Published in final edited form as:

Exp Neurol. 2012 February ; 233(2): 662–673. doi:10.1016/j.expneurol.2011.09.019.

A re-assessment of the effects of intracortical delivery of inosine on transmidline growth of corticospinal tract axons after unilateral lesions of the medullary pyramid

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Abstract

This study was undertaken as part of the NIH “Facilities of Research Excellence-Spinal Cord Injury”, which supports independent replication of published studies. Here, we repeat an experiment reporting that intracortical delivery of inosine promoted trans-midline growth of corticospinal tract (CST) axons in the spinal cord after unilateral injury to the medullary pyramid. Rats received unilateral transections of the medullary pyramid and 1 day later, a cannula assembly was implanted into the sensorimotor cortex contralateral to the pyramidotomy to deliver either inosine or vehicle. The cannula assembly was attached to an osmotic minipump that was implanted sub-cutaneously. Seventeen or 18 days post-injury, the CST was traced by making multiple injections of miniruby-BDA into the sensorimotor cortex. Rats were killed for tract tracing 14 days after the BDA injections. Sections through the cervical spinal cord were stained for BDA and immunostained for GAP43 and GFAP. Our results revealed no evidence for enhanced growth of CST axons across the midline of the dorsal column in rats that received intracortical infusion of inosine. Possible reasons for the failure to replicate are discussed.

Keywords

CNS injury; Spinal cord injury; Biotinylated dextran amine; BDA; Pyramidal tract; Corticospinal tract; Sprouting; Plasticity; Lesions; Medullary pyramid; Regeneration; Rat

Introduction

In 2003, the National Institute of Neurological Disorders and Stroke launched a program called “Facilities of Research Excellence-Spinal Cord Injury” which supports independent replication of published studies. Here, we repeat an experiment reporting that intracortical delivery of inosine promoted transmidline growth of corticospinal tract axons after unilateral injury to the medullary pyramid (Benowitz et al., 1999).

There is great interest in promoting regenerative growth of the CST after brain and spinal cord injury. In people, the CST is the primary pathway responsible for the control of voluntary motor function, especially learned and skilled functions involving the upper

extremity. It is thought that even minor amounts of regenerative growth of CST axons could contribute to recovery of motor function.

The report of Benowitz et al. described robust growth of CST axons across the midline in the dorsal column after unilateral destruction of the medullary pyramid. Trans-midline growth was seen at mid-cervical levels, but not at other levels. The growth was somewhat surprising because the growth occurred in white matter (the dorsal column) and appeared to involve extension of CST axons across the glial boundary at the midline.

Growth of new re-crossing CST connections is of considerable potential importance because if the axons form functional connections, this could restore CST input to the side of the spinal cord that had lost its input due to the pyramidotomy. It is well known that one side of the cortex can, under some circumstances, take over the functions normally mediated by the opposite side, and the enhanced transmidline sprouting described by Benowitz would represent a physical substrate for mediating motor recovery.

Growth that leads to formation of new crossing connections could be of particular functional importance following unilateral brain injuries, such as occur with strokes. Accordingly, the original report motivated follow-up studies to assess whether inosine delivery would promote CST growth and recovery of motor function after experimental stroke in rats (Chen et al., 2002). Chen et al. reported that inosine-treated rats exhibited the same sort of trans-midline sprouting in the dorsal column at the cervical as seen in the original study, as well as enhanced transmidline crossing at the level of the red nucleus. Inosine-treated rats also showed enhanced recovery of motor function in several tasks. A more recent study in the stroke model assessed the effects of inosine alone, and inosine combined with a Nogo receptor blocking peptide (NEP1-40 (Zai et al., 2011)). This study reported that both inosine and NEP1-40 and to an even greater extent the combination, led to an increased number of labeled CST axons in the dorsal column opposite to the main tract (as in the original study), an increased number and length of CST axon arbors in the gray matter, and enhanced recovery of motor function.

One potentially important technical difference between the original and follow-up studies is the positioning of the intracranial cannula for delivery of inosine. In the original study, the cannula tip was reported to be at a depth of 1.5 mm, which would place the tip in the middle of the sensorimotor cortex. In the first stroke study (Chen et al., 2002), the cannula tip was reported to be in the cisterna magna (coordinates are not given). In the latest paper (Zai et al., 2011) the cannula tip was reported to be in the cerebral ventricle (depth in mm is not reported). None of these studies including the original provided histological verification of the position of the cannula tip, however.

The extension of CST axons across the midline in the dorsal column described by Benowitz et al., 1999 remains somewhat surprising given the inhibitory nature of white matter and the fact that axons would have to cross the midline glial boundary. Accordingly, our goal here was to repeat the original study with as much fidelity as possible, and focus especially on the midline crossing. We report, however, that inosine-treated rats did not exhibit the transmidline sprouting reported in the original study. Possible reasons for the failure to replicate are discussed.

Materials and methods

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Experimental animals were male Sprague–Dawley rats from Charles River Laboratory that weighed 250–300 g at the start of the experiment.

Pyramidotomy surgery

Our research team had no prior experience with pyramidotomy surgery. We contacted Dr. Benowitz, but his laboratory had not done pyramidotomy surgeries for several years and had no active IACUC protocol. Thus, we asked Dr. Ken Kadota from UCSD to come to our Center to provide a training session on the surgery, which he kindly agreed to do.

The overall replication has involved 5 rounds of surgery described in detail below and summarized in Table 1, the first round of which was in November 2010. As explained further in the text, there was attrition during the surgery mainly due to respiratory complications resulting from retraction of the trachea. Also, after the first round of experiments had commenced, discussions with the original authors revealed that there was some uncertainty about the exact position intra-cranial cannula in the original study. The methods of the original paper reported that the tip of the cannula was 1.5 mm deep to the cortical surface whereas the investigators involved in the original study recalled that the depth was 3.5 mm, which would have placed the tip of the cannula in the ventricle. Because of this uncertainty, a second round of surgeries was carried out in April 2011, as described further below.

