UNIVERSITY OF CALIFORNIA SAN DIEGO

Exploration of Synaptic Silencing by Gabapentin to Promote Forelimb Corticospinal Tract Regeneration After Spinal Cord Injury

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by

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The thesis of Elizabeth Marie Riley is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would like to dedicate this to my great grandmother who was denied entrance to her schoolhouse in the Lemon Grove Incident; to my grandmother and mother who postponed their education so my sisters and I could further ours. “Sí, se puede”

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ABSTRACT OF THE THESIS

Exploration of Synaptic Silencing by Gabapentin to Promote Forelimb Corticospinal Tract Regeneration After Spinal Cord Injury

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Professor Pengzhe Lu, Chair

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Although our previous studies demonstrate robust regeneration of corticospinal tract (CST) into neural progenitor cell (NPC) grafts placed into spinal cord injury (SCI) site, our recent preliminary data unexpectedly found that only hindlimb CST regenerated and forelimb CST rarely regenerated after mid-cervical SCI. This limited regeneration may be due to small proportional injury of forelimb CST and remaining synaptic connections above SCI, which is consistent with a recent new hypothesis, synaptic suppression of axon regeneration. In order to promote forelimb CST regeneration, we targeted to silence synaptic activity transiently by administration of Gabapentin, a pharmacological agent that blocks the Alpha2delta2 subunit of voltage-gated calcium channels involved in regulation of synaptic activity. Adult rats received mid-cervical SCI and NPC graft-expressing green fluorescent protein (GFP) followed by Gabapentin administration for two weeks. The forelimb-CST were specifically traced by injection of adeno-associated virus (AAV) expressing red fluorescent protein (RFP) and animals survived additional 3 weeks. The experimental group receiving Gabapentin did not show a dramatic increase of forelimb-CST regeneration as hindlimb CST, but a trend toward more regeneration compared to the control group. Further experiment with higher dosage and more frequent administration needs to confirm whether Gabapentin can elicit forelimb CST regeneration and has potential for treatment of SCI.

INTRODUCTION

Spinal Cord Injury (Epidemiology)

Spinal cord injury (SCI) is a prevalent issue, as in the United States alone, there are approximately 18,000 new SCI cases every year (NSCISC, 2020). Globally, the frequency of SCI cases can be as high as 500,000 new cases every year (Bennett et al., 2022). SCI damages both local neural cells and axonal tracts that carry signals between the brain and the rest of the body. One axonal tract of particular interest is the corticospinal tract (CST), the primary voluntary motor control axis for humans (**Figure 1**). The CST carries motor signals from the cerebral cortex to the spinal cord. Similar to other central nervous system (CNS) components, the CST remains largely refractory to regeneration following SCI (Poplawski et al., 2020).

The two steps of SCI involve primary and secondary injuries. The primary injury is the initial trauma that occurs at the time of SCI which bruises or tears the spinal cord and is irreversible (Alizadeh et al., 2019). The secondary injury is a process that begins after the initial SCI. It furthers the initial damage and limits regeneration, resulting in inflammation, necrosis, cell death, and axon die-back, though it can be reversible (Sun et al., 2021). Currently, treating SCI after the primary injury takes precedence, and therapeutics that augment the limited regeneration from secondary injury are underway.

Mechanism: Why CNS Cannot Regenerate

There are two main theories about the mechanism behind the limited CNS regeneration following SCI (Afshari et al., 2009). The first one is the extrinsic factor or injury environment that limits the CNS regeneration. The second one is intrinsic or endogenous factors that limit CNS regeneration.

In the early time, the extrinsic factor theory was dominant in the SCI research field. This includes myelin that is produced by oligodendrocytes and contains inhibitory factors and glial scar formation around the SCI site. The discovery of myelin inhibition is from an *in vitro* study that shows myelin inhibition of neurite growth in neuron culture (Schwab, 1990). Later, there were specific inhibitor molecules found in myelin, including NogoA, MAG, and OMgp which act partly via the Nogo receptor (Schwab, 2004). Besides myelin inhibition, the glial scar is traditionally recognized as a major extrinsic inhibitor not only as a physical barrier for axon regeneration, but also due to production of extracellular inhibitory molecules (Silver and Miller, 2004). The major inhibitory molecules are chondroitin sulfate proteoglycans (CSPGs). In a healthy, uninjured CNS, CSPGs are produced by neurons and astrocytes (Sofroniew, 2018). These CSPGs along with their receptors, protein tyrosine phosphatase-sigma (PTPσ) and Nogo-receptors (NgR), help form the perineuronal net (PNN). The PNN helps regulate synaptic plasticity in both the developing and adult CNS, where NgR acts to inhibit axon growth (Sofroniew, 2018). After SCI, CSPGs are one of the main components of the glial scar formed around the injury site and prevent axonal growth (Rosenzweig et al., 2019).

