**ONLINE SUPPLEMENT**

**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 $Na_V$ 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-$\alpha$2 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 phospate 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-$Na_V$1.6 (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

549, 1:300). 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 [6] 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 $\mu m^2$ 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 $Na_V1.6$ and ankG length was performed by examining five, 100X fields/animal of layer 5 cortical neurons in motor (sham) and peri-infarct cortex. $Na_V1.6$ 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.**

| AIS label | Sham (microns) | Peri-infarct (microns) | Difference (%/microns) | Significance |
|---|---|---|---|---|
| ankyrinG | 20.72 +/-0.21 | 17.82 +/-0.13 | 14.0/ 2.90 | p<0.00001 |
| beta-IV spectrin | 21.33 +/-0.22 | 18.04 +/-0.13 | 15.4/ 3.29 | p<0.00001 |
| $Na_V$ 1.6 | 17.22 +/-0.33 | 15.54 +/- 0.28 | 9.8/ 1.68 | p<0.00001 |
| beta-IV spectrin in YFP-positive Layer 5 cells | 26.19 +/-0.41 | 22.48 +/-0.34 | 14.1/ 3.71 | p<0.00001 |

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. $Na_V1.6$ labeling demonstrates a similar decrease in length in stroke (n=4/131) compared to sham (n=4/136) while the $Na_V1.6$/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**

| Cell type | AIS length (microns) | % change | Significance |
|---|---|---|---|
| BDA-positive cells | 18.36 +/-0.38 | 33.0% | P<0.0001 |
| BDA-negative cells | 27.42 +/-0.36 | n.a. | n.a. |

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).

