabilized in 10% normal donkey serum with 0.3% Triton X-100 for 30 min and transferred to MOM block (Vector Labs) for 1 hr at room temperature (RT). After 3 additional washes in PBS, primary antibody cocktails were diluted in blocking solution and left overnight at RT. The following antibodies were used: mouse anti-ankyrinG (1:200, clone N106/36; UC Davis/NIH Neuromab facility), rabbit anti-beta-IV spectrin (1:400)4, rabbit anti-NaV1.6 (1:200)5; mouse anti-caspr (1:500, clone K65/35; UC Davis/NIH Neuromab facility); rabbit anti-neurofilament 200 (1:500, Sigma); mouse anti-NeuN (1:500, Sigma). After three washes in 1X PBS, sections were incubated for 1 hr at RT in donkey anti-mouse IgG (Dylight 649, Jackson Immunoresearch, 1:300) and donkey anti-rabbit IgG (Dylight
Floating sections were then mounted on gelatin-coated glass slides, air dried and dehydrated in alcohols and xylene then coverslipped. Control sections without primary antibodies were performed for all immunostains concurrently. For immunofluorescence in tissue from white matter stroke animals, fifty-micron sections were rinsed twice in PBS, blocked and permeabilized as above and incubated with rabbit anti-beta-IV spectrin and strepavidin-405 (2 μg/mL) (Life Technologies, Inc.) overnight at RT. Washing, secondary antibody labeling, dehydration and mounting were performed as above.

**GABAR-α2 subunit immunofluorescence**

Tissue processing and staining for GABAR-α2 subunits was performed using a separate cohort of both sham (n=5) and stroke mice (n=5). Rabbit anti-GABAR-α2 (1:500, Synaptic Systems) and mouse anti-ankyrinG were used to co-label synaptic densities labeling for GABAR-α2 along the ankyrinG-positive initial segment with confocal imaging. Quantitation was performed by counting GABAR-α2-positive boutons per ankyrinG-positive length in 5 cells from 3 different sections per animal through motor cortex to generate an average AIS length between axoaxonic boutons ratio.

**Quantitation of axon initial segment morphology**

From each animal, three 40 μm cryostat sections from a 160 μm series were immunostained for ankG and beta-IV spectrin. Stitched 0.5 mm x 0.7 mm 100X confocal z-stacks corresponding to 7.5 μm optical sections were obtained from peri-infarct cortex or sham motor cortex (Nikon C2 laser scanning confocal microscope). The 200 μm peri-lesional area adjacent to the stroke was cropped, rotated when necessary, and a grid divided into 100 μm squares overlayed. Using a random number generator, five areas of 100 μm² each were selected for initial segment measurement from each section and independently for each immunolabel. Initial segment length was measured in pixels (NeuronJ:Fiji) for both immunolabels. All linear structures within each measured field in which the beginning and end could be determined were measured, excluding nodes of Ranvier. This included a fraction of (short-appearing) initial segments not completely within the actual or optical section but this fraction did not differ between control and stroke. Pixel lengths were converted to microns based on the optical calibration.

Quantitation of NaV1.6 and ankG length was performed by examining five, 100X fields/animal of layer 5 cortical neurons in motor (sham) and peri-infarct cortex. NaV1.6 and ankyrinG lengths were measured in 5-10 cells/section (NeuronJ:Fiji) as above.

In YFP-positive cells, AIS length was measured by tracing the labeled initial segment of YFP-positive cells using the multipoint line feature of NIS Elements software (Nikon). The peri-infarct region of 200 μm lateral to the stroke edge was imaged from two sections from a 120 μm series. AIS length in 4-15 YFP-positive cells/animal was measured compared to a corresponding region of motor cortex from sham animals.

Axon initial segment number was quantified using the same 0.5 mm x 0.7 mm stitched confocal images used for length measurements. These images included the upper boundary of cortical layer 2 to the lower boundary of layer 6 and included the lateral edge of the stroke. The region of interest for AIS number was defined as the region between 100 and 200 μm lateral to the stroke edge. Within this region of interest, all beta-IV spectrin-positive structures were
counted that had a typical AIS appearance: more than 5 μm in length with a wide base and tapered end. AIS number was binned according to 100 μm squares.

For AIS length measurement after white matter stroke, three 50 μm sections from a 150 μm series in each of five animals were imaged using a 0.5 mm x 0.5 mm stitched 100X confocal z-stack of the motor cortex overlying the stroke. BDA-positive cells were counted and their AIS length measured as above (NeuronJ:Fiji). Percent change in AIS length was calculated by measuring AIS length from unlabeled cells within the same region.

For length measurements in both focal cortical and white matter stroke, statistical significance was determined using Welch’s t-test assuming unequal variance with a p-value <0.05. For AIS number analysis, a Bonferroni correction was applied for multiple comparisons and established statistical significance at p<0.007 (Microsoft Excel).
Table S1. Peri-infarct changes in AIS length.