The above extrinsic factors are present around SCI site. The progressive cell death in the epicenter of SCI often results in cystic cavitation along with fibrotic scar formation, both of which also contribute to the inhibitory environment (Zhu et al., 2015).

Recent studies emphasize intrinsic factors that limit axon growth and regeneration (He and Jin, 2016). The basic theory is that adult neurons shut down the growth or regeneration programs that are active during development. Studies have demonstrated that intrinsic signals play critical roles in axon regeneration. Several molecules, such as Phosphatase and tensin homolog (*Pten*), suppressor of cytokine signaling 3 (SOCS3), and Kruppel Like Factor 7 (*KLF7*)

have been discovered to modulate CST regrowth (Liu et al., 2010; Blackmore et al., 2012; Park et al., 2015). *Pten* and SOCS3 play a role in intrinsic reconnection and regeneration of the host CST following SCI. *Pten* is a tumor suppressor gene which negatively regulates the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway (Sun et al., 2021). The mTOR pathway is associated with developmental stages of CST neurons, and both aging and SCI are associated with downregulation of mTOR (Liu et al., 2010). Interestingly, in cortical neurons one year post-SCI, deletion of *Pten* genes could elicit regeneration of injured CST axons (He and Jin, 2016). This indicates the importance of genetic factors in limited axonal growth following SCI. SOCS3 also limits neuronal growth after SCI, but through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Polizzotto et al., 2000). One knock down model of SOCS3 elicited increased regeneration of dendrites and prevented axon demyelination following SCI (Park et al., 2015). Taken together, these studies with *Pten* and SOCS3 emphasize the importance of addressing issues with endogenous manipulation of important genes that can promote CNS regeneration after SCI.

Strategies to Promote CNS Regeneration

The intrinsic factors can be modified by deletion of the *Pten* gene, which leads to the up-regulation of growth machinery. In one study, genetic deletion of *Pten in vivo* in mouse models of T8 dorsal hemisection resulted in the main CST axons extending to the lesion margin (Liu et al., 2010). CST axons that regenerated past the lesion grew bilaterally, suggesting regenerative growth that cannot be attributed to spared axons (Liu et al., 2010). This indicates the potential of *Pten* deletion as a regenerative growth mechanism. Additional studies have co-deleted *Pten* and SOCS3, resulting in enhanced sprouting of CST neurons (Jin et al., 2015). These methods hold promise for resolving the intrinsic issue of axon growth following SCI.

One method studied to promote extrinsic CNS regeneration is the targeting of myelin in the SCI environment. Nogo-A is a factor enriched in myelin which, in conjunction with its receptor NgR1, limits the growth of neurons (Schwab et al., 2014). This makes Nogo-A and NgR1 attractive candidates for silencing. One study found Nogo knockout lines lacking Nogo-A partially blocked the inhibition of neuron growth caused by Nogo-A *in vitro*, but *in vivo* this data was not significant (Geoffroy et al., 2015). These results suggest deleting Nogo in models of spinal cord injury does not produce a marked effect on CST regeneration.

Another method to promote CNS regeneration is targeting the CSPG component of the glial scar in the extrinsic environment. The glial scar results from an extrinsic signal, and typically this astrocyte reactivity is reversible (Escartin et al., 2021). There are many non-astrocyte cells that produce CPSGs at the site of SCI, and preventing glial scar formation does not decrease the amount of CSPGs produced (Anderson et al., 2016). The CSPGs which help form the astrocyte wall of the glial scar can be degraded by Chase ABC (ChABC). Enzymatic delivery of ChABC at an ideal time and site of astrogliosis can improve CST axon regeneration in a mouse hemisection injury model (Tran et al., 2018). These results emphasize the importance of decreasing axon-inhibitory proteins for the extrinsic issue of decreased axon growth following SCI (Tran et al., 2022).