Round 1, November 2010

For the pyramidotomy surgery, rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg). The fur on the ventral side of the neck was shaved and the area was washed with Betadine. A midline incision was made, and the muscle overlying the trachea was bluntly dissected. The blood vessels penetrating the larynx on the right side were cauterized, and the trachea was retracted. Blunt dissection was continued to the skull base. A small section of the skull base was removed with rongeurs to expose the basilar artery and extending at least 2 mm laterally. The dura mater was cut with a 30-gauge syringe needle to expose the medullary pyramid. The right pyramid was transected to a depth of approximately 0.5 mm either using a microknife or microscissors (the microscissors were much more satisfactory as discussed further below). The transections extended from just adjacent to the basilar artery to about 1.2 mm lateral. After the transection, a small amount of Avitene (Davol, Warwick, RI; product number: 1010090) was placed in the craniotomy site. The skin was closed with silk suture. After the completion of the surgery, rats were kept on a water-jacket heating pad in a cage with fresh bedding until they recovered consciousness. Once they were fully recovered, rats were housed singly. The reason is that when rats with cannula assemblies are housed together, there is a risk that they may chew each other's cannula tubing.

On our first independent surgery session on 11/22/2010, we initially attempted to transect the medullary pyramid using a microknife as described in Benowitz et al. Of the 16 rats operated on the first surgical day, the operative procedure for 4 rats was terminated prior to completion of the lesion due to excessive bleeding (2 rats) or damage to the brainstem during removal of the bone (2 rats). Surprisingly, 3 of the rats with brainstem injuries survived without noticeable deficits. Four rats died as a consequence of respiratory distress after the trachea was retracted but before completion of the lesion (animals suddenly stopped breathing and could not be resuscitated), and 4 rats died within minutes after the transection of the pyramid (again, animals stopped breathing and could not be resuscitated). Overall, only 2 rats survived the transection with the microknife.

We noticed that there was considerable movement of the brainstem as the microknife was drawn through the pyramid. We speculated that the movement of the brainstem might be the cause of the sudden respiratory arrest after the transection. Accordingly, in 2 rats, the transection was carried out with microscissors (FST, cat# 15005-08). This was the

procedure used by Dr. Kadoya during his training session. Both of the rats that received lesions with microscissors survived. Thus, overall success rate on the first day of surgery was 4/17. The 4 rats that survived transections in the first surgery session did not receive cannula implants but did receive BDA injections 18 days post-lesion.

The second surgery session on 11/23/2010 involved a total of 13 rats. All transections were done with microscissors. The surgery on 2 rats was terminated prior to completion of the lesion due to excessive bleeding (one of these rats survived; one died), and 2 rats died from anesthetic complications. All 9 rats that survived successful transections in the second surgery session were alert and appeared healthy when they were examined early in the morning after the surgery; these rats received cannula implants on the day following the pyramidotomy.

A third round of pyramidotomy surgeries involving 20 rats was carried out on 12/14/2011. Six rats died as a consequence of respiratory distress after the trachea was retracted but before completion of the lesion; one rat died as a result of uncontrolled hemorrhage. All rats that survived the surgery were alert and appeared healthy when they were examined early in the morning after the surgery except for one rat that exhibited symptoms of damage to the olivary nucleus (rolling toward the side of the lesion). Nevertheless, one rat died suddenly of unknown causes in the later morning prior to cannula implantation.

Cannula and mini-pump implantation

Mini-pumps were filled with a syringe with either 10 mM Inosine (Sigma-Aldrich, product number: I4125) in 1×PBS, or the vehicle only. Pumps and catheters were primed by soaking in saline overnight.

On the day following the pyramidotomy, rats were re-anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) for the surgeries on 11/24/2010 or 3–4% isoflurane for implantation surgery on 12/15. The fur on the head was shaved and the scalp was washed with Betadyne and placed in a stereotaxic apparatus. The scalp was incised at the midline, and a burr hole was placed at the point of cannula insertion (0.8 mm posterior to bregma, 2.2 mm lateral on the left side, contralateral to the pyramidotomy). The cannulae from brain infusion kits (Alzet Osmotic; Durect Corp, Palo Alto; product number: 8663) were assembled with 4 spacers so as to leave approximately 2.5 mm of exposed cannula. The assembly was inserted into the skull using a microelectrode holder, and glued in place using cyano acrylate (Alzet Osmotic; Durect Corp, Palo Alto; product number 8670). The mini-pump was inserted beneath the skin of the neck into the region of the back between the shoulders. The incision was closed with silk sutures, and rats were kept on a water jacketed heating pad in a cage with fresh bedding until they recovered consciousness.

All rats recovered from the pump implantations with minimal impairments, but one of the rats was perfused early due to 22% weight loss 8 days post injury, one rat developed a skin lesion in the genital area (treated with antibiotics) and one rat was found dead in the morning of the 13th day post-injury. This was over the weekend, and the rat appeared healthy on the Friday before death.

BDA injections

Injections to trace CST axons from the side opposite to the original pyramidotomy were done 17 or 18 days post lesion on 12/09/10 and 12/10/10. Rats were anesthetized as above, the scalp was opened around the implanted cannula, and the cannula was detached by inserting a scalpel blade under the edge of the cannula and applying lever action by turning the blade. The cortex overlying the area to be injected was exposed by removing the skull with a dental drill. BDA injections were made at 11 sites, delivering 70 nl of BDA at 3

depths per site (0.5, 1.0, and 1.5 mm deep to the cortical surface). Coordinates for the sites in mm were: 3.8 L/1.2A, 3.4 L/0.7A, 3.4 L/0.2A, 3.6 L/0.3P, 3.2 L/0.8P, 3.4 L/1.3P, 2.2 L/1/3P, 2.2 L/1.8P, 2.2 L/2.3P, 2.2 L/2.8P, and 3.0 L/3.3P. A/P coordinates are with respect to Bregma.

Each 70 nl injection was made over a 1-minute period, so the time required to inject each site was approximately 4–5 min. The total time required to complete the series of injections in each animal was about 45 min. After completion of the injections, the scalp was sutured.