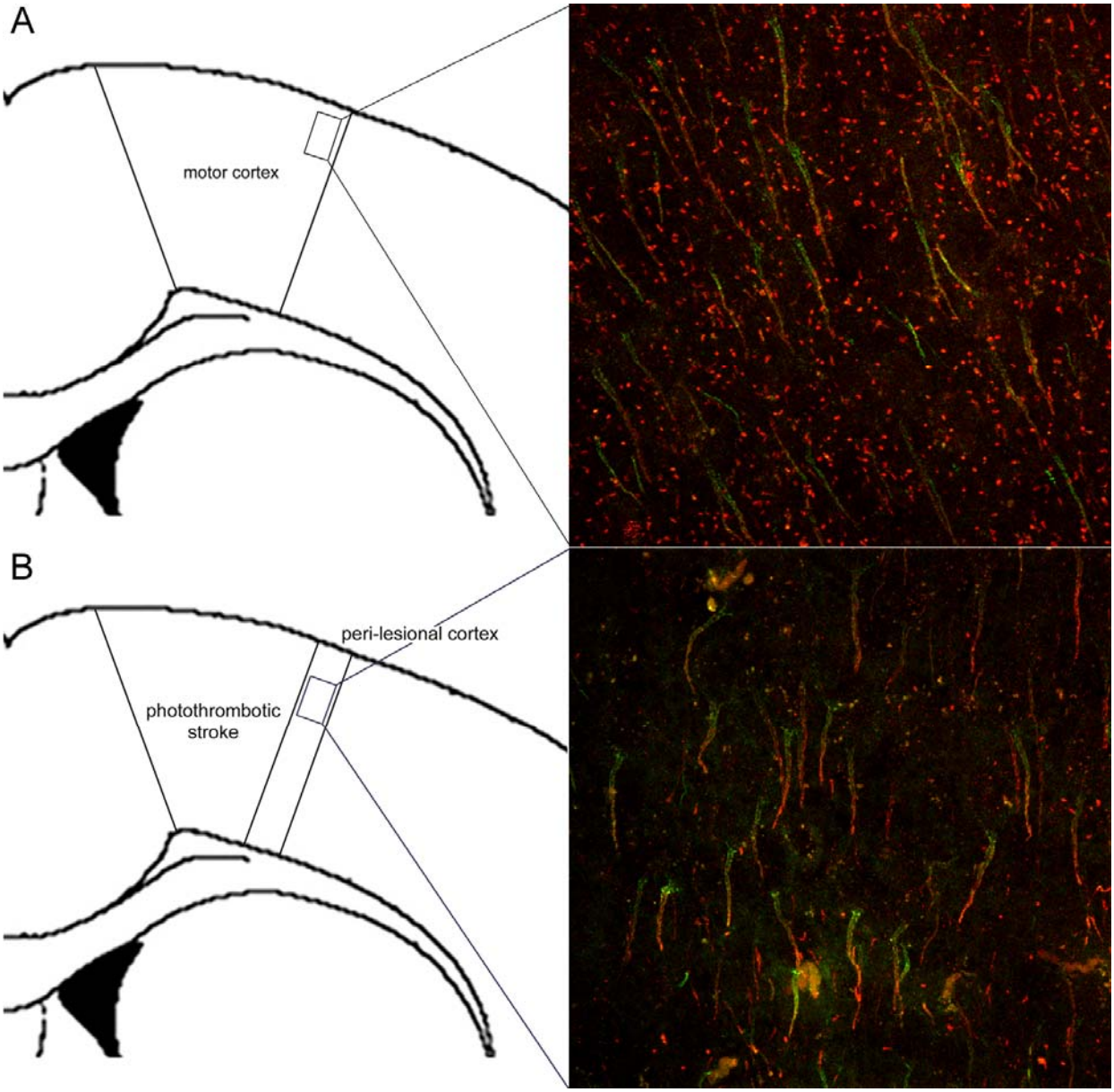


**Figure S1.** Peri-infarct shortening of the AIS occurs from its distal end and decreases $Na_V1.6$. In both sham and stroke animals, $Na_V1.6$ labeling (red) is restricted to the distal two-thirds of the AIS whereas ankG (green) labels the entire length of the initial segment. Schematics are provided 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 $Na_V1.6$/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 $Na_V1.6$/ankG ratios is provided in Supplemental Table 1. 100X digital image; scale bar = 5 μm.

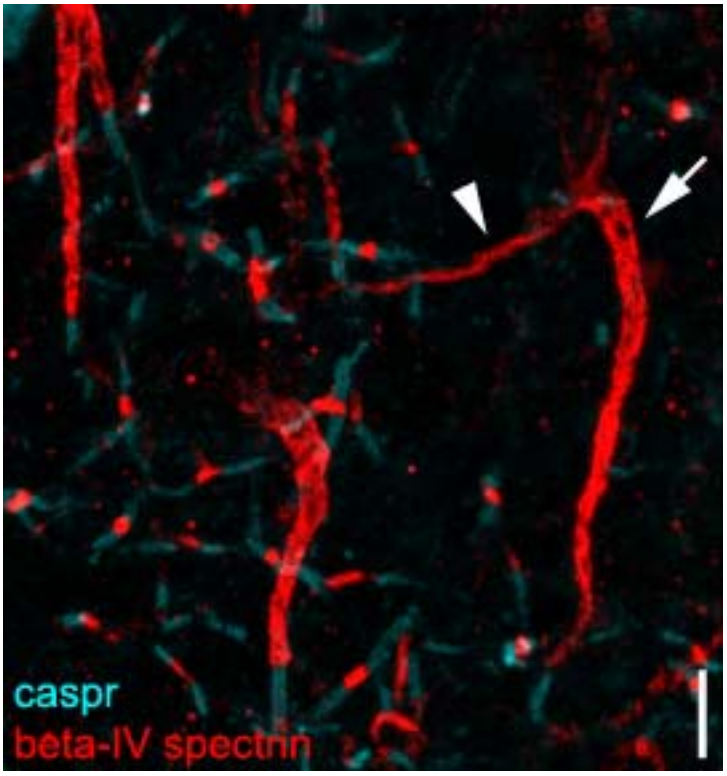


**Figure S2.** Supernumerary AIS in peri-infarct cortex. The increase in AIS number in peri-infarct cortex may, in part, be secondary to supernumerary AIS formation from surviving neurons. In this example, one beta-IV spectrin-positive (red) AIS projects inferiorly towards the white matter (arrow), while 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


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