Our lab recently focuses on neural stem/progenitor cell (NSC/NPC) transplantation as a strategy to repair SCI. Transplanted NSCs/NPCs can differentiate into neurons and glia that replace lost neural cells and provide a permissive cellular environment to attract injured axons to regenerate. More importantly, our recent studies demonstrate that transplant derived neurons can extend their own axons into the host for long distance (Lu et al., 2012; 2014). Therefore, transplanted NSC/NPC could function as neuronal relays to reconnect damaged circuitry after

SCI. These grafts stimulate the internal axonal growth programs required for regeneration and sprouting while also providing a favorable extrinsic environment for axons to grow into, minimizing myelin and CSPG interference (Lu and Tuszynski, 2017).

NPC Graft and Robust CST Regeneration

Recently our studies demonstrated robust regeneration of CST after SCI using spinal cord derived NPC grafts (Kadoya et al., 2016; Poplawski et al., 2020). Notably, regenerating CST axons make synaptic connections with graft-derived neurons in the appropriate space (Dulin et al., 2018; Kumamaru et al., 2019). In addition, optogenetic stimulation of CST axons that regenerate into NPC grafts elicits distinct and segregated neuronal network responses (Ceto et al., 2020). Moreover, stimulation of NPC graft-derived axons that extend caudally into the denervated spinal cord also trigger local host neuronal network responses (Ceto et al., 2020). These results indicate that graft-derived neurons could serve as neuronal relays to transmit motor signals from regenerated CST axons above the injury site into host spinal cord neurons below the injury. Furthermore, a recent study shows that injured CST neurons revert transcriptionally to an embryonic state for a short period of time after SCI and transplanted NPCs can sustain this reversed transcriptome, indicating that NPCs can affect neuronal intrinsic growth program (Poplawski et al., 2020).

Discovery of Regenerative Discrepancy Between Forelimb and Hindlimb CST

Given the remarkable regeneration and connectivity of CST axons into NPC grafts that in turn extend a large number of axons into host spinal cord for long distances (Lu et al., 2012), we sought to study whether regeneration of CST axons into NPC grafts could enhance skilled forelimb motor function such as reaching and grasping, which is critical for daily independent life in quadriplegics after cervical SCI (Anderson, 2004). Forelimb function is largely controlled

by the forelimb CST (F-CST) which projects to the cervical spinal cord. We hypothesize that both F-CST and hindlimb CST (H-CST) regenerate into the NPC graft after mid-cervical SCI since both F-CST and H-CST are transected. To our great surprise, we found the F-CST rarely regenerated into the NPC graft, while hindlimb CST axons robustly regenerated in NPC grafts in the mid-cervical lesion site when we separately labeled F-CST and H-CST axons (**Figure 2**). While this phenomenon has been observed, there is currently no explanation for this difference in the robust regeneration of the H-CST compared to the minimal regeneration of the F-CST. Given that both the F-CST and H-CST have the NPC graft as a permissive environment to regenerate, there may be an alternative hypothesis that has not yet been considered. The traditional intrinsic and extrinsic factor theory cannot explain why the F-CST does not regenerate as robustly as the H-CST. The mechanism as to why the F-CST does not regenerate well is still elusive.

Synaptic Suppression of Axon Regeneration Hypothesis

In order to understand the potential mechanism why F-CST does not regenerate, we explored the major differences between F-CST and H-CST after mid-cervical SCI. The major difference is that cervical SCI transects all H-CST axons, including those collaterals and synaptic connections in the lumbar cord below cervical SCI. In contrast, mid-cervical SCI transects proportionately fewer F-CST axons that project below the injury, and those projecting above SCI are not destroyed. Indeed, our lab recently mapped the entire projectome of the F-CST neurons and found that it has numerous sustaining collateral branches in the upper spinal cord, brainstem and brain that form synapses: 80% of the F-CST projects to regions of the upper cervical spinal cord, brain and brainstem (Sinopoulou and Rosenzweig, unpublished data). In contrast, we find that H-CST neurons have very few collateral branches in the brain and brainstem. This suggests

that these sustaining collaterals and synaptic connections in the upper cervical spinal cord, brainstem and brain of the F-CST may limit their regeneration in the spinal cord.