Animal care

Following each surgery, the rats were immediately placed on a water circulating jacketed heating pad. After recovering from the anesthetic, animals were housed individually. The analgesic Buprenex (Buprenorphine, 0.01 mg/kg) was given prior to surgery and then Caprofen (0.04 mg/kg) was given via their water bottles 48 h post surgery. Rats received lactated ringers (5 mg/100 g, sub-cutaneously) for hydration for 3–7 days. Rats also received Baytril (Enrofloxacin 2.5 mg/kg, subcutaneously) for 3–7 days for prophylaxis against urinary tract infections (UTI's).

Animals were housed individually with food on the bottom of their cages and long sipper tubes to access the water. Rats were monitored twice daily for general health, coat quality (indicative of normal grooming activity) and mobility within the cage. In addition, signs of paralysis were monitored, including lack of forelimb movement, tail flaccidity, and instability/uncoordinated movement. Animals were also monitored for signs of skin lesions on the limbs or autophagia of the toes. None of the animals exhibited autophagia throughout the experiment. Bladders were manually expressed for the 4 days following dorsal column lesion. Animals were monitored for urinary tract infections (UTI's) for the entire duration of the experiment; no UTI's were observed. Rats were weighed prior to injury and at 4 days post-injury as a measure of general health.

Round 2, April 2011

After the experiment was underway, continuing discussions with Dr. Benowitz revealed that there was some uncertainty in his group about the coordinates for the cannula in the original study. In current studies in the Benowitz lab, two spacers are used for the cannula assembly so that the cannula tip is positioned at a depth of approximately 3.5 mm. Also, the individual who actually did the implantation surgery for the original study believed that the implants had been done with two spacers. This is in contrast to what is stated in the Methods of the original paper, where the cannula tip was reported to be 1.5 mm deep to the cortical surface, which would require the use of 4 spacers. Accordingly, a second round of experiments was initiated in which the cannula was prepared with 2 spacers so as to position the tip at 3.5 mm deep to the cortical surface.

Pyramidotomy surgery

On 04/05/11, all transections were done with microscissors. Ten out of 22 rats survived transections. Five rats died shortly after the trachea was retracted to expose the skull base (animals suddenly stopped breathing and could not be resuscitated). The surgery on 2 rats was terminated prior to completion of the lesion due to surgical errors (damage to the brainstem as the bone at the skull base was being removed), 2 rats died during surgery due to excessive bleeding, 2 rats died post lesion, and 1 rat died from anesthetic complication. Two of these rats exhibited uncontrolled rolling when they recovered from the anesthetic, likely due to injury to the olivary nucleus.

The 10 surviving rats with successful transections received cannula implants on the day following the pyramidotomy (4/6/2011). The mini-pumps were prepared as described previously. In contrast to Round 1, however, 2 spacers were used rather than 4 so that the depth of the cannula would be 3.5 mm rather than 1.5 mm. 2 animals died post cannula implantation, leaving a total of 8 rats (all inosine-treated).

Eight additional rats were prepared for pyramidotomy surgery on 04/06/11; all transections were done with microscissors. Of the total of 8 rats, 1 died immediately after receiving the anesthetic, 1 died during surgery due to surgical error (damage to the brainstem during bone removal), 2 additional rats suffered bleeding during bone removal. The bleeding was successfully stopped, but the pyramidotomy surgery was terminated (so these are sham pyramidotomies). These two rats later received cannula placements for inosine delivery and then received BDA injections. Thus, 4/8 rats survived pyramidotomy surgery.

Cannula implants

8 rats that received lesions on 04/05 and 6 rats that received lesions or sham surgeries on 4/6 received cannula implants on the day following the pyramidotomy (04/06 or 04/07 respectively). Of the 8 rats that received cannula implants on 04/06, 2 died during the cannula implantation surgery due to anesthetic complications. All of the rats that received cannula implants on 04/07 survived the surgery, but the 2 rats that were exhibiting uncontrolled rolling were later euthanized when the rolling behavior did not subside and they began to exhibit deteriorating health.

Tract tracing surgery

Surviving rats with pyramidotomies (n=10) and the two rats with sham surgery received BDA injections into the sensorimotor cortex as above 16 days post-lesion (4/21/11 and 4/22/11). During the tract tracing surgeries, two animals from group 4 that received their pyramidotomies on 04/05 died during the surgery due to anesthetic complications. One of these animals had experienced significant weight loss after the pyramidotomy (24% of original body weight).

After attrition due to all causes, 8 rats that had been treated with inosine and 2 rats with sham surgeries that received inosine were euthanized on May 5 and 6, 2011, and prepared for histology as above.

Perfusions and histology

Animals received a lethal dose of Euthasol (100 mg/kg) and were perfused transcardially with 4% paraformaldehyde 2 weeks after BDA injections. The brain and spinal cord were removed and stored in 4% paraformaldehyde. Brains and spinal cords were cryoprotected in 27% sucrose overnight, embedded in OCT, then flash frozen. Transverse sections throughout the rostro-caudal extent of the spinal cord were taken at 20 μm with a cryostat. Brains were sectioned in the coronal plane at 20 μm .

One series of transverse sections through the spinal cord taken every 500 μm was stained for BDA with avidin-biotin-peroxidase complex (ABC). A series of sections at 500 μm intervals through the cervical enlargement was stained for BDA using Cy3 amplification followed by immunostaining for GFAP. A third series was stained for GAP-43. Selected sections (3 per rat) were stained using the Fink–Heimer technique (Fink and Heimer, 1967). One cryostat section from the mid-cervical region of each rat in Round 1 was embedded in plastic, sectioned at 1 μm and stained for toluidine blue. Sections through the brainstem (one every 500 μm) were stained with hematoxylin and eosin (H&E) and Luxol Fast Blue. Sections through the forebrain (one every 500 μm) were stained for BDA (ABC), and

selected sections through the region of the cortex that contained the track of the cannula were stained with Cresyl violet.

