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Axon sprouting in adult mouse spinal cord after motor cortex stroke

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ABSTRACT

Functional reorganization of brain cortical areas occurs following stroke in humans, and many instances of this plasticity are associated with recovery of function. Rodent studies have shown that following a cortical stroke, neurons in uninjured areas of the brain are capable of sprouting new axons into areas previously innervated by injured cortex. The pattern and extent of structural plasticity depend on the species, experimental model, and lesion localization. In this study, we examined the pattern of axon sprouting in spinal cord after a localized lesion which selectively targeted the primary motor cortex in adult mice. We subjected mice to a stereotaxic-guided photothrombotic stroke of the left motor cortex, followed 2 weeks later by an injection of the neuronal tracer biotinylated dextran amine (BDA) into the uninjured right motor cortex. BDA-positive axons originating from the uninjured motor cortex were increased in the gray matter of the right cervical spinal cord in stroke mice, compared to sham control mice. These results show that axon sprouting can occur in the spinal cord of adult wild-type mice after a localized stroke in motor cortex.

Stroke is one of the leading causes of long-term disability in the United States [32]. Fortunately, many stroke patients experience some degree of recovery. One mechanism thought to underlie recovery is axon sprouting and the formation of new axon pathways. Imaging studies have shown reorganization of cortical areas following brain injury in humans, including stroke (reviewed in [9]), and in many cases this plasticity is correlated with recovery (reviewed in [30]). Recovery after motor cortex injury is of particular interest because this region is commonly affected in human strokes, and often results in devastating functional deficits related to impaired control of the contralateral face, arm, and leg [24]. While it is possible that changes in activation patterns seen with MRI, PET, and transcranial magnetic stimulation after stroke could be due solely to changes in existing synapses, neuronal tracer studies in rodents indicate that new anatomical connections are formed after stroke.

There is considerable evidence from rat models that axon sprouting can occur in untreated animals after stroke. Stroke in sensorimotor cortex induced sprouting of axons from contralateral cortical neurons into the peri-infarct cortex and underlying denervated striatum [6,25,39]. Liu et al. [21] showed that rat middle cerebral artery occlusion (MCAo) was followed by sprouting of axons from the uninfarcted hemisphere into denervated spinal cord, even in untreated control rats. The MCAo model injured both cortical and subcortical structures, and it is not known whether a lesion confined to cortex alone would be sufficient to trigger sprouting in spinal cord. These studies demonstrate unique patterns of distal axon sprouting after different types of ischemic lesions in rats.

While most experimental stroke studies have been performed in rats, mouse models provide opportunities for studying recovery mechanisms in transgenic animals. Mouse stroke models are more technically challenging and fewer studies of post-stroke axon sprouting are available. Riban and Chesselet [29] observed sprouting in mouse striatum after a focal lesion in the ipsilateral sensorimotor cortex. Lee et al. [16] described sprouting in spinal cord of Nogo-A/B or Nogo receptor heterozygous mice after a photothrombosis lesion.

We set out to assess whether spontaneous spinal axon sprouting also occurs in wild-type mice, after a focal ischemic lesion anatomically targeted to primary motor cortex. The rodent MCAo model may induce considerable variability in infarct sizes, and does not consistently injure the primary motor area (unpublished observations and [3,22,31,36,38]). Therefore, we used a photothrombosis model, in which transcranial illumination by a stereotaxic fiber optic probe activates a systemically administered dye, rose bengal, triggering intravascular coagulation. Some of this work has been presented in abstract form [15].

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For this study, 10 adult male C57Bl/6 mice (14 weeks old, Jackson Laboratory, Bar Harbor, ME) were used: five underwent stroke surgery, and five underwent sham surgery. One stroke mouse was excluded because the tracer injection did not label layers II/III through V, one stroke mouse was excluded because the lesion did not completely injure layer V, and one sham mouse died. In total, 7 mice were analyzed for sprouting in spinal cord (4 sham and 3 stroke mice). All procedures involving vertebrate animals were performed in accordance with the Institute of Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals, and procedures were approved by the Institutional Animal Studies Committee at Washington University School of Medicine.

The photothrombotic stroke model used was based on that of Watson et al. [40] and Schroeter et al. [34]. Mice were anesthetized with 1.75% isoflurane in room air and body temperature maintained at 37.0 ± 0.5 °C. The animal was positioned in a three-point fixation stereotaxic frame and a midline incision was made in the scalp. One end of a 1.0 m long fiber optic cable with 2 SMA-type ends and a 1.5 mm diameter (0.37 numerical aperture) core (BFH37-1500, Thorlabs Inc., Newton, NJ) was attached to a lamp housing (Fostec) with EKE-type halogen bulb (150 W) and the other end was attached to the stereotaxic frame. A hot mirror (passes ~375–750 nm; Part 21002b; Chroma Technology Corp., Rockingham, VT) was placed within the path of light to reflect ultra-violet and infrared light and reduce the amount of heat delivered to the brain. After placing the fiber optic (with light off) on the skull directly above the left motor cortex (coordinates: anterior/posterior (a/p): +1.1 mm, medial/lateral (m/l): +1.5 mm; [20,27]), the mouse received an intraperitoneal injection of 200 μL of 20 mg/mL rose bengal dye (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein sodium salt; Sigma, St. Louis, MO) in phosphate buffered saline (PBS). After 1 min, the lamp was turned on and skull overlying the motor cortex was illuminated for 15 min. Sham mice were treated the same, except the lamp was not turned on.