Our theory is consistent with a recent hypothesis "synaptic suppression of axon regeneration" stating that excess synaptic connectivity, which suppresses axonal growth mechanisms during development, are also relevant for axon regeneration (Meves and Zheng, 2016). The above hypothesis is supported by evidence that the presence of sustaining axonal collaterals suppress regeneration of injured sensory axons (Lorenzana et al., 2015). Specific elimination of either the ascending or descending branch of a sensory axon using a localized laser injury led to a poor regenerative response, while eliminating both led to a strong regenerative response (Lorenzana et al., 2015). This result indicates that a surviving intact branch suppresses regeneration of the injured branch because the intact branch preserves the remaining axon architecture. Two additional recent studies support this hypothesis: the first study used transcriptomic analysis and bioinformatics to identify *Cacna2d2* as a developmental switch that limits axon growth and regeneration (Tedeschi et al., 2016). *Cacna2d2* encodes the Alpha2delta2 subunit of voltage-gated calcium channels that regulate synaptic activity, and deleting or silencing the *Cacna2d2* gene promotes axon growth *in vitro* (Tedeschi et al., 2016). Pharmacological blockade of Alpha2delta2 with gabapentinoid enhances sensory axon regeneration and CST axon regenerative sprouting after SCI in mice (Sun et al., 2020). This is hypothesized to be due to inhibition of the Alpha2delta2 subunits, thereby reducing synaptic activity or silencing synapses, and thus eliminating synaptic suppression of cell growth and enhancing axonal regeneration after SCI. Indeed, a recent retrospective study suggested a potential beneficial effect of gabapentinoid anticonvulsants on motor recovery in humans after SCI (Warner et al., 2020). A recent study identifies Munc13, a protein essential for synaptic

vesicle release, to suppress axonal growth (Hilton et al., 2022). Deletion of *Munc13* gene promotes axon growth and regeneration both *in vitro* and *in vivo*, and pharmacological depression of synaptic transmission with Baclofen enhances axon regeneration after SCI in mice. The potential mechanism is inhibition of synaptic transmission, which reduces synaptic activity or silences synapses, thereby eliminating synaptic suppression of axonal regeneration and enhancing growth and regeneration of injured axons after SCI.

Silencing Synapses to Promote F-CST Regeneration

Based on above studies, we hypothesize that extensive collaterals and synaptic activity in F-CST axons above a mid-cervical injury limits regeneration. To maintain translational relevance, we cannot simply eliminate these collaterals and their synaptic connections above the injury. However, there are existing tools for transient and reversible silencing of glutamate receptor synaptic activity, including pharmacological agents (Wiegert et al., 2017). Gabapentin is a pharmacological agent that blocks Alpha2delta2, which is involved in synaptic transmission. Gabapentin is a well-known drug used to prevent seizures and relieve pain for certain nervous system conditions (Quintero, 2017). Therefore, we will determine if transient blockade of F-CST synaptic activity using Gabapentin can enhance F-CST regeneration following SCI in *in vivo* rat models.

To test the above hypothesis, we performed a cervical level 4 (C4) dorsal column lesion that completely transected the main CST, including the F-CST. We transplanted NPCs into the injury site to provide a permissive cellular environment and then experimentally silenced F-CST neurons using Gabapentin to determine if it improved regeneration compared to the graft-only group. We hypothesize that this would silence synaptic activity from those F-CST collaterals above the SCI site and enable better regeneration of the F-CST. This experiment can help us to

elucidate the mechanisms preventing F-CST regeneration, and progress toward the goal of enabling F-CST regeneration to reestablish lost fine motor hand control in humans.

METHODS

Animals

Adult female Fischer 344 rats were the main subjects in this study. Average weight was 130g (n=17). The Institutional Animal Care and Use Committee of the Department of Veteran Affairs San Diego Healthcare System approved all animal housekeeping and surgeries in accordance with AAALAC requirements. Animals had free access to food and water throughout the study and daily health checks were performed. All surgeries were performed under an anesthetic cocktail (2 mL/kg) of ketamine (25 mg/mL), xylazine (1.3 mg/mL) and acepromazine (0.25 mg/mL). Animals were given injections of a mixture of banamine, ampicillin, and Lactated Ringers solution for three days postoperatively.