For BDA staining with avidin-biotin-peroxidase, sections were washed in PBS with 0.1% Triton X-100 three times then incubated overnight in avidin-biotin-peroxidase complex (Vector Laboratories, PK-6100) in PBS with 0.1% Triton X-100 at room temperature. The following day, sections were washed in PBS three times, then stained in nickel enhanced diaminobenzidine solution (Vector Laboratories, SK-4100) 25 min. Sections were washed in PBS then mounted on gelatin-subbed slides, air dried, dehydrated, cleared, then coverslipped.

For BDA staining with Cy3 amplification and GFAP, sections were washed in PBS, quenched in 1% hydrogen peroxide in PBS 15 min, washed 3 times in PBS, then incubated overnight in 1:1000 anti GFAP antibody (Dako Z0334) in PBS with 5% NGS. The following day, sections were washed in PBS 3 times, incubated in 1:250 dilution goat anti-rabbit Alexafluor488 conjugated antibody in PBS with 5% NGS 2 h and washed again in PBS 3 times. To detect the BDA, the sections were then incubated in 1:200 dilution streptavidin-HRP (Perkin Elmer NEL75000-1ea) in PBS 2 h, washed 3 times in PBS, then reacted with TSA-Cy3 (1:100) in the supplied amplification diluent (Perkin Elmer SAT704A001ea). Sections were washed 2 times in PBS, then mounted on gelatin subbed slides, and coverslipped with Vectashield (Vector labs H-1000).

For immunostaining for GAP43, spinal cord sections were washed in PBS three times, blocked in PBS with 5% normal goat serum 1–2 h, and then incubated in 1:2500 dilution rabbit-anti-GAP-43 in PBS with 5% normal goat serum overnight at room temperature. The following day, sections were washed in PBS three times, incubated in 1:250 dilution goat anti-rabbit Alexafluor 488 conjugated antibody (Invitrogen, Molecular Probes, A-11034) 2 hours, then washed in PBS three times. Sections were then mounted on gelatin-subbed slides, air-dried, then coverslipped with Kaiser's glycerol jelly mounting medium.

Selected sections (3–4 per case) through the cervical region of the spinal cord were stained for degenerating axons using the Fink–Heimer technique (1967) using the Pope–Giolli modification. Briefly, sections were washed, immersed in 0.05% calcium hydroxide for 5 min, washed, immersed in 0.5% nitric acid for 5 min, washed, then immersed in 10% uranyl nitrate for 4 min, washed, then immersed in 2.5% silver nitrate for 10 min, washed, then immersed in freshly made ammoniacal silver (2.6% silver nitrate; 28% ethanol; 0.1% strong ammonia; and 0.1% sodium hydroxide solution) for 4.5 min. Sections were transferred to Nauta-Gygax reducer (0.03% citric acid; 1.05% formaldehyde; 0.94% ethanol solution) for 4 min, washed in water, and then were mounted on gelatin-subbed slides, dried, then coverslipped with DPX.

Quantitative assessment of the extent of damage to the pyramid

To determine the extent of the damage to the medullary pyramid, Luxol fast blue sections were examined to identify the section in which the lesion was at its maximal extent. Images were captured and the area of the uninjured left pyramid was calculated. If there was a spared portion of the right pyramid, the spared area was used to calculate the % lesion (see Table 1).

Quantitative analysis of BDA labeled CST axons

BDA labeled axons in the dorsal CST ipsilateral to the cortex of origin (left side) were counted in five sections taken at 2 mm intervals centered on the cervical enlargement. Sections were viewed on an Olympus Provis AX-80T using a 40× objective. Counts ranged from 0 to 42. To count labeled axons in the densely labeled dCST contralateral to the cortex

of origin (right side), the most rostral of the 5 sections was digitally imaged using a 40× objective to create multiple overlapping images, focusing on either the top or bottom edge of the section. In this way, the cut tip of the labeled axon passing through the section can be visualized. The images were stitched into a montage with Adobe Photoshop CS5 v.12 software and labeled axon tips were manually counted with Photoshop's counting tool (counts ranged from 682–3130).

Results

Round 1: Cannula placed 1.5 mm deep to the cortical surface (4 spacers in the cannula headstage)

Lesion verification—Examination of H&E and Luxol fast blue-stained sections through the medulla revealed that in most rats, the lesion extended from near the ventral midline to approximately 1.5 mm lateral to a depth of about 400–500 μm , completely transecting the pyramid on the right side. Fig. 1A illustrates a section taken at the level of the lesion in a rat with a complete lesion. In 13/28 rats, there was some sparing of axons in the pyramid (Fig. 1B, arrow with asterisk). There was a varying degree of additional damage to the medullary tegmentum in the different rats. Some rats exhibited a narrow lesion that extended dorsally near the midline on the right side with a distribution that suggested an ischemic lesion due to interruption of penetrating blood vessels from the basilar artery (Fig. 1C).

To quantitatively assess the extent of the lesion in the different rats, the area of the pyramid on each side in the section with the largest lesion was measured, and the percent damage was calculated (Table 2). In considering lesion extent, however, it is important to recall that we stained sections every 500 μm , and thus may not have the section in which the lesion is maximal.

Degeneration of CST axons was documented by assessing toluidine-blue stained thin sections through the cervical enlargement and by staining sections through the spinal cord using the Fink–Heimer technique.

In toluidine blue-stained thin sections through the cervical region, there was a clear line of demarcation between the intact dCST axons in the ventral part of the right dorsal column and degenerating axons on the left. Fig. 2A and C illustrates a section from a rat that received a pyramidotomy but no cannula implant (122410A #3). In this case, there were very few intact axons in the area of the dCST on the left side following the right pyramidotomy. In other rats, there were a few more non-degenerating axons on the left indicating partial sparing of the CST. For example, Fig. 2B and D illustrate a rat that received a pyramidotomy and saline infusion (122110 #22), in which intact axons can be seen amongst the debris of degenerating axons. Although there were some non-degenerating axons in the left CST in some cases, degenerating axons far outnumbered intact axons in all cases.