<table>
<thead>
<tr>
<th>AIS label</th>
<th>Sham (microns)</th>
<th>Peri-infarct (microns)</th>
<th>Difference (%/microns)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ankyrinG</td>
<td>20.72 +/-0.21</td>
<td>17.82 +/-0.13</td>
<td>14.0/ 2.90</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>beta-IV spectrin</td>
<td>21.33 +/-0.22</td>
<td>18.04 +/-0.13</td>
<td>15.4/ 3.29</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>NaV 1.6</td>
<td>17.22 +/-0.33</td>
<td>15.54 +/- 0.28</td>
<td>9.8/ 1.68</td>
<td>p&lt;0.00001</td>
</tr>
<tr>
<td>beta-IV spectrin in YFP-positive Layer 5 cells</td>
<td>26.19 +/-0.41</td>
<td>22.48 +/-0.34</td>
<td>14.1/ 3.71</td>
<td>p&lt;0.00001</td>
</tr>
</tbody>
</table>

Quantification of AIS length in peri-infarct cortex 2 weeks after photothrombotic stroke. Control ankG (n=2(animals)/618 (segments)) and beta-IV spectrin (n=2/503) immunoreactive AIS lengths were measured in sham and stroke (ankG n=4/1100; beta-IV spectrin n=4/1125). Both ankyrinG and beta-IV spectrin demonstrate ~15% reductions in AIS length after stroke that were statistically significant. NaV1.6 labeling demonstrates a similar decrease in length in stroke (n=4/131) compared to sham (n=4/136) while the NaV1.6/ankG ratio does not change indicating that the distal end of the initial segment is preferentially affected by post-stroke remodeling. Beta-IV spectrin AIS labeling in YFP-positive layer 5 pyramidal neurons demonstrates a similar 14.1% decrease in AIS length within peri-infarct cortex (n=7/183) compared to sham (n=4/88).
Table S2. Decreases in AIS length after white matter stroke

<table>
<thead>
<tr>
<th>Cell type</th>
<th>AIS length (microns)</th>
<th>% change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDA-positive cells</td>
<td>18.36 +/-0.38</td>
<td>33.0%</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>BDA-negative cells</td>
<td>27.42 +/-0.36</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Beta-IV spectrin-immunoreactive AIS lengths were measured in five different animals from neurons that were BDA-positive (n=5 animals/288 segments) and thus had axonal injury vs. neighboring neurons in motor cortex that were BDA-negative (n=5/372).
Figure S1. Peri-infarct shortening of the AIS occurs from its distal end and decreases Na\textsubscript{v}1.6. In both sham and stroke animals, Na\textsubscript{v}1.6 labeling (red) is restricted to the distal two-thirds of the AIS whereas ankG (green) labels the entire length of the initial segment. Schematics are provided to demonstrate approximately where the representative images were taken from in sham (left panel, A) and stroke (left panel, B). When compared to sham (A), the Na\textsubscript{v}1.6/ankG ratio in peri-infarct is unchanged (B) but overall AIS length remains decreased indicating that the post-stroke remodeling occurs at the distal end of the AIS. Quantification of Na\textsubscript{v}1.6/ankG ratios is provided in Supplemental Table 1. 100X digital image; scale bar = 5 µm.
Figure S2. Supernumerary AIS in peri-infarct cortex. The increase in AIS number in peri-infarct cortex may, in part, be secondary to supernumerary AIS formation from surviving neurons. In this example, one beta-IV spectrin-positive (red) AIS projects inferiorly towards the white matter (arrow), while a second, smaller caliber AIS projects laterally (arrowhead). The primary AIS (arrow) is myelinated at its distal end, evidenced by immunoreactivity for caspr (cyan) just after the distal end, while the new, supernumerary AIS (arrowhead) is not myelinated at its distal end. 100X digital confocal z-stack image representing 1.0 micron optical thickness. Scale bar = 5 μm.
Supplemental References


脳卒中後の脳卒中における軸索起始部リモデリング
Remodeling of the Axon Initial Segment After Focal Cortical and White Matter Stroke

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Abstract

背景および目的: 脳卒中からの回復には、近傍の残存皮質の神経可塑性を回復させなければならない。軸索起始部（AIS）は活動電位発火部位であり、ニューロンの興奮性を調整する上で重要な部位である。AISのリモデリングは脳卒中後の神経可塑性に重要と思われる。

方法: 前肢運動を担うマウス大脳皮質部位で脳卒中をphotothrombosis（光増感反応血栓モデル）によって誘発し、対照群と比較した。白質脳卒中は血管収縮薬の定位注入により作成し、ビオチン化デキストランアミンの同時注入により、損傷している皮質ニューロンを逆行性に標識した。脳卒中発症2週間後に免疫蛍光法と共焦点顕微鏡検査でAISの長さ、形、および数を測定した。

結果: 白質脳卒中後、梗塞周囲の皮質内のAISの長さはAISの形の変化に伴って短くなった。梗塞周囲の皮質では、AISの長さは脳卒中後に遠位端から短くなり始め、AISに沿って軸索軸索間シナプスにあるγアミノ酪酸受容体A-α2サブユニットが有意に減少した。さらに、梗塞周囲皮質の第2/3層に小さな未成熟の起始部が有意に増加しており、軸索発芽の成熟と、残存ニューロンからの新しい起始部の作成が反映されていた。

結論: 梗塞周囲の皮質と白質脳卒中によって損傷したニューロンの軸索遠位部の神経可塑性は梗塞によって変える。AISに認められたこれらの変化は、ニューロンの興奮性の調整において重要な役割を果たし、回復のニューロンの興奮性を変えることから、脳卒中によって影響を受けた生存組織の神経可塑性の改善に重要と考えられる。