Spinal Cord Lesion

All rats received a bilateral dorsal column wire-knife lesion to transect the main dorsal column corticospinal tract. This was done by performing a laminectomy at cervical level 4, positioning the wire-knife at 0.6 mm to the right of the spinal cord central axis, lowering the wire-knife 1.2 mm ventrally from the dorsal surface of the spinal cord, and extruding the 1.5 mm-wide wire-knife hook in accordance with Weidner et al., 1999 (**Figure 3**). The knife was then stereotaxically raised 0.2 mm at a time while a bent blunt needle tip was pressed against the white matter from the dorsal surface, ensuring all axons were lesioned above the knife hook. Once the wire-knife hook was highly visible near the surface of the spinal cord, the wire-knife hook was retracted, and the entire wire-knife was removed from the spinal cord. This surgical procedure severed more than 90% of the corticospinal tract axons.

NPC Transplant

Neural progenitor cell grafts were implanted into the lesion site one week after the spinal cord lesion (**Figure 4**). Embryonic day 13 spinal cords from transgenic Fischer 344-Tg (EGFP) rats which ubiquitously express GFP under the ubiquitin C promoter served as donor tissue for the grafting. E13 spinal cords were dissected and dissociated using the same methods followed in Lu et al., 2012. Dissociated E13 cells were resuspended at a concentration of 250,000 cells/μL in a fibrin matrix (25 mg/mL fibrinogen and 25 units/mL thrombin Sigma-Aldrich) containing a ten- growth factor cocktail to support graft survival. This growth cocktail consisted of BDNF, neurotrophin-3, platelet-derived growth factor, insulin-like growth factor 1, epidermal growth factor, basic fibroblast growth factor, acidic fibroblast growth factor, glial-cell-line-derived neurotrophic factor, hepatocyte growth factor, and calpain inhibitor in accordance with Lu et al., 2012. The neural progenitor cell and growth factor cocktail was then loaded into a pulled glass micropipette using a Hamilton syringe, and 1.5 μL were injected into the cervical level 4 lesion cavity using a PicoSpritzer II (General Valve Inc., Fairfield, New Jersey).

Gabapentin administration

Gabapentin administration began in the experimental group (n=7) 3 days following neural progenitor cell grafting (**Figure 4**). Gabapentin injections were administered subcutaneously 3 times per day at 9am, 1pm, and 5pm for 14 days. Gabapentin in drink water was provided to the single housed experimental animals overnight from 5pm to 9am to be consumed ad libitum. The gabapentin water consumption overnight was measured and recorded every morning when the gabapentin water was removed from the subject's cage.

Gabapentin injections were prepared at a dosage of 133 mg/kg, totaling 400 mg/kg in 3 injections per rat per day. 50 mL of sterile distilled water were added to a sterile conical tube,

and 500 mg of gabapentin (Millipore-Sigma, U.S.) was weighed out and added into the conical tube (10 mg/ml). The injection solution was loaded into syringes and the amount of injection volume was calculated according to the body weight of the rat at the time of injection to achieve the dosage of 133 mg/kg/time.

Gabapentin in drink water was prepared to target at a dose of 100 mg/kg per rat per night. 90 mL of 5% sucrose water was added to a container. 10 mL of gabapentin oral solution [50 mg/mL] (Amneal Pharmaceuticals, U.S.) was added to this water in the container, and the container was shaken until the solution was homogeneous (5 mg/ml). 20 mL of this solution was loaded into a 50 mL conical tube and a nozzle with a long spout was placed as the cap so the animals could drink the solution ad libitum overnight. The amount of Gabapentin water consumed by a rat was determined by subtracting the amount of drink water left from the initial 20 ml loaded.

Anterograde forelimb CST tracer injections

All animals received injections of AAV8 viral vectors expressing membrane targeted red fluorescent protein (RFP) under control of CAG promoter at titer of $5x10^{12}$ transducing units/mL into the forelimb motor cortex in the brain in both right and left hemispheres. 2 µL of viral vectors was injected into 5 sites per primary forelimb motor hemisphere (0.4 µL/site). The coordinates were as follows: anterior-posterior (mm)/medial-lateral (mm) (2.8/±2.2; 1.5/±2.3, ±3; 0.8/±2.3, ±3) for the left (+mm) and right (-mm) hemispheres, respectfully (**Figure 5**). All injections were done at a depth of 1.2mm dorsoventral.

Perfusion

All rats were perfused with 4% paraformaldehyde three weeks following their cortical brain injections. Their central nervous system tissue was harvested and stored in 30% sucrose

until histological processing.