Fink–Heimer staining of sections through the cervical spinal cord revealed extensive degeneration of CST axons in the ventral part of the dorsal column contralateral to the pyramidotomy in all rats. An example is shown in Fig. 1D. There were also a few scattered degenerating axons in the CST ipsilateral to the pyramidotomy. These are likely due in part to damage to the cortex during BDA injections, and in part reflect the presence of a few ipsilaterally descending axons in the dCST.

There was evident shrinkage of the ventral part of the dorsal column on the left, due to Wallerian degeneration of CST axons (Fig. 2). Because of this shrinkage, the intact tract on the right bulged to the left, leading to a leftward shift of the midline glial boundary between

the left and right dCST (further documented below by GFAP staining). The long arrow in Fig. 2A and B extends from the central canal ventrally to the midline of the dorsal part of the dorsal column. At the level of the dCST, the midline is shifted to the left (indicated by short arrows). Shrinkage of the injured track, bulging of the intact tract and left shift of the midline glial boundary was seen in all rats with pyramidotomies, and represents a useful surrogate marker of CST degeneration.

As expected based on the coordinates, the cannula tip in Round 1 was at a depth of about 1.5–1.8 mm from the cortical surface. Fig. 3A illustrates a BDA stained coronal section through the cannula track in a case that received inosine. It is noteworthy that although one of the BDA injection sites was at the same coordinates as the cannula, BDA labeling in the area surrounding the cannula is not as heavy as at other injection sites. A nearby section stained for cresyl violet (Fig. 3B) revealed minimal cellular injury surrounding the cannula; indeed, healthy looking neurons were evident within 50 μ m of the cannula track.

Distribution of BDA-labeled axons from the intact CST following unilateral pyramidotomy—In control rats (sham pyramidotomy), BDA injections into the sensorimotor cortex led to labeling of large numbers of CST axons in the wedge-shaped ventral part of the dorsal column on the side contralateral to the injection (Fig. 4A). There were also a small number of labeled CST axons in the dorsal column ipsilateral to the cortex of origin. A few CST axons on the “wrong” side are typically seen in tract tracing experiments in uninjured rats. BDA labeled axons were also present in the dorsal part of the lateral column representing the dorsolateral CST (dlCST) and in the ventral column ipsilateral to the cortex of origin representing the ventral CST (vCST).

BDA-labeled axon arbors were also present in the gray matter of the spinal cord on the same side as the labeled tract (Fig. 4A). Axon arbor density was greatest in the dorsal horn, but labeled axons were also seen in the ventral horn, especially in the cervical enlargement. A few BDA labeled axons were also present in the gray matter on the side contralateral to the main labeled tract. Some of these had a trajectory suggesting that they originated from BDA labeled axons in the ventral CST that descends ipsilateral to the cortex of origin (not shown).

In control rats that received unilateral pyramidotomy and then saline infusions into the sensorimotor cortex followed by BDA injections, labeled CST axons were almost exclusively restricted to their normal location in the dorsal column contralateral to the injection (Fig. 4B). As in uninjured rats, a few BDA labeled axons were scattered in the dorsal column on the ipsilateral side.

Distribution of CST axons in rats that received inosine—Benowitz et al. (1999) reported that in rats treated with inosine, CST axons in the undamaged tract extended across the midline from their normal location in the dorsal column contralateral to the cortex of origin into the dorsal column that contained degenerating CST axons. This extension of CST axons across the midline was reported to be prominent throughout the cervical enlargement, but absent at high cervical levels and at thoracic levels and below.

In contrast to what was reported in Benowitz et al., however, none of the inosine-treated rats in our study exhibited this abnormal recrossing of CST axons. Instead, as in normal rats or the control rats in the present study treated with saline, labeled CST axons were almost exclusively restricted to the right dorsal column contralateral to the cortex of origin, except for the few axons sprinkled throughout the left dorsal column that are also seen in normal rats. Fig. 4C–F illustrates the pattern of labeling in 4 inosine-treated rats in Round 1.

Shrinkage of the ventral part of the right dorsal column containing the degenerating CST and bulging of the intact tract toward the left was evident in all cases. This raises the question, exactly where is the midline astroglial boundary between the degenerating and intact tracts? To address this question, sections were stained for BDA using Cy3 amplification to generate a red fluorescent signal and co-immunostained for GFAP to reveal the astroglial boundary at the midline. As illustrated in Fig. 5A, Cy3-labeled CST axons were restricted to the right dorsal column and did not extend beyond the GFAP stained astroglial boundary at the midline (arrows in Fig. 5B).

Benowitz et al. also report extension of GAP43-positive axons from the intact CST across the midline into the area containing degenerating CST axons. Fig. 6 illustrates an example of GAP43 staining from inosine-treated rats in the present study. Normal CST axons in uninjured animals are GAP43-positive, so in intact rats, the two sides of the dCST stain equivalently (Fig. 6A). This is the same case illustrated in Fig. 4A. Fig. 6B illustrates red fluorescence from BDA-miniruby in the same section. Unilateral degeneration of the CST after pyramidotomy leads to a profound loss of GAP43 staining on the injured side (Fig. 6C). Here again, bulging of the intact side due to shrinkage was evident. There was no evidence for extension of GAP43 positive axons across the midline into the degenerating tract, however, or evidence for extension of miniruby-labeled axons from the intact tract on the right (Fig. 6D).

Round 2: Cannula placed 3.5 mm deep to the cortical surface (2 spacers in the cannula headstage)

Because of the uncertainty regarding the placement of the cannula in the original study, we carried out a second round of experiments in which 2 spacers were used in the cannula headstage, which would put the cannula tip at a depth of approximately 3.5 mm. Sections through the cortex stained for BDA or cresyl violet revealed that the cannula extended through the cerebral cortex into the white matter and in some cases, into the cerebral ventricle (Fig. 3C and D).

