

UNIVERSITY OF CALIFORNIA, SAN DIEGO

**Chondroitinase ABC Administration in a Non-Human Primate Model of Spinal
Cord Injury: Functional and Anatomical Assessment**

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The thesis of Justine Jane Liang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2016

DEDICATION

I would like to dedicate this thesis to my parents, who have fully supported me in all my educational pursuits.

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I would finally like to thank Dr. Kathleen French and Dr. Stefan Leutgeb for serving on my committee.

ABSTRACT OF THE THESIS

Chondroitinase ABC Administration in a Non-Human Primate Model of Spinal Cord

Injury: Functional and Anatomical Assessment

by

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Several factors contribute to the lack of repair following spinal cord injury, such as the lack of growth-promoting factors, poor intrinsic central nervous system regeneration abilities, and inhibition due to the glial scar¹. The glial scar contains a class of inhibitory extracellular matrix molecules, chondroitin sulfate proteoglycans (CSPGs), which create a physical and chemical barrier to axon regeneration and sprouting. Previous studies showed that administration of an enzyme, chondroitinase ABC (ch'ase), can remove this inhibition and promote functional and behavioral recovery in smaller

animal models. To aid in translation to clinical trials, we examined whether ch'ase administration in a rhesus monkey model of spinal cord injury promoted such recovery. As of now, 9 of the 12 subjects were analyzed and we found a trend towards anatomical and behavioral recovery in ch'ase administered subjects. The trend toward increased corticospinal tract sprouting in ch'ase subjects may demonstrate that the success of ch'ase in smaller animal models is not unfounded. However, the behavioral recovery is not as distinct, and perhaps more optimization of ch'ase treatment is required in the monkey model before translation to clinical trials. Further conclusions can be drawn once the remaining 3 subjects have been analyzed and assessed. However, despite the incomplete dataset and low n, there are interesting trends in the data that may influence whether the ch'ase treatment will move on to clinical trials.

I: Introduction

Limitations to spinal cord injury therapies

300,000 people in the United States live with spinal cord injury (SCI); the majority of SCIs are the result of motor vehicle accidents². SCI can result in: loss of movement, loss of sensation, and heightened chronic pain and spasticity. This debilitating disorder is both costly and taxing for patients and family members. The damage following injury is often permanent; current treatments rely on alleviating symptoms in lieu of repairing the damaged circuitry. Despite advancements in rehabilitation and support for people with SCI, there is still no effective treatment for SCI. This is largely due to the limited regeneration potential of the adult central nervous system (CNS) that prevents axonal regrowth¹ and subsequent repair of neural circuitry.

Inhibition due to the glial scar

After initial neuron injury and axon death, glial scar formation is one of the main components that inhibit regeneration in an injured spinal cord. Glial cells accumulate and surround the injury site to stabilize and seal the wound³. However, this creates both a chemical and physical barrier to regenerating axons. Inhibitory molecules are secreted by this glial scar, of which one class are chondroitin sulphate proteoglycans (CSPGs)⁴. CSPGs, which include brevican, versican, neurocan, and phosphacan, are an abundant class of proteins found in the nervous system. These proteoglycans are extracellular matrix molecules that are upregulated following CNS injury, which then create a growth-inhibitory matrix⁵. CSPGs are made of a glycoprotein core with covalently attached chondroitin sulfate glycosaminoglycan (GAG) side chains^{3,5}. This GAG sugar chain is the major inhibitory component of this protein. CSPG expression increases immediately post-injury⁶ and forms lattice-like structures called perineuronal nets (PNNs)⁷. PNNs physically wrap around neurons and further inhibit growth and plasticity.

Removal of inhibitory glial scar promotes regeneration in smaller animal models

One method to remove this inhibitory CSPG barrier is the application of an enzyme, chondroitinase ABC (Ch'ase), which can digest the inhibitory extracellular matrix. Ch'ase is a bacterial enzyme, derived from *Proteus vulgaris*, which cleaves the inhibitory GAG sugar chain from the CSPG protein core⁵. It has been shown that deglycosylation promotes sprouting in corticospinal and serotonergic descending pathways following dorsal column-lesioned rats on both intact and injured sides⁸, as well as collateral sprouting from the intact side. Therefore, to assess the potential of ch'ase administration as a strategy to promote sprouting in an injured spinal cord in a larger animal model, we will utilize ch'ase treatment in an adult rhesus monkey model of spinal cord injury.

Rhesus monkey model of SCI and corticospinal tract layout

Over the years, our lab here at UCSD collaborated extensively with UCSF, UC Davis, UCLA, and UC Irvine in creating a rhesus monkey model for various therapies in SCI. As there are distinct differences between rodent and primate spinal cord systems, a nonhuman primate model is critical to assess a potential therapy before moving on to clinical trials⁹. Several distinct differences between rodent and primate spinal cord systems include: size, corticospinal tract (CST) distribution, function, and inflammatory response^{10,11}. In the intact primate CST, many axons branch and decussate across the cervical spinal cord midline, which creates bilateral terminations. Each primate CST axon extends multiple collaterals, which is different in rats, as 96-98% of their CST projections decussate in the medullary pyramids and midline crossing is minimal¹².

Over the years, we have developed a clinically relevant C7 hemisection lesion model. We found that primates that received C7 hemisections that removed 90% of

projecting CST axons displayed a remarkable degree of spontaneous plasticity and reconstituted the CST axon density in the gray matter below the lesion to a remarkable 60% of normal levels¹². This is entirely different compared to the corticospinal tract layout in rats, as they reconstitute very little (3-5%) of the CST axon density after a cervical hemisection. In the primate, CST axons extensively decussate across the spinal cord midline, allowing for a retention of supraspinal influence below a lateral hemisection¹².

Assessment of chondroitinase ABC administration in a monkey model of spinal cord injury

This project evaluates the outcomes of ch'ase administration, and whether it has the potential to move forward to future clinical trials. Previous studies¹² have demonstrated that the corticospinal tract does sprout and regenerate following SCI in monkeys. Therefore, perhaps ch'ase administration during this period of plasticity following SCI can enhance those effects. To do so, we observed whether spinal cord injured rhesus monkeys improved anatomically and functionally following ch'ase treatment. Anatomical recovery was assessed by examining sprouting in the corticospinal tract and raphespinal (serotonergic) tracts. In addition, our subjects underwent behavioral training and testing to assess for improvement in hand function following a right C7 hemisection lesion. Our results from the incomplete data set (9 of 12 subjects) indicate a trend towards increased anatomical and behavioral recovery in ch'ase administered subjects without statistical significance. This opens further questions as to whether removing CSPG inhibition is sufficient to promote growth in the intrinsic regeneration-averse programming in the larger animal adult spinal cord. Given that there are other factors that contribute to the lack of regeneration following SCI,

perhaps ch'ase administration will be more effective and pronounced in combination therapies such as with neural stem cell grafts. However, our results still provide a useful piece of analysis that can aid in future assessments of ch'ase in the monkey model as well as consideration of whether this treatment can move on to clinical trials.

II: Materials and Methods

Subjects

12 male rhesus monkeys (*Macaca mulatta*) were used as subjects in this study (6-11 years old; mean age 8.6 years \pm 1.35 years). 9 have been fully processed and analyzed; the remaining 3 (2 control, 1 ch'ase) have received tracer injections and will be sacrificed at the end of July. Of the 9 animals, they were divided into two groups: Group 1 was ch'ase (n=5) and Group 2 was control (n=4). All surgical and experimental procedures in these experiments were carried out using the principles outlined by Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee (IACUC). Subjects were housed at the UC Davis National Primate Research Center.