Histology and Immunohistochemistry

All spinal cords were blocked 5 mm caudal and 5 mm rostral to the SCI/graft site and sectioned into 30 μm sagittal sections on a microtome. From the remaining rostral section of the tissue, another block 1 mm above the graft site at C2 level was sectioned into 30 μm coronal sections on a microtome. Sections were stored in a 24 well plate in tissue collection solution (TCS) buffer at 4°C. 1-in-3 series free-floating sections were incubated with primary antibodies from chicken against GFP (at 1:1000 to label graft cells), rabbit against RFP (at 1:500 to label CST axons) and guinea pig against NeuN (at 1:2000 to label neurons) for 3 days at 4°C. Sections were then incubated in Alexa 488 (anti-chicken, 1:500), Alexa 647 (anti-guinea pig 1:500), and Alexa 568 (anti-rabbit 1:1000) conjugated donkey secondary antibodies for one day at 4°C. The sections were then washed, and cover slipped with a mounting medium.

The spinal cords of the three intact animals were blocked at C4, C8, T6, and L3 in 1 mm segments and sectioned into 30 μm coronal sections on a microtome. Sections were stored in a 24 well plate in TCS buffer at 4°C. 1-in-6 series free-floating sections were incubated with primary antibodies from rabbit against RFP (at 1:500 to label CST axons) and guinea pig against NeuN (at 1:2000 to label neurons) for 3 days at 4°C. Sections were then incubated in Alexa 488 (anti-guinea pig, 1:500) and Alexa 568 (anti-rabbit, 1:1000) conjugated donkey secondary antibodies for one day at 4°C. The sections were washed, mounted and cover slipped as above.

The brains of the two animals perfused early on were blocked in 2 mm segments containing the rostral forelimb region and sectioned into 30 μm coronal sections on a microtome. Sections were stored in a 12 well plate in TCS buffer at 4°C. 1-in-6 series free-floating sections were incubated with primary antibodies from rabbit against C-Fos (at 1:3000 to label neurons)

for 3 days at 4°C. Sections were then incubated in Goat HRP (anti-rabbit, 1:200) secondary antibody for 1 day at 4°C. The sections were incubated for 10 minutes in DAB Substrate Kit, Peroxidase with Nickel (Vector Laboratories, U.S.). The sections were washed and mounted as above, dehydrated through a series of graded ethanol, and cover slipped with permount.

Imaging

All microscope slides originally labeled with the animal number were covered with random letters of the alphabet to prevent bias in later quantification steps. The letters were removed, and the images were decoded after quantification was complete. Images of the medial corticospinal tract sagittal sections were taken using CellSens on a fluorescent BX53 Olympus microscope at 40x and 100x magnification. The images of RFP-labeled corticospinal tract were taken using a red channel and the images of GFP labeled neural progenitor cell graft were taken using a green channel. The raw images were merged using the red and green channels in Photoshop.

Images of the corticospinal tract coronal sections at C2 level above SCI site were taken using CellSens on a fluorescence BX53 Olympus microscope at 200x magnification. The raw images were processed using ImageJ software. Images of the coronal brain sections were taken using CellSens on a fluorescence BX53 Olympus microscope at 100x magnification. The raw images were processed using ImageJ software.

Quantification

To quantify the number of regenerated F-CST axons into NPC graft, we used a modified method from Kadoya et al., 2016 to count the number of regenerated axon profiles at different locations crossing the rostrocaudal axis of the NPC graft. Two medial sections containing the main tract of CST were selected. A virtual line was drawn mediolaterally onto 40x images at

distance of 0.25, 0.5, 1, 1.5, 2mm from the rostral host/graft interface and any regenerated RFP-labeled CST axonal profiles that touched the line within NPC graft were counted. The average of the two section counts per animal was calculated and then used for the comparison between experimental group (n=6) and control group (n=6).

The number of CST axons in the main tract per coronal section (C2) above SCI were counted using the analyze particles function of ImageJ software (8 pixel threshold) as a normalization factor to count for different CST anterograde labeling efficiency. This was done for two of the most symmetrically-labeled coronal sections per animal. The average of the two section counts per animal was calculated. The average of the regenerated raw axon counts per animal were divided with the raw number of axons labeled in the main tract of the CST from C2 coronal sections for that animal to obtain normalized regenerated CST axons, or CST regeneration index as referenced in Schwab, 1990.