Overall, the number of BDA labeled CST axons was somewhat greater in the cases prepared for Round 2. Fig. 7 illustrates 6 of the 8 cases. As in the inosine-treated cases from Round 1, BDA-labeled CST axons were restricted to the right dorsal column contralateral to the cortex of origin, except for a few axons scattered throughout the dorsal column ipsilateral to the injection. There did appear to be a larger number of BDA labeled CST axons on the ipsilateral side than in Round 1, however. Also, in two cases, some of the labeled axons on the ipsilateral side were very near the midline, and a few looked as if they might be crossing over from the opposite side.

If axons are extending across the midline, they ought to be seen at the midline only in the section in which they cross over. If, however, the axons are extending along the dorsal column, they will be seen in approximately the same position from section to section. To distinguish between these possibilities, we examined sections taken at 200 μm intervals along the rostro-caudal axis. Comparisons across sections revealed that BDA labeled axons could be seen in the separate sections in approximately the same location near the midline. Fig. 8 illustrates 2 sections separated by 800 μm . Although the number and location of BDA labeled axons in the different sections is not identical, they are similar. This suggests that the BDA labeled axons near the midline that appear to be crossing may be extending longitudinally along the dorsal column next to the midline.

As in the cases in Round 1, immunostaining for GAP43 revealed loss of staining on the denervated side, but again there was no evidence of the extensive re-crossing axons of CST axons reported by Benowitz et al. (Fig. 6E and F).

Quantitative analysis of BDA labeled axons—To obtain meaningful estimates of CST axon numbers in the (denervated) tract, it is important to control for the overall extent of labeling across cases. The best measure of overall labeling is the number of labeled axons in the main dCST. Table 2 illustrates the number of BDA labeled axons on the two sides in the different cases. Because there were no apparent differences in the distribution of labeled axons in Round 1 vs. Round 2, quantitative analyses were carried out by combining data sets.

Although the overall extent of labeling appeared qualitatively greater in the cases in Round 2, the counts of labeled axons revealed that this was more a matter of differences in staining intensity than differences in axon numbers. The mean number of labeled axons was 1154 ± 377 in Round 1 and 1779 ± 655 in Round 2.

One way to determine whether there are more axons crossing the midline in rats treated with inosine is to express the counts of BDA labeled axons in the denervated tract as a ratio of the number of axons in the main tract. In both groups, the number of axons in the denervated tract was less than 1% of the number in the main tract (see Table 2, I/C ratios). Averaging across cases in both Rounds 1 and 2, this ratio was virtually identical in controls vs. rats treated with inosine ($5.85 \times 10^{-3} \pm 4.52 \times 10^{-3}$ in controls vs. $8.40 \times 10^{-3} \pm 5.77 \times 10^{-3}$ in rats treated with inosine). The controls here include both rats with pyramidotomies treated with saline and rats without pyramidotomies. Considering only the cases in which the lesions of the pyramid were complete yields a similar conclusion ($5.07 \times 10^{-3} \pm 3.86 \times 10^{-3}$ in controls vs. $6.93 \times 10^{-3} \pm 4.77 \times 10^{-3}$ in rats treated with inosine).

The total number of labeled axons in the main tract ranged from 682 to 3130 (see Table 2). One potential caveat of a simple ratio is that there may be a floor or ceiling effect that obscures a difference between groups. To assess this, we plotted the data from the two sides in a scatterplot (Fig. 9). If there was a difference between groups in the relative number of labeled axons on the denervated side, the points from the inosine treated group should separate from the points from the controls. As illustrated in Fig. 9, the points in the scatterplot from inosine treated rats are co-distributed with points from controls.

Discussion

The present results revealed no evidence that intracortical delivery of inosine triggered extensive growth of CST axons across the astroglial boundary at the midline of the dorsal column following unilateral pyramidotomy, as reported by Benowitz et al. (1999). Growth of this sort is of great interest because it could restore CST input to the side of the spinal cord that had lost its normal CST input. This initial observation motivated subsequent studies in stroke models, which also reported enhanced CST growth and recovery of motor function with inosine treatment (Chen et al., 2002; Zai et al., 2011). Other studies have reported that inosine treatment also promoted motor recovery and CST sprouting after unilateral injury to the motor cortex (Smith et al., 2007).

It is important to note that the original paper reports trans-midline sprouting of CST axons in the white matter of the dorsal column, which would be remarkable because it involves growth in white matter and involves crossing the midline astroglial boundary. This is quite different than the growth of CST axons in the gray matter that is reported more commonly, including in the recent studies in a stroke model (Zai et al., 2011), and the study involving unilateral cortical injury (Smith et al., 2007). It is important to note that the present study did not assess formation of additional ipsilateral arbors in the gray matter.

There are no obvious technical explanations for our failure to detect the growth reported in Benowitz et al. In what follows, we consider some of the technical details of the present paper vs. Benowitz et al.

Experimental animals and experimental procedures

Both our study and Benowitz et al. used male Sprague–Dawley rats from Charles River Labs weighing 250–300 g at the start of the experiment. Although the strain and vendor is the same, it is conceivable that there has been genetic drift in the approximately 13 years separating the two studies. Housing conditions are not specified in Benowitz et al., but subsequent discussions with the authors revealed that their rats were housed 3 per cage. Here, rats were housed singly. The differences in housing conditions would provide different opportunities for social interactions and physical activity, which could affect sprouting of CST axons after injury.

In the study by Benowitz et al., rats were anesthetized for surgery by metofane inhalation following by i.p. sodium pentobarbital. Here, rats were anesthetized with ketamine/xylazine. Differences in anesthetic could affect secondary responses during the early post-injury period including secondary degeneration. There is no obvious reason why this would have major effects on CST growth responses, but the possibility cannot be excluded.

Lesions

The lesions in our study destroyed most of the medullary pyramid in all animals, but did spare a small portion of the pyramid in some animals. Lesions were not shown in Benowitz et al. (1999) and so it is not possible to directly their lesions with ours. In addition to differences in the extent of damage, it is also possible that the rostro-caudal location of the transection differed in the two studies.