The light power was adjusted prior to placement of the fiber using a LaserCheck light meter (Coherent Inc., Santa Clara, CA) calibrated to the wavelength corresponding to the maximum absorption peak of the rose bengal dye (562 nm) [4]. For these experiments, the lamp was set to provide 50 mW output light intensity, with a light fluence of 2.8 W/cm^2 and total exposed energy density of 2.52 kJ/cm^2. The effective photothrombotic dose is proportional to the fluence within the rose bengal dye absorption band, which can be approximated by normalizing the full fluence by the ratio (0.08) of the spectral width of the rose bengal absorption (30 nm) [14] to the spectral width of the illumination light (375 nm). Using this normalization the effective light levels are a fluence of 224 mW/cm^2 and a total dose of 202 J/cm^2.

Two weeks after photothrombosis, mice were anesthetized as described above and placed in the stereotaxic frame. A 1 mm burr hole was made in the skull above the right motor cortex (a/p: +1.1 mm, m/l: −1.5 mm, dorsal ventral (d/v): −1.6 mm [20,27]. A 5 μL Hamilton microsyringe with a 33 gauge needle with 45° tip (Hamilton Co., Reno, NV) was controlled by a nanoinjector pump (Stoelting Co., Wood Dale, IL) and used to inject 1.5 μL of 10% biotinylated dextran amine (10,000 MW BDA; Molecular Probes, Carlsbad, CA) in PBS at 0.15 μL/min. The needle was withdrawn 5 min post-infusion.

Mice were sacrificed 2 weeks post-injection of BDA (4 weeks after photothrombosis). Mice were anesthetized with ketamine and xylazine then transcardially perfused with PBS followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hartfield, PA) in 0.1 M phosphate buffer. Brain and spinal column were fixed overnight in 4% paraformaldehyde. The next day, the spinal cords were dissected out of the vertebrae and brains and spinal cords were separately coded to blind the experimenters to the experimental group. Tissue was cryoprotected through graded concentrations of sucrose, embedded in OCT (Sakura Finetek U.S.A., Inc., Torrance, CA) and frozen. Tissue was sectioned coronally at 16 μm on a cryostat microtome. Every 12th brain section was stained with cresyl violet Nissl stain to visualize infarcts. Briefly, frozen sections were rinsed in water, incubated for 10 min in 0.25% cresyl violet acetate solution (pH 3.6), rinsed in water, incubated in 95% ethanol, 100% ethanol, xylenes, then coverslipped with Permount (Fisher Scientific). Every 50th cervical spinal cord section was incubated as follows to visualize the BDA: 3% hydrogen peroxide in 90% methanol to block endogenous peroxidases, streptavidin–HRP (horseradish-peroxidase; diluted 1:500; PerkinElmer) followed by 0.05% 3,3′-diaminobenzidine tetrahydrochloride, dihydrate (DAB), and 0.003% hydrogen peroxide.

Digital photographs of tissue were taken using a light microscope (Nikon TE300) and Spot Camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI). Images of Nissl-stained brain sections (every 12th section) were taken as montages with a 4× objective and infarct area in the left hemisphere was measured with Metamorph Imaging software.

Montage digital photographs of the cervical spinal cord (every 50th section, 6–11 sections per mouse) were taken with a 10× objective. Experimenters were further blinded from whether images were from the same mouse. BDA$^+$ axon length (in μm) was quantified in the right and left gray matter and dorsal corticospinal tract (dCST) using Metamorph Imaging software. In addition, a thresholding function was used to highlight BDA$^+$ pixels and measure total BDA$^+$ surface area (in μm^2) in right and left cervical dCST. To account for possible variability in injection volume between animals, the amount of BDA label in each section was expressed as a ratio: right/left (ipsilateral to injection/contralateral). The ratios were averaged for each animal and then sham and stroke groups were compared using a Student's *t*-test. Errors are standard error of the mean.

At 35 days after photothrombotic stroke, ischemic lesions were visible in an anatomical area corresponding to forelimb motor cortex [20,27] (Fig. 1). Sham-treated brains did not show any signs of injury (Fig. 1). Nissl staining showed infarcts with a maximum width of 1.2 ± 0.2 mm (medial to lateral) (Fig. 1B) and an average length of 1.2 ± 0.2 mm (anterior to posterior; Fig. 1C). Therefore, the average infarct size was slightly smaller than the diameter of the 1.5 mm fiber optic. The lesion reached through at least layer V in the 3 stroke mice analyzed. The photothrombosis lesions resulted in a cystic core surrounded by increased cellularity as identified by a nuclear stain (data not shown) or Nissl stain (Fig. 1B). Many of these cells were Iba-1-positive microglia (data not shown).

The neuronal tracer BDA typically labeled cells throughout all layers of the cortex and the BDA injections did not appear to spread to the underlying white matter. All mice analyzed had BDA-labeled cell bodies in layer V that were located at the same anterior–posterior level as the lesion.

Previous studies have shown post-stroke axon sprouting after cortical lesions in spinal cord of rats [21] and genetically modified mice [16], so we asked whether spinal axon sprouting also occurs in wild-type mice. Four weeks after photothrombosis of left motor cortex and 2 weeks after injection of BDA into right motor cortex, BDA$^+$ axon length was measured in the right and left halves of cervical spinal cord gray matter. In shams, neurons labeled with BDA in the right motor cortex sent axons that projected mainly through the left dCST, with just a few axon fragments visible in right dCST (Fig. 2A and C). Axons in sham animals projected to the gray matter within the cervical spinal cord, mainly to the left half of the spinal cord (Fig. 2A and C). Very few BDA$^+$ axons were seen at thoracic levels of spinal cord (data not shown). The average BDA$^+$