Statistics

The number of regenerated CST between the experimental group that received Gabapentin and the control group was compared using a two-tailed Student T test. All statistics were performed by JMP software (SAS, Cary, North Carolina) with a designated significance level of 95%. Data are presented as mean ± s.e.m. As stated above, the observer was blinded to group identity while performing quantifications.

For each animal in the experimental group that received gabapentin, the number of axons that regenerated into the neural progenitor cell graft was tested for bivariate fit against the average amount of gabapentin consumed each night. All statistics were performed by JMP Software (SAD, Cary, North Carolina) with a designated significance level of 95%.

RESULTS

Specific anterograde labeling of F-CST (3 intact rats with labeling)

The CST are topographically divided into different regions that control forelimb and hindlimb movement (Oza et al., 2015). Therefore, the F-CST can be specifically traced by injection of AAV-expressing RFP into the rostral motor cortex area that controls forelimb movement. Three intact rats were used to test whether injection of anterograde AAV expressing RFP in the forelimb region of their brain as described in the methods (**Figure 5**) can specifically label F-CST. **Figure 6** demonstrates specific labeling of the main tract of CST in the dorsal column at cervical level, including C4 (**Figure 6A**) and C8 (**Figure 6B**) region. There were only a few sparsely labeled CST axons in the mid-thoracic level (T6) (**Figure 6C**) and there was no labeling of the F-CST in the lumbar region (**Figure 6D**). These results indicate that our coordinates for the cortical injections in the F-CST region of the brain were highly specific for F-CST axons. Therefore, our axonal labeling and quantification of the experimental animals receiving gabapentin and their respective controls is representative of F-CST axons with little extraneous labeling.

Gabapentin trended toward promoting moderate F-CST regeneration

To test our hypothesis that silencing synaptic activity from those F-CST collaterals above SCI site to enable their regeneration into NPC graft placed in SCI, we transiently administered Gabapentin which can block alpha2delta2 involving in synaptic activity for two weeks after SCI and NPC grafting. We chose transient and reversible synapse silencing because chronic synaptic silencing may entirely suppress neuronal activity and prevent newly regenerated F-CST axons from forming synaptic connections with grafted neurons. To maximize the effect of Gabapentin, we chose a high dosage of Gabapentin in human clinical practice (3600 mg/kg/day) and

converted it to rat dosage of about 400 mg/kg/day for three-time injections during daytime (Nair et al., 2016). In addition, we administered Gabapentin in drink water overnight to ensure continuous suppression of synaptic activity during night-time.

Due to the voluntary nature of Gabapentin water consumption, it was important to track the amount of Gabapentin each individual animal consumed. The data in **Figure 7** show that water consumption per animal varied greatly each night. Furthermore, the average water consumption across all animals over two weeks varied from 1 mL to 18 mL per night. This indicates that animals were receiving at least 35 mg/kg, and at most 500 mg/kg of Gabapentin overnight. Given the large variability in overnight water consumption, it is possible that the regeneration of the corticospinal tract could vary across the experimental animal group.

Histological analysis showed rare regeneration of F-CST axons into the NPC graft in the control group without Gabapentin administration, confirming the early preliminary experimental results (**Figure 8A, C**). In the experimental group receiving Gabapentin, no robust regeneration of F-CST was observed (**Figure 8B, D**) as reported in our previous studies (Kadoya et al., 2016; Dulin et al., 2018; Poplawski et al., 2020). However, there was a moderate regeneration in the experimental group receiving Gabapentin (**Figure 8B, D**). The length of regenerated F-CST axons was mostly within 1 mm into the NPC graft although a few regenerated axons reached as far as 2 mm into the graft.

We then did a statistical comparison of the regeneration of the F-CST axons between the experimental group treated with gabapentin (orange) with the control group (blue) (**Figure 9**). Although the number of regenerated F-CST axons appeared high in several distances in the experimental group compared to the control, statistical analysis showed these did not reach a significant difference except at a distance of 1 mm (**Figure 9A**). A similar result was obtained

when the normalized data of regenerated F-CST was analyzed, wherein the raw data was divided by the average number of axons labeled in the coronal C2 sections. The data indicate that Gabapentin administered at the dosage and frequency in this study did not promote F-CST axon regeneration although there was a trend toward more regeneration compared to the control group.