BDA labeling

Tracer—The Methods section in Benowitz et al. indicates that BDA from Molecular Probes was used for tracing CST axons. The catalog number is not given, but this is presumably regular BDA. We used miniruby-BDA because miniruby fluorescence can be visualized without additional staining in sections that are prepared for immunofluorescence (as in Fig. 6). There is no obvious reason why this difference could account for our failure to detect the form of transmidline sprouting described by Benowitz et al., but it is important to fully consider possibilities.

Extent of labeling—BDA injections led to the labeling of many hundreds to thousands of CST axons in our cases. Comparisons with the images presented in Benowitz et al. suggest that the overall extent of labeling might have been greater in their experiments. This could be due to actual differences in the number of axons that were labeled, differences in section thickness (40 μm in Benowitz vs. 20 μm here), or differences in the staining procedures such that their sections were more darkly stained.

Labeling of axons from neurons that were affected by inosine—In both studies, multiple BDA injections were distributed throughout the sensorimotor cortex and were not targeted to the site of inosine delivery. Examination of the cannula track and BDA injection sites in the cortex reveals that in some cases, the area immediately surrounding the cannula was not heavily labeled with BDA. This raises a question: did BDA injections label the axons of cortical motoneurons that were affected by inosine? This question is impossible to answer because it is not known how or where inosine acts, or how far inosine diffuses through the cortex under the present conditions. One possibility that must be considered is that the spread of BDA miniruby is less than the spread of regular BDA. In this case, our

injections might have labeled fewer axons from neurons in the area of inosine delivery. Benowitz et al. do not illustrate BDA injection sites in the cortex, so it is not possible to directly compare the apparent spread of BDA in their experiments vs. miniruby BDA here.

Possibility of a BDA labeling artifact—We recently discovered that intracortical injections of BDA made on the same day as a spinal cord injury can lead to artifactual labeling of axons in the spinal cord (Steward et al., 2007). This labeling artifact likely accounts for at least some of discrepancy between different studies of Nogo knockout mice. Could there have been artifactual labeling in the original study that did not occur in the replication?

Although we did not fully define the mechanisms that led to artifactual labeling, we did identify the conditions that produced it. First, artifactual labeling was seen when BDA injections were made on the same day as the spinal cord injury, but not when injections were made 4 weeks post-injury. Intermediate time points were not examined. Second, artifactual labeling occurred as a result of BDA getting into the CSF. Third, artifactual labeling had a distinctly different appearance than bona fide labeling in that it appeared to stain the myelin sheath rather than the axon proper.

It seems unlikely that the BDA labeling artifact that we identified would have occurred in the original experiment by Benowitz et al. because BDA injections were made over 2 weeks after the lesion. Nevertheless, the possibility of artifactual labeling in the original study cannot be completely excluded.

Conclusion—The present results fail to confirm the report of Benowitz et al., that intracortical delivery of inosine following unilateral pyramidotomy triggered growth of corticospinal tract axons across the midline of the dorsal column. There were no obvious technical explanations for the differences in findings. We did not assess other forms of CST axon growth, for example formation of new trans-midline axon collaterals in the gray matter of the spinal cord, which has been reported in animal models of stroke following inosine treatment (Zai et al., 2011). Our findings are limited to a very specific type of post-lesion growth in a particular injury model, and should not be taken as evidence against a role for inosine treatment in improving function or enhancing axon growth after nervous system injury. Our results do imply, however, that additional studies are required to sort out the anatomical consequences of inosine treatment and define how these consequences relate to recovery of function.

Acknowledgments

Supported by HHSN271200800039C to O.S. Thanks to Ilse Sears Kraxberger, Ardi Gunawan, Laine Butler, and Jamie Mizufuka for technical assistance. Special thanks to Dr. Ken Kadoya, University of California San Diego, for instruction on the surgical methods.

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Fig. 1.

Examples of lesions of the medullary pyramid. A–C) H&E stained sections at the level of the lesion. Arrowheads indicate the intact pyramid on the left; arrows indicate the lesion on the right. A) Case in which lesion is complete. B) Case in which the medial part of the pyramid is spared. Arrow with asterisk indicates spared region; C) case in which there is a wedge-shaped infarct extending dorsally through the tegmentum. D) Fink–Heimer stained section through the dorsal horn. The degenerating axons are on the left. Scale bar in B=500 μm , and applies to A–C. Scale bar in D=100 μm .

Fig. 2.

Toluidine blue stained sections through the dorsal column at a mid-cervical level. Degenerating CST is on the left. A) Animal #3, complete lesion of pyramid. B) Animal #22, 72% lesion of the pyramid. C and D higher magnification views of midline junction between the CST on the left and right. The area normally occupied by CST axons on the left side contains degeneration debris characteristic of axons undergoing Wallerian degeneration. CST axons on the right are normal in appearance. The long line with arrowheads at each end extends between the central canal (cc) to the midline of the dorsal part of the dorsal column. Left pointing arrows indicate the shift of the midline at the level of the CST. Arrows in C and D indicate the midline. Scale bar in B=250 μm , and applies to A and B. Scale bar in D=50 μm and applies to C and D.

Fig. 3.

Cannula placement in the sensorimotor cortex. A) BDA stained coronal section through the cannula track in a case from Round 1 that received inosine. B) Nearby section stained for cresyl violet. Note healthy looking neurons within 50 μm of the cannula track. C) BDA stained section through the cannula track in a case from Round 2 that received inosine. D) Nearby section stained for cresyl violet. Scale bar=1 mm.

Fig. 4. Distribution of BDA-labeled CST axons in control vs. inosine-treated rats from Round 1. A) BDA-labeled CST axons in the dorsal column in a control rat (#75) that received a sham pyramidotomy. B) BDA-labeled CST axons in a rat that received unilateral pyramidotomy and then saline infusions into the sensorimotor cortex (#24). C–F) BDA labeled CST axons in 4 rats from Round 1 (#27, 29, 34, and 40) that received unilateral pyramidotomy and then inosine infusions into the sensorimotor cortex. Scale bar=100 μ m.

Fig. 5.