Lesion Surgery

All subjects were sedated with 1 mg/kg ketamine intramuscularly, and anesthetized with 1.5–2.5% isoflurane. During surgery, body temperature, heart rate, respiration rate, and blood pressure were closely monitored and maintained. The caudal portion of the C5 dorsal lamina and the entire C6 dorsal lamina were removed. The dura was slit along the midline and retracted gently. A surgical micro-knife was mounted on a stereotaxic arm, positioned at the spinal midline, midway between the C5 and C6 dorsal laminae. This corresponds to the C7 spinal cord segment. The stereotaxic manipulator was used to lower the blade through the entire dorsoventral extent of the spinal cord, without severing the ventral artery. This initial cut established the medial position of the lesion. The lesion was then completed using microscissors under microscopic observation by the surgeons (M.H.T.; Y.S.N.) to ensure lesion completeness laterally and ventrally. All subjects retained bowel, bladder, and autonomic function after SCI.

Ch'ase Treatment

Four weeks after SCI, the lesion site was re-exposed, and additional laminectomy and longitudinal dural incision was performed to expose the C7-C8-T1 spinal cord. Hand-assembled nested silica cannulae (240 and 150 μm outside diameter, www.molex.com) were attached to a NanoFil 100 μl syringe (www.wpiinc.com) filled with 20 U/ml chondroitinase ABC (a gift of Acorda Therapeutics, Ardsley, New York) in ice-cold saline solution. The syringe was mounted in a syringe pump attached to a stereotaxic frame. 5 μl of chondroitinase was injected into the spinal cord parenchyma at each of 10 sites caudal to the SCI site. Rostrocaudal position: the first site was 1 mm caudal to the lesion border, each additional site was 1.5 mm further caudal. Mediolateral position: 0.7 mm medial to the medial edge of the dorsal root entry zone (DREZ) on the right side of the spinal cord. At each of these sites, the cannula was lowered into the spinal cord parenchyma and the 5 μl of chondroitinase was injected in two portions (2.5 μl each): one injection 2.5 mm deep, then a second injection 3.5 mm deep. Injection rate was 0.5 $\mu\text{l}/\text{min}$. After the deeper injection at each site, we waited 2 min before withdrawing the cannula. As with the initial SCI, the dura was sutured, then the overlying muscle and skin were sutured in layers.

Three control subjects received injections of saline only, and one control subject did not undergo the treatment surgery.

Anterograde tracing of corticospinal tract axons

Subjects were sedated with 1 mg/kg ketamine intramuscularly and anesthetized with 1.5-2.5% isoflurane. A frontoparietal craniotomy was performed to expose the left motor cortex. 300 nL of lysine-fixable biotinylated dextran amine (BDA; 10% in PBS; 10,000 molecular weight; Molecular Probes, Eugene, OR) was injected with a pulled

glass micropipette (outside diameter at tip $\approx 40 \mu\text{m}$) attached to a picospritzer (Parker Hannifin, Fairfield, NJ). The injection sites were areas of the motor cortex that innervated: the hand and arm (84 injections in 28 locations, 1.5 and 2.5 mm anterior to the central sulcus, spanning a territory from 13.3 to 24.5 mm lateral to the sagittal sinus and at depths in each site of 2.0, 3.5, and 5.5 mm), the trunk (11 injections in 11 locations, 1.5 mm anterior to the central sulcus, spanning a territory from 3.9 to 12.5 mm lateral to the sagittal sinus and at a depth of 2.2 mm), and the foot and lower extremities (32 injections were made at 20 locations, 2 and 3 mm lateral of the sagittal sinus, spanning a territory from 1 to 11 mm anterior to the junction of the central sulcus and the sagittal sinus and at depths ranging from 1.8 to 6.5 mm). After tracer injection, the excised piece of cranium was replaced and the incision closed. Of the 9 subjects, 8 received BDA injections; one of the ch'ase subjects was too sick to undergo the tracer surgery.

Sacrifice and tissue processing

Six weeks after tracer injections, subjects were sedated with 1 mg/kg ketamine administered intramuscularly, deeply anesthetized with intraperitoneal pentobarbital (25–30 mg/kg, to effect) and transcardially perfused for 10 minutes with 1% paraformaldehyde at 4°C (in 0.1 M phosphate buffer, pH 7.4), then for 50 minutes with 4% paraformaldehyde at 4°C (total perfusion volume of $\approx 8 \text{ L}$). The entire spinal cord was dissected from the spinal column. The spinal cord was postfixed in 4% paraformaldehyde for 5–6 hours, transferred to 10% glycerin (in 0.1 M phosphate buffer) for 12–24 hours, then placed in 20% glycerin.

The spinal cord dura was removed, and the spinal cord was cut in the transverse plane into blocks 1–1.5 cm long. Blocks containing segments C3 and C8–T1 were

sectioned in the transverse plane on a freezing microtome set at 40- μ m intervals. Tissue sections were stored at -20°C in TCS (25% glycerin, 30% ethylene glycol in 0.05 M phosphate buffer).

Transverse sections from segments C3 and C8 (three sections per segment per animal), were selected for BDA labeling.

Behavioral testing

Bimanual Brinkman Board

A custom-made plexiglass box with two small openings (i.e., one for each arm) was hung at the front of each animal's cage. The opening for the right hand had access to 5 interchangeable Brinkman boards of increasing difficulty, starting with a plate that contained 2-sloped wells (easiest), 2-non_sloped wells, 4-non_sloped wells, 6-non_sloped wells and 9-non_sloped wells (hardest). Using their left hand (unimpaired), animals pushed a lever that opened the lid covering each of the plates on the right side, allowing for bimanual engagement and food item retrieval with right hand only. All animals ($N = 8$) were scored while they were in-cage. They were scored as: no attempt (0 points), attempt (1 point), pickup and drop (2 points) and successful transfer to mouth (3 points). Scores were calculated as successful transfer to mouth and expressed as a percentage. All animals were given one minute to clear each board. For time to clear analysis, only 6 of 8 subjects had recorded data.

E-cage

All subjects were scored in an open field task that was developed previously to assess general motor function and locomotion, climbing, and object manipulation⁹. Animals entered a large open wire-mesh cage (7 feet \times 10 ft \times 6 ft [height, width, depth]) through a chute where they could ascend a series of four elevated perches to reach a

food reward placed inside a hollow rubber toy (filled with food such as peanuts, raisins, small pieces of dried fruits, etc.; The Kong Company, Golden, Co), on the top most perch. Locomotor activity was evaluated while the animals traversed the perches, as well as on the floor of the open cage. Climbing was assessed during food retrieval from a series of five cups hanging at different heights (1.5, 2.7, 3.5, 4.3, and 4.7 ft) on the front of the cage. Object manipulation was assessed during manipulation of the Kong[®] (hollow toy) and during the consumption of a large food item (e.g., apple or orange). Body and limb posture, wrist, hand, and individual finger movements were assessed during manipulation of the objects both on and off the ground. Scoring of functional recovery was done by our UC Davis collaborators¹³. Scoring in the exercise cage was recently described by Salegio et al (2016), and details a 72-point scale where animal can score a maximum of 34 points during general locomotion, climbing 16 points and object manipulation 22 points. All scores were expressed as percent of functional recovery. Animals were placed in the exercise cage 2 times/week with at least one session/week scored live.

Immunohistochemistry

For WFA detection, sections were washed in TBS and then quenched at 0.6% H₂O₂ in TBS for 30 minutes at room temperature. The sections were then washed with TBS and incubated in WFA-biotin (1:1000; reduced form; sigma L1766) and TBS-containing 0.25% Triton-X100, overnight at 4°C. Afterwards, sections were washed in TBS and incubated with Vector Elite ABC kit for 1 hour at room temperature. The sections were then washed in TBS and developed with DAB and NiCl₂. Sections were dehydrated and coverslipped with DPX mounting medium.

Nissl: transverse sections were taken in 1-12 throughout the entire cord for nissl. The sections were washed in TBS and fixed in buffered 4% paraformaldehyde for 1 hour at room temperature. Then, the sections were washed and mounted to dry overnight before nissl staining in 0.25% thionin.

BDA: sections were washed in TBS and incubated in 0.6% H₂O₂ in 100% methanol for 30 minutes at room temperature. After another wash with TBS, sections were incubated in Vector ABC elite solution in TBS containing 0.25% Triton-X100 overnight at 4C. After washing, sections were developed with DAB and NiCl₂. Sections were mounted, dehydrated, and coverslipped with DPX mounting medium.

5HT, TH: Transverse sections near C8 (hand area) were pre-treated with 50% methanol for 30 min at room temperature, washed in TBS, and blocked with TBS containing 5% normal donkey serum and 0.25% Triton-X100, for 1 hour at room temperature. Sections were incubated overnight in primary antibodies against serotonin (5HT; ThermoFisher; 1:5000; goat) and tyrosine hydroxylase (TH; ThermoFisher; 1:250; mouse) in TBS containing 0.25% TX100 and 5% donkey serum. Sections were washed with TBS and incubated in ThermoFisher Alexa-Fluor (594 and 488 nm at 1:500)-conjugated donkey secondary antibodies for 1 hour. Sections then were washed with TBS, mounted on slides, and coverslipped with Mowiol mounting medium.

Image capture and manipulation of images

Images were captured on the Keyence BZ-X700 (Keyence, Woodcliff Lake, NJ) digital microscope using the z-stack function and xy stitching. Raw images were then stitched together using the Keyence Analysis software and merged through the full-focus image analysis software (BZ-II, Keyence). For immunofluorescent 5HT analysis purposes, the images were captured with a haze reduction setting to reduce background

level labeling. Brightness and contrast were adjusted for optimal viewing using Adobe Photoshop CC 2014 (Adobe Systems, San Jose, CA).

Quantification of axonal growth (BDA, 5HT)

All quantifications were performed with the treatment groups blinded, using ImageJ 1.41c (Wayne Rasband, National Institutes of Health, rsb.info.nih.gov/ij/). A custom-written script was created (J.J.L.), derived from the methods used in Rosenzweig et al (2009). Regions of interest were drawn using border-defining images taken to highlight the gray matter. A total of 5 ROIs were drawn, which include the right gray matter, right dorsal horn, right ventral gray matter, right motor pool, right intermediate zone, and left dorsolateral tract. Right intermediate zone and right motor pool are subsets of right ventral gray matter; right dorsal horn and right ventral gray are subsets of right gray matter. The image was auto-thresholded and measured in each ROI for the pixel density. BDA densities were then normalized to the averaged left dorsolateral tract axon counts taken from the cervical 3 segment of each corresponding animal. The C3 axon counts were performed on ImageJ using CellCounter. BDA axon length was quantified by using the skeletonize function on ImageJ. 5HT axon density quantifications were conducted in a similar manner. All images were corrected for brightness and contrast equally to reduce background noise. A modified script (J.J.L.) was used to quantify the 5HT sections. Using nissl images to help identify motor pools, the left and right motor pools were drawn to create ROIs.

Statistical analysis of results

Graphs were generated and statistical analysis was performed using GraphPad Prism v7 (GraphPad Software Inc, La Jolla, CA).

III: Results

Administration of ch'ase in rhesus monkeys

One month after receiving right C7 hemisection lesions, subjects received 10 injections (ch'ase or saline) throughout the C8-T1 segments of the spinal cord (Figure 1). Three months later, all subjects received BDA tracer injections in the left motor cortex to label the right descending corticospinal tract. Six weeks after the tracer injections, subjects were sacrificed and their tissue processed and analyzed. All hemisected subjects showed complete disruption of corticospinal tract projections to the right spinal cord (Figure 4). The lesion resulted in loss of right hand function and leg function post-injury. Left hand fine motor control and locomotion was unaffected.

Lesion reconstructions demonstrate that lesions were consistent between and within subjects (Figure 2). In addition, all 10 injection sites below the lesion through C8-T1 were found in every subject. Subjects did not suffer from detectable increased damage due to injections. Thus, differences between subjects in anatomical and behavioral data are not due to experimental methods and treatment delivery.

In a preliminary study where subjects were sacrificed two weeks after receiving ch'ase injections, we observed CGPG removal (Figure 3) on the entire right side of the spinal cord below the lesion. However, as expected long term, these inhibitory perineuronal nets have reconstituted; the mean gray values are no different between ch'ase and that of control (Figure 3).

Anatomical analysis of corticospinal tract sprouting caudal to injury

Anatomical analysis revealed no significant increase in corticospinal tract axons in ch'ase compared to control subjects. Three months after subjects received injections of ch'ase or saline, they received 127 injections of biotinylated dextran amine (BDA) in the left motor cortex, to label the right corticospinal tract. Six weeks later, they were

sacrificed and their tissue processed. BDA-labeled axons were quantified by subdividing a transverse section of the spinal cord below the lesion (around the C8 hand area) into five regions of interest in the right gray matter (Figure 4). In this analysis, 8 of the 9 subjects were used (Ch'ase = 5, control = 3) as one of the control animals had complications and was sacrificed before the BDA was able to trace the corticospinal tract to term. The author created a custom-written script that would trace the BDA-labeled axons and eliminate background signal. Quantifications were normalized to axon counts of areas outside the effect of the treatment within subjects. Quantifications of corticospinal tract growth indicated that there was a trend towards a higher CST axonal growth in ch'ase subjects compared to control subjects (Figure 5A; right IZ $p = 0.30$), but these values are not statistically significant. However, there is a notable 1.5-fold increase in axon growth in ch'ase administered subjects compared to that of control. Corticospinal tract axon density was also assessed to determine if there was an increase in axon caliber (Figure 5B). However, while there was increased CST density in the right intermediate zone in that of ch'ase compared to control, the values are not statistically significant and do not show a trend as seen in the axon length (Figure 5A).

Anatomical analysis of other descending motor systems caudal to injury

As corticospinal tract sprouting is often the least likely – and last – to occur following SCI, we examined whether sprouting due to other descending motor systems could account for any observed improvements in behavior. Another descending motor pathway is the raphespinal tract, which originates from the brain stem. These pathways are serotonergic positive, which we labeled for using immunofluorescence (Figure 6A, 6B). 5HT axon terminals are often localized to the dorsal horn, ventral horn, and intermediate zone¹⁴. They contribute to the neuronal network of the central pattern

generator as well as specific locomotion programs required to determine the specific velocity, magnitude, and duration of movement¹⁴. Therefore, main right and left motor pools were quantified for any increased serotonergic axon density (Figure 6B, 6D). Regions of interest were drawn based upon adjacent nissl sections that marked the motor pools. There was no significant difference in 5HT axon density in ch'ase (n=4) versus control subjects (n=5). However, in both groups, the right motor pool density is similar to that of the left motor pool (Figure 6C; ratio of right MP to left MP = 0.92, 0.91; ch'ase, control respectively). Ch'ase administration does not appear to have a significant difference in increased serotonergic axon sprouting.

Assessment of functional recovery in ch'ase versus control

To determine whether ch'ase subjects improved more compared to control subjects, we tested the subjects in a behavioral exercise cage. Subjects (n=8) were placed in an open field where they could walk, climb, and manipulate food or objects spontaneously. Although both groups improved over time, ch'ase subjects were not significantly better at these tasks (Figure 7A). In all four scores (of overall, climbing, object manipulation, and locomotion), both groups improved over time ($p < 0.0001$; repeated measures ANOVA), but no significant differences were observed between groups. Scores taken from weeks 15-22 (last binned timepoint) were not statistically significant (unpaired two-tailed t-test; overall $p = 0.24$; Figure 7B). However, the data shows a possible trend toward improved behavioral function in ch'ase versus control.

The subjects were also tested in a bimanual Brinkman board. Subjects had to push a lever with their left hand to allow the right hand to access the brinkman board filled with treats. The board was composed of wells of varying difficulty (sloped/non-sloped). The brinkman board retrieval rate data was very bimodal (Figure 8A).

Therefore, we examined the time to clear the board as well as an indication of whether the subjects improved in completing the task quicker (Figure 8B). However, as with the retrieval rate, the data is extremely variable within conditions, and there is no detectable difference with the data that has been processed. In addition, some of this time to clear data had not been recorded for two ch'ase subjects. An unpaired two-tailed t-test taken from behavioral sessions from weeks 16-22 indicates that there was no statistically significant difference (n=6). Therefore, the bimanual Brinkman board analysis shows that there was no increased functional recovery in ch'ase versus control.

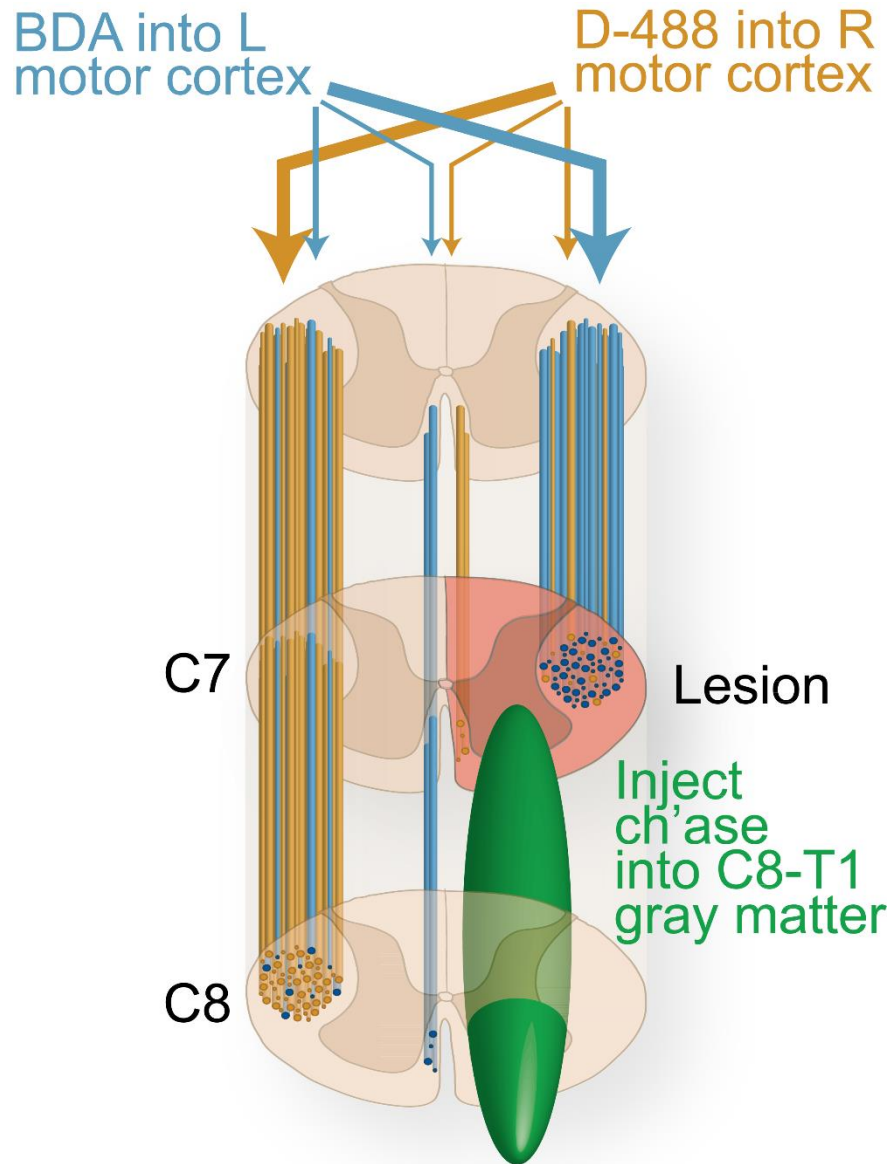


Figure 1. Schematic of ch'ase delivery and experimental design. 12 rhesus monkeys received right C7 hemisection lesions. One month later, they received 10 injections of either ch'ase or saline 1mm below the site of the lesion in the gray matter in areas C8-T1 of the spinal cord. Three months after the injections, the subjects received injections of BDA into the left motor cortex to label the right corticospinal tract (subjects also received injections of D-488 into the right motor cortex to label the left CST, but those quantifications were not performed in this thesis). In this thesis, 9 of the 12 subjects have been processed and analyzed, and 8 of them have complete behavioral and anatomical analyses.

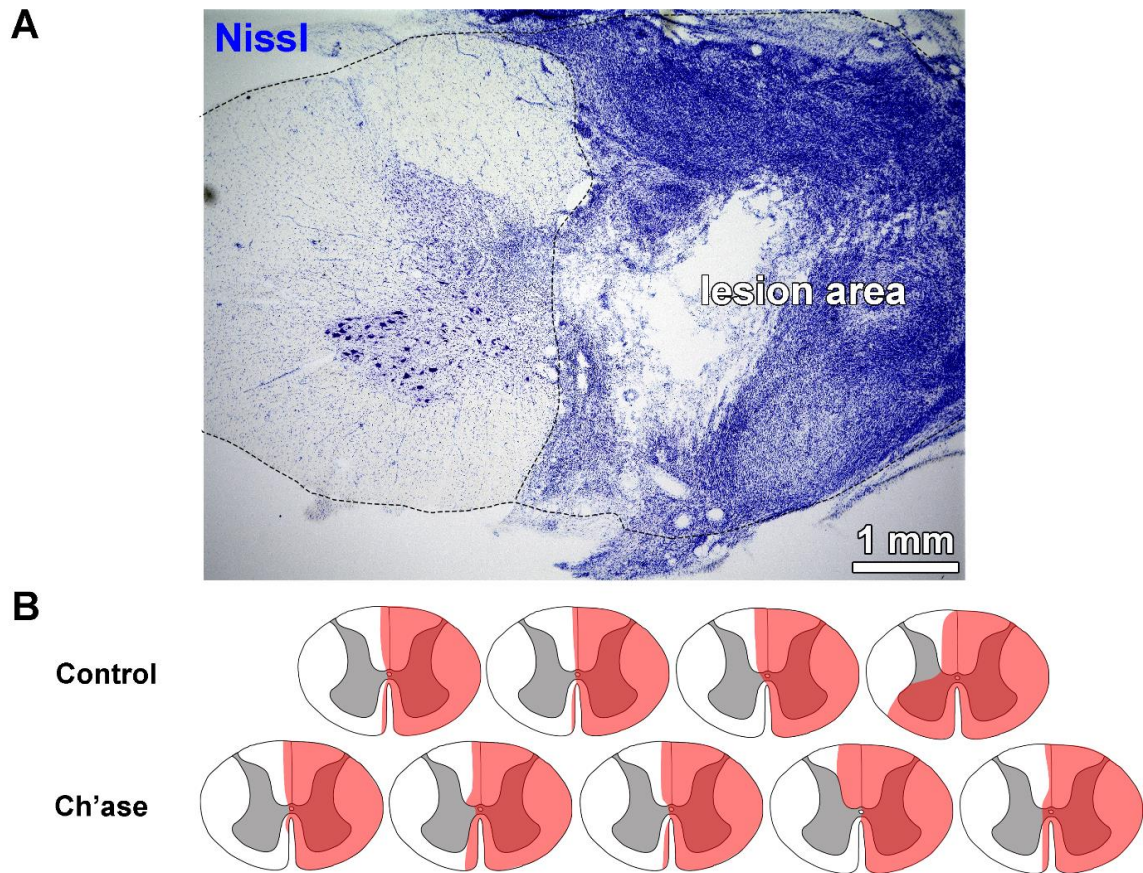


Figure 2. Lesion extents are consistent between and within ch'ase and control subjects. (A) shows a representative image of the lesion extent in an animal. The image was taken from a transverse section of the spinal cord at the C7 lesion area. The lesion spans 4 nissl sections, which are spaced in a 1:12 series. (B) shows the reconstructions that were created from the nissls and combined to show the maximal extent of damage. Controls and ch'ase subjects were consistent in lesion extent.

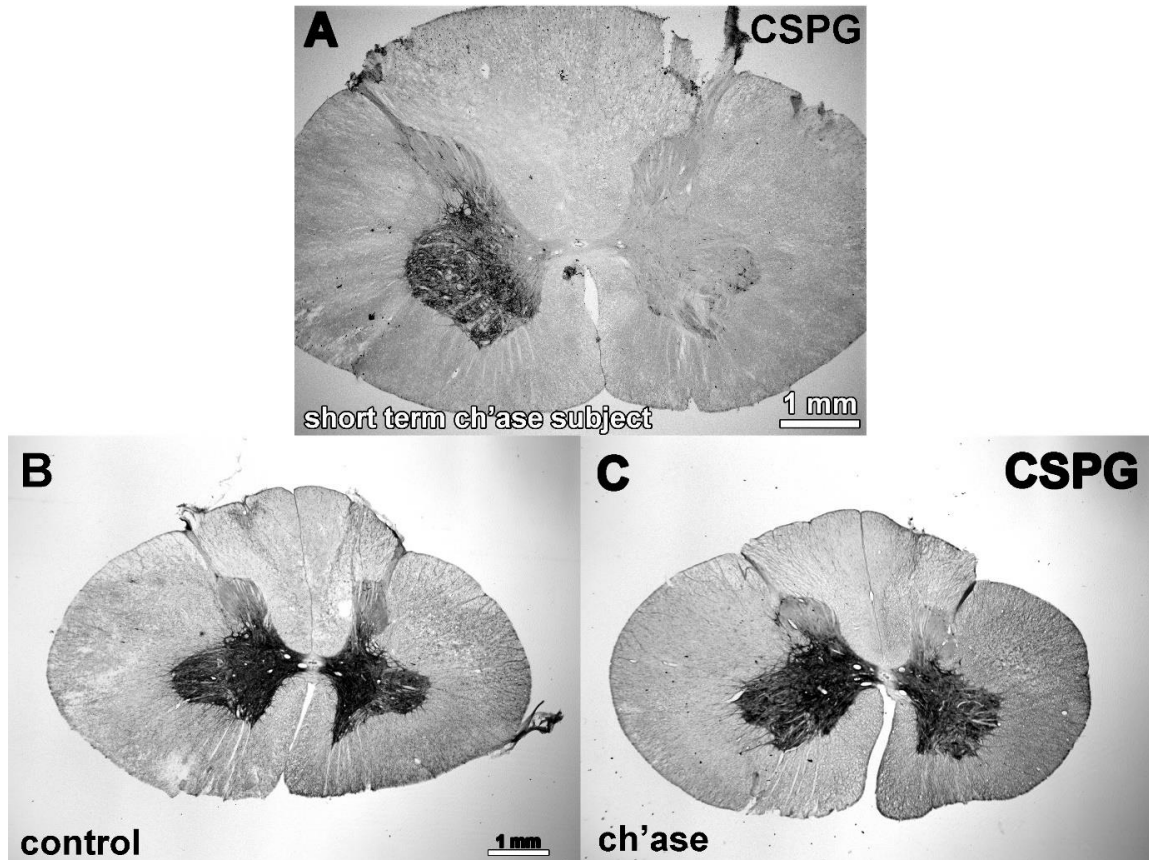


Figure 3. CSPGs are removed 2 weeks after ch'ase injections, but as expected, CSPGs reconstitute by 6 months post-injection. CSPGs were labeled with WFA antibody. Images were captured caudal to the lesion and near the site of injections. **(A)** shows a subject that was enrolled in a previous short-term study that was sacrificed 2 weeks after receiving the 10 ch'ase injections. The subject had received a right C7 hemisection lesion and received the ch'ase injections caudal to the lesion 1 month later. **(B and C)** show representative subjects from the current study (long-term). These subjects were sacrificed 6 months post-lesion. CSPGs reconstitute by 6 months post-injury.

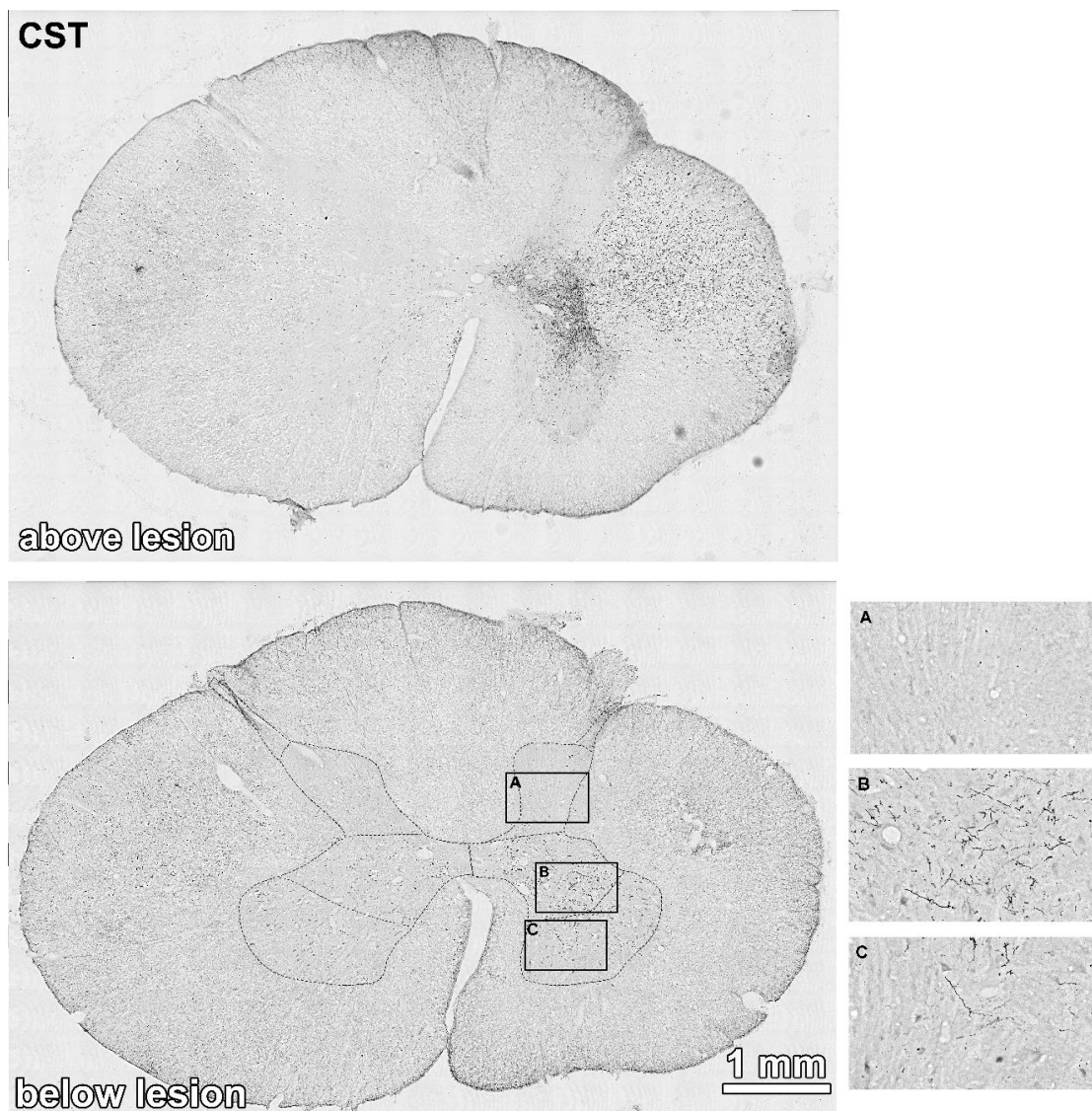


Figure 4. CST distribution and regions of interest to assess increased axonal growth. The figure shows the CST distribution above and below the lesion in a representative subject. CST was labeled with BDA injected into the left motor cortex. Above shows a typical CST distribution that is more concentrated around the intermediate zone and right dorsolateral tract. As expected, below the lesion a majority of the right dorsolateral tract axons were cut and not visible. Dotted lines highlight the regions of interest (ROIs) that were drawn for subsequent analysis. However, in each of the ROIs, there is variability between axon density. Panel **(A)** shows the dorsal horn, **(B)** shows the intermediate zone, and **(C)** shows the motor pool. As seen in above lesion, the intermediate zone **(B)** has the highest CST density.

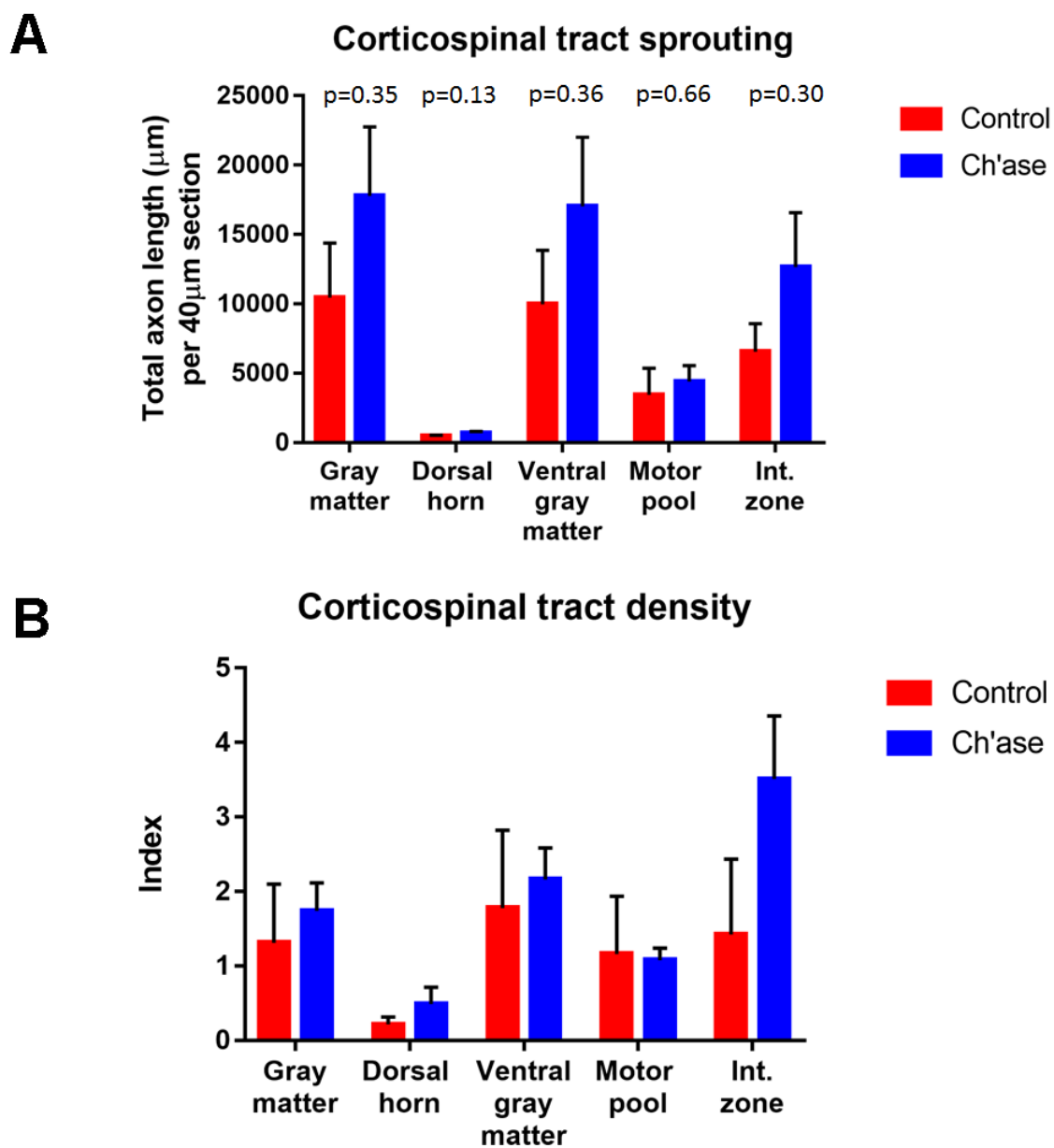


Figure 5. Trend towards increased corticospinal tract sprouting in ch'ase versus control. We derived 5 ROIs to examine the CST axonal growth (shown in Figure 4). The gray matter ROI includes the sub-ROIs of dorsal horn and ventral gray matter, and the ventral gray matter contains sub-ROIs of the motor pool and intermediate zone. Values obtained from the ImageJ macro was normalized to the left dorsolateral tract hand-counted axons. An unpaired two-tailed t-test showed that there was no statistical significance. **(A)** shows the axonal length per section, and **(B)** shows the pixel density. There was no statistical significance in increased CST density as well.

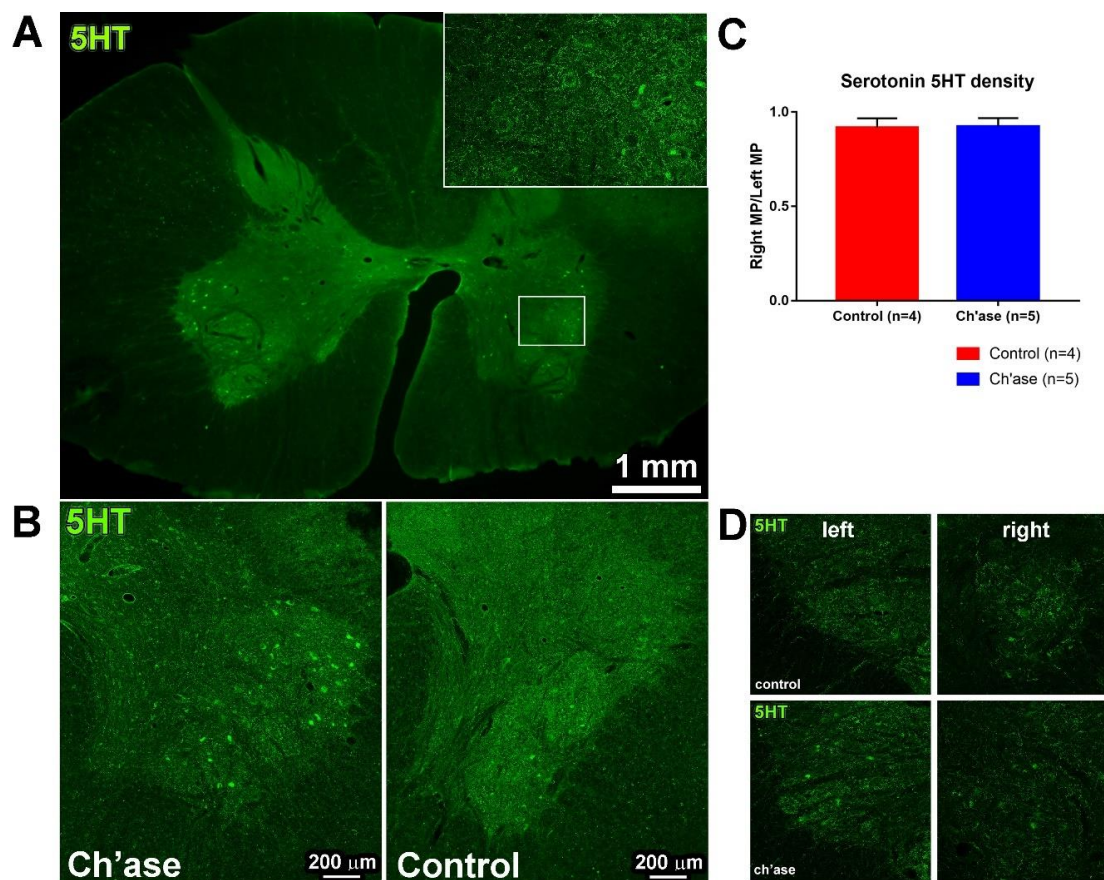


Figure 6. No increased raphespinal tract sprouting in ch'ase versus control.

Sections were taken around the C8 region to assess any increases in 5HT-labeled axons of the raphespinal tract. **(A)** shows the overall image, the white box displays the right motor pool where quantifications were performed. Inset shows the close up of 5HT-positive fibers. **(B)** shows the qualitative similarity between ch'ase and control right motor pools in 5HT density. It is important to note that the brightly labeled circles are the autofluorescence from motor neurons. The custom-written ImageJ script filtered out these autofluorescence and quantified only the fibers. **(C)** shows the quantifications that were obtained from the script. The right motor pool was normalized to the left (shown as "right MP/left MP"). There was no statistical significant difference between the two (unpaired two-tailed t-test; $p=0.92$). **(D)** shows the difference between the left and right motor pools in a subject from each group. There is no visible difference in axon density.

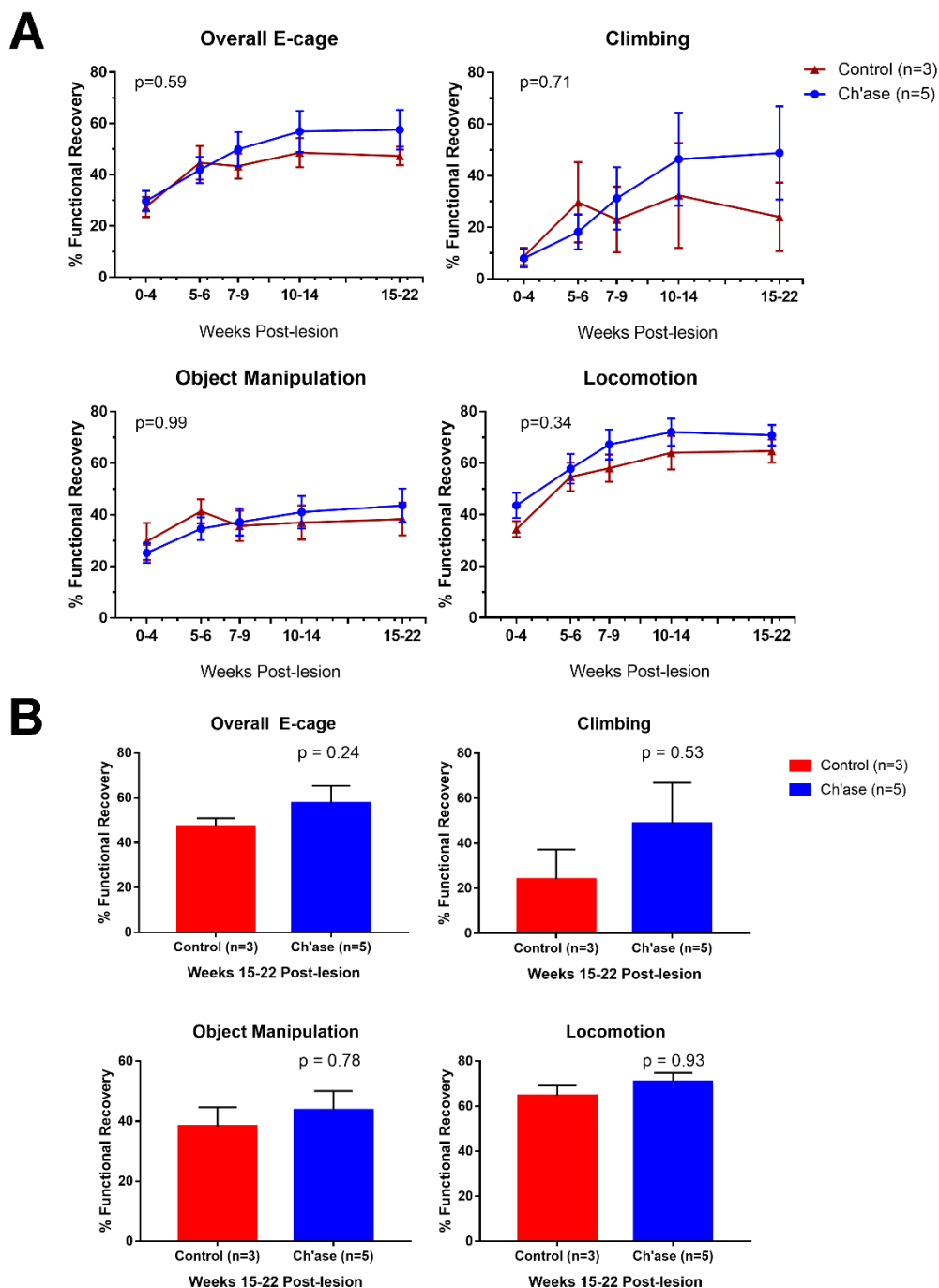


Figure 7. E-cage shows possible trend toward increased functional recovery in ch'ase versus control. The e-cage overall score is made of sub-scores of climbing, object manipulation, and locomotion. **(A)** shows that both groups improve significantly over time but there is no statistically significant difference between the ch'ase and control. (Repeated measures two-way ANOVA). **(B)** shows the last binned data point of combined behavioral sessions from weeks 15-22 post-lesion in control versus ch'ase. Again, there was no significant difference, however there is a possible trend towards

increased ch'ase recovery, especially in the overall, climbing, and object manipulation scores but not in locomotion.

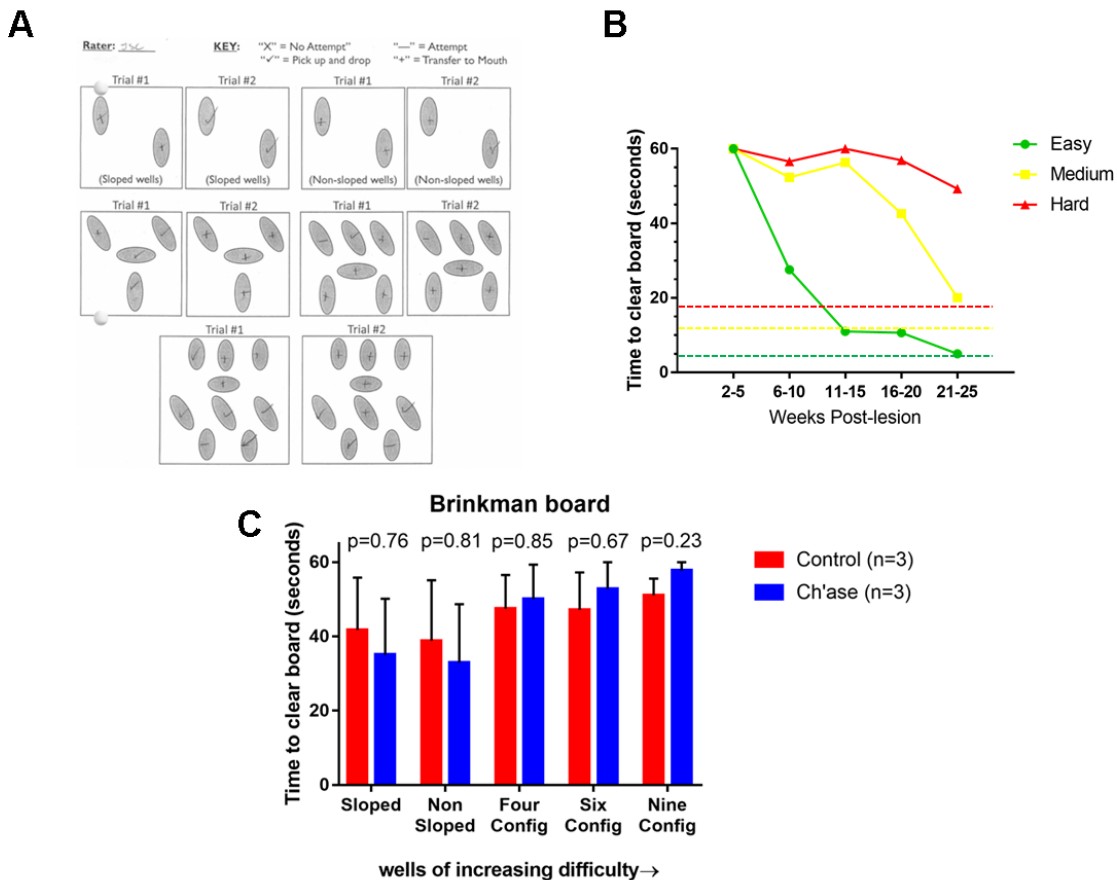


Figure 8. Brinkman board analysis shows no increased functional recovery in ch'ase versus control. (A) shows a typical raw data sheet from one representative subject to demonstrate the scoring of the Brinkman board percent retrieved data. The board is made up of varying wells (e.g. sloped, non-sloped, four-configuration, six-configuration, and nine-configuration). Data from the percent retrieved was very bimodal and not a sensitive measure of recovery. Therefore, we examined the time to clear the board as a means of detecting any differences in functional recovery. **(B)** shows a plot from a representative animal. The wells "sloped" were plotted as "easy", "four-configuration" as "medium", and "nine-configuration" as "hard". This subject in particular improved over time. The dashed dashed lines represent the pre-lesioned average time to clear for each of the difficulties (green, easy, sloped, 4 seconds; yellow, medium, four-configuration, 11 seconds; red, hard, nine-configuration, 17 seconds). **(C)** compares the time to clear the Brinkman board. There is no measurable statistical significance and no clear trend in ch'ase or control behavioral recovery.

IV: Discussion

We have demonstrated that with the 9 subjects that have been processed and analyzed, there is a trend of increased corticospinal tract axon growth in ch'ase compared to control, and a possible trend in functional recovery, but no measurable increased improvement in behavioral outcomes. After the three remaining subjects (ch'ase = 1; control = 2) are sacrificed and processed, we can reach a more definite conclusion on ch'ase effects on functional and anatomical recovery. However, it is important to note that ch'ase did not have any adverse effects either on both anatomical and behavioral measures.

Our anatomical assessment of ch'ase administration showed that there was a trend toward increased CST axon growth in ch'ase compared to control. Previously, we found that the rhesus monkey CST exhibits significant and substantial spontaneous plasticity following spinal cord injury. This intrinsic sprouting is mainly due to collaterals of the intact side that have either already been present on the right side. This is strikingly different in the rat CST, where axons simply do not regenerate. The human CST is similar to that of the monkey, and our experimental design has taken in translational difference between rats and monkey models. However, given our study with a very low n, we still observed a trend towards increased CST axonal growth in ch'ase treated subjects. While it is not significant, once the remaining 3 subjects are sacrificed and processed, we can reach a more definite conclusion. The strongest trend toward increased axonal growth was especially in the intermediate zone, an area where is also densest prior to injury. The intermediate zone is a region where many motor neurons are present. This increase is promising in the context of both a low N and that the CST is thought to be the most relevant to functional recovery. A power analysis was conducted to determine the number of subjects required for a statistically significant result. 52 total subjects would need to be enrolled, 26 ch'ase and 26 control. Further quantifications of

midline crossers will determine whether there are collateral sprouts from the intact side to examine other sources of sprouting. In addition, synapse analysis through colabeling of synaptophysin and BDA-labeled CST will examine whether there are functional synaptic connections in the potential circuitry.

Anatomical analysis of 5HT-expressing raphespinal tract fibers in the motor pools did not show an increased density in ch'ase versus control. In humans, some raphespinal tract axons make monosynaptic connections with alpha motor neurons, which can contribute to the spinal circuitry through collaterals¹⁵. In addition, while previous studies of ch'ase in rat models of SCI have shown an increased 5HT density, in the human and primates, the raphespinal tract is smaller. Perhaps there was no observable increased density in this particular descending motor system, but analysis of other descending motor systems can determine if there are other effects as well. For example, tyrosine-hydroxylase labeling of the cerulospinal tract, which were assessed in other SCI studies, can be conducted to quantify any increased density as well.

E-cage behavioral assessment of ch'ase administration showed that there was a possible trend towards increased functional recovery in ch'ase compared to control. Given that there is no observed significance as seen by the high variability in the subjects' behavioral outcomes, once the 3 subjects are processed, a clearer conclusion can be drawn. Subsequent analysis through a principal components test will reduce variability and elucidate the relationship between functional and anatomical measures. However, subjects do significantly improve over time and there is no observed detrimental effect of ch'ase in any of the behavioral measures. This lends to the potential safety of ch'ase injections and that our methods of delivery of the bacterial enzyme did not introduce increased damage. While groups appeared to plateau at different points towards the last binned data session (Weeks 15-22 post-lesion), an unpaired two-tailed

t-test demonstrated that the last data point was not significant, lending to the variability between subjects. However, there was no observed increase in locomotion, which corresponds to the sites of ch'ase delivery, which were centered primarily in the C8-T1 area of the spinal cord that is responsible for hand function.

The Brinkman board analysis showed no increased functional recovery in ch'ase compared to control. Assessment of the time to clear the board showed no statistical significance in the differences between groups. While the Brinkman board is a behavioral test of hand pincer function, it appears that there was no observable effect. Two subjects that have complete anatomical and e-cage behavioral data did not have the time to clear recorded, and thus with a total n=6, it seems that a definitive conclusion is premature. The remaining subjects enrolled in the study will be analyzed and processed by Fall 2016.

Despite our incomplete dataset, there are interesting trends in the data. As these results will influence whether ch'ase moves on to clinical trials, it introduces important analyses and considerations. First, given that our method is a one-time injection of ch'ase delivered intraparenchymally, it may be likely that the increased CST axonal growth may not be enough to promote functional plasticity that is observable by behavioral measures. Previous rat models of ch'ase delivery was primarily intrathecal and utilized ports with repeated infusions of ch'ase. Given the possible high-dosage delivered intraparenchymally, and complete CSPG degradation 2 weeks after injection, it is plausible that ch'ase is sufficient for some anatomical recovery but not enough time for functional recovery. As CSPGs do reconstitute after, perhaps prolonging the period of optimal plasticity will improve the actual functional connections that are created. Second, while we waited 1 month after injury to deliver ch'ase, as it was clinically relevant to the typical recovery following SCI, previous studies administered ch'ase following a few days

after injury. Perhaps an earlier treatment timepoint will show a more pronounced effect due to ch'ase. Third, there are other factors that contribute to lack of repair following SCI, which lends to why SCI treatments are so difficult to develop and optimize. Factors include: the inactive growth program of CNS neurons, myelin-associated inhibitors, and lack of neurotrophins (growth-promoting factors)¹. Perhaps targeting the inhibitory molecules that make up the glial scar is not sufficient to promote observable functional recovery in the rhesus monkey model. Ch'ase may be more effective in combination therapies such as with human neural stem cell grafts. Our lab is optimizing and evaluating human neural stem cell grafts in a rhesus monkey model of SCI, and it may be a potentially promising combination therapy, given the increased anatomical CST plasticity and possible trend towards functional recovery observed in ch'ase-administered subjects.

In conclusion, despite the low n, we still observed trends that could potentially lead to observable anatomical and behavioral effects of ch'ase-treated subjects. Monkey studies are extremely costly and time-consuming, given the support, care, and treatment necessary for these animals. However, despite the variability between subjects and complications that arose throughout the study, it will be interesting to re-evaluate the data once the other three subjects have been assessed. Still, there are other assessments that can be built upon the work thus far, which can influence whether ch'ase administration will move on to clinical trials.

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