We performed another statistical correlation analysis of the number of regenerated F-CST axons with the amount of gabapentin water consumed in each experimental animal overnight (**Figure 10**). Although there was a negative correlation of -0.43, statistical analysis showed that there was no strong correlation between the two. The data indicate that the amount of gabapentin water consumed overnight by each experimental animal did not have a significant effect on the regeneration of the axons of the F-CST.

Examination of neuronal activity

One hour after the last injection of gabapentin for the experimental group at 9am, one experimental rat and one control rat were perfused. Their central nervous system tissue was harvested as described in the methods and stained for neuronal activity with C-Fos. As expected, the control animal exhibited dense and dark staining in the neurons of cortical layer 5, indicating normal cortical neuronal activity (**Figure 11A**) (Perrin-Terrin, et al., 2016). The experimental animal showed similar C-Fos staining as the control and there was no apparent difference of C-Fos staining pattern between the experimental group and control (**Figure 11B**). This indicates that gabapentin may not effectively silence normal neuronal activity of cortical layer 5 neurons on a broad spectrum. Besides neuronal activity observed using C-Fos staining, whether Gabapentin effectively silences synaptic activity of F-CST at axonal terminals is unknown.

FIGURES

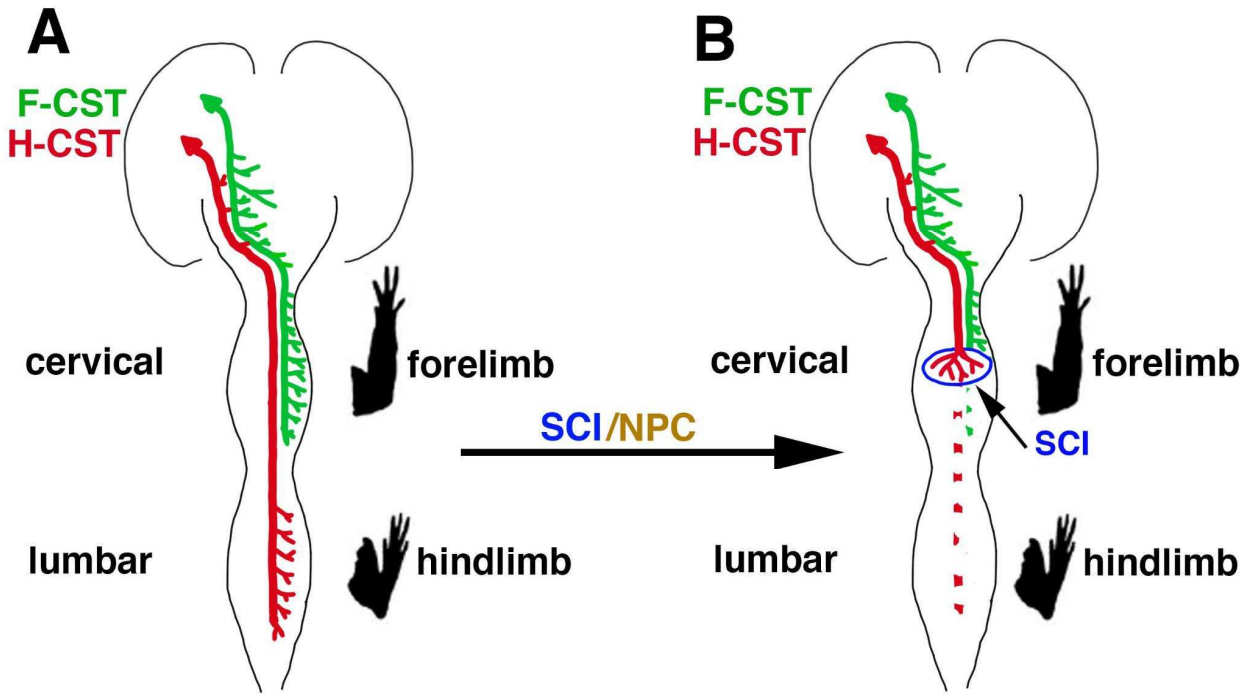

Figure 1: F-CST and H-CST Innervation of the spinal cord before and after SCI and NPC Graft. (**A**) shows collaterals in the brain and their innervation of the forelimb corticospinal tract (F-CST) and hindlimb corticospinal tract (H-CST) in an intact rat model. (**B**) shows the regenerative capabilities of the H-CST following a mid-cervical spinal cord injury and repair with neural progenitor cell graft, though not the F-CST (Adapted from Lu, P. Unpublished data).