BDA-labeled CST axons do not cross the astroglial boundary at the midline. A) BDA-labeled CST axons revealed using Cy3 fluorescence amplification. B) Same section showing immunofluorescence for GFAP to reveal the astroglial boundary at the midline. Note that Cy3-labeled CST axons are restricted to the right dorsal column and did not extend beyond the GFAP stained astroglial boundary at the midline (arrows). Scale bar=100 μ m.

Fig. 6.

Immunostaining for GAP43. A) Immunofluorescence for GAP43 in the dorsal column in a control rat (#75) that received a sham pyramidotomy. B) BDA-labeled CST axons in the same section shown in A as revealed by miniruby fluorescence. C) Immunofluorescence for GAP43 in the dorsal column in a rat that received a unilateral pyramidotomy and then inosine infusions into the sensorimotor cortex (#29 from Round 1). Note loss of GAP43 staining on the injured side (arrows). D) BDA-labeled CST axons in the same section shown in C as revealed by miniruby fluorescence. E) Immunofluorescence for GAP43 in the dorsal column in a rat that received a unilateral pyramidotomy and then inosine infusions into the sensorimotor cortex (#69 from Round 2). Arrows indicate the area containing degenerating CST axons. F) BDA-labeled CST axons in the same section shown in E as revealed by miniruby fluorescence. Scale bar=100 μ m.

Fig. 7. Distribution of BDA-labeled CST axons in control vs. inosine-treated rats from Round 2. A–F) BDA labeled CST axons in 6 rats from Round 2 (#52, 57, 59, 66, 69, and 78) that received unilateral pyramidotomy and then inosine infusions into the sensorimotor cortex. Scale bar=100 μ m.

Fig. 8.

Comparison of the distribution of BDA labeled CST axons in the dorsal column ipsilateral to the cortex of origin in 2 sections separated by 800 μm . Arrows indicate groups of axons that appear to be extending across the midline; “n” refers to the number of axons in the group. The presence of what appears to be the same groups of axons in sections separated by 800 μm suggests that the axons are extending along the dorsal column near the midline rather than crossing over. Scale bar=100 μm .

Fig. 9.
Scatterplot of the relationship between the number of BDA labeled axons on the denervated side vs. the total number of BDA labeled axons in the main tract.

Table 1

The table lists all animals prepared for the study, the treatment delivered, date of surgery, pump implantation, BDA injection and euthanasia and depth of cannula.

Group ID	Pyramidotomy	Implantation of pumps	BDA	Euthanized	Final n	Treatment	Depth of cannula
Group 1	11/22/10	N/A	12/10/10	12/24/10	3	N/A	
Group 2a	11/23/10	11/24/11	12/09/10	12/23/10	4	Inosine=4, PBS=4	1.5 mm
Group 2b	11/23/10	11/24/11	12/10/10	12/24/10	4		
Group 3	12/14/10	12/15/11	1/3/11	1/17/11	9	Inosine=5, PBS=4	1.5 mm
Group 4	04/05/11	04/06/11	04/21/11	05/05/11	6	Inosine=6	3.5 mm
Group 5a	04/06/11	04/07/11	04/22/11	05/06/11	4	Inosine=4	3.5 mm
Group 5b	N/A	04/07/11	04/22/11	05/06/11		Inosine=2	3.5 mm

Table 2

The table lists the animal ID numbers, treatment, the percent damage of the medullary pyramid, and the number of BDA labeled axons in the denervated dorsal CST (dCST) in comparison to the total number of BDA labeled axons in the main tract, and finally the I/C ratio of axon numbers in each case.

Animal #	Treatment	Percent damage of pyramid	Axons in denervated dCST	Axons in dCST	I/C ratio ($\times 10^{-3}$)
3	Injury, no cannula	100%	5.2	1367	3.80
11	Injury, no cannula	48%	7.4	1898	3.90
17	Injury, no cannula	100%	10.4	961	10.82
19	Injury, PBS	86%	8	1139	7.02
22	Injury, PBS	72%	28.8	1729	16.66
23	Injury, PBS	100%	4.8	753	6.37
24	Injury, PBS	100%	13.2	1475	8.95
46	Injury, PBS	87%	0.4	974	0.41
48	Injury, PBS	53%	0.2	684	0.29
49	Injury, PBS	100%	3	983	3.05
50	Injury, PBS	100%	4.4	1675	2.63
75	No injury, inosine	0%	15.6	1998	7.81
80	No injury, inosine	0%	4.4	1012	4.35
Mean $\pm$ SD			8.14 $\pm$ 7.70	1280 $\pm$ 439	5.85 $\pm$ 4.52
Complete lesions only			5.75 $\pm$ 3.23	1247 $\pm$ 341	5.07 $\pm$ 3.86
26	Injury, inosine	100%	3.6	682	5.28
27	Injury, inosine	100%	7.4	975	7.59
28	Injury, inosine	100%	1	1033	0.97
29	Injury, inosine	100%	4.6	1329	3.46
32	Injury, inosine	100%	22.8	1646	13.85
34	Injury, inosine	65%	3	760	3.95
35	Injury, inosine	85%	1.8	791	2.28
40	Injury, inosine	62%	11.6	956	12.13
43	Injury, inosine	77%	9.4	972	9.67
52	Injury, inosine	81%	20.2	1942	10.40
57	Injury, inosine	100%	5.2	1191	4.37
59	Injury, inosine	100%	6.4	1336	4.79
66	Injury, inosine	100%	34.8	2320	15.00
67	Injury, inosine	100%	5	1612	3.10
69	Injury, inosine	100%	34	3130	10.86
76	Injury, inosine	73%	26.4	1143	23.10
78	Injury, inosine	57%	25.4	2107	12.06
Mean $\pm$ SD			13.10 $\pm$ 11.57	1407 $\pm$ 654	8.40 $\pm$ 5.77
Complete lesions only			12.48 $\pm$ 12.96	1525 $\pm$ 683	6.93 $\pm$ 4.77

The table lists the animal ID numbers, treatment, the percent damage of the medullary pyramid, and the number of BDA labeled axons in the denervated dorsal CST (dCST) in comparison to the total number of BDA labeled axons in the main tract, and finally the I/C ratio of axon numbers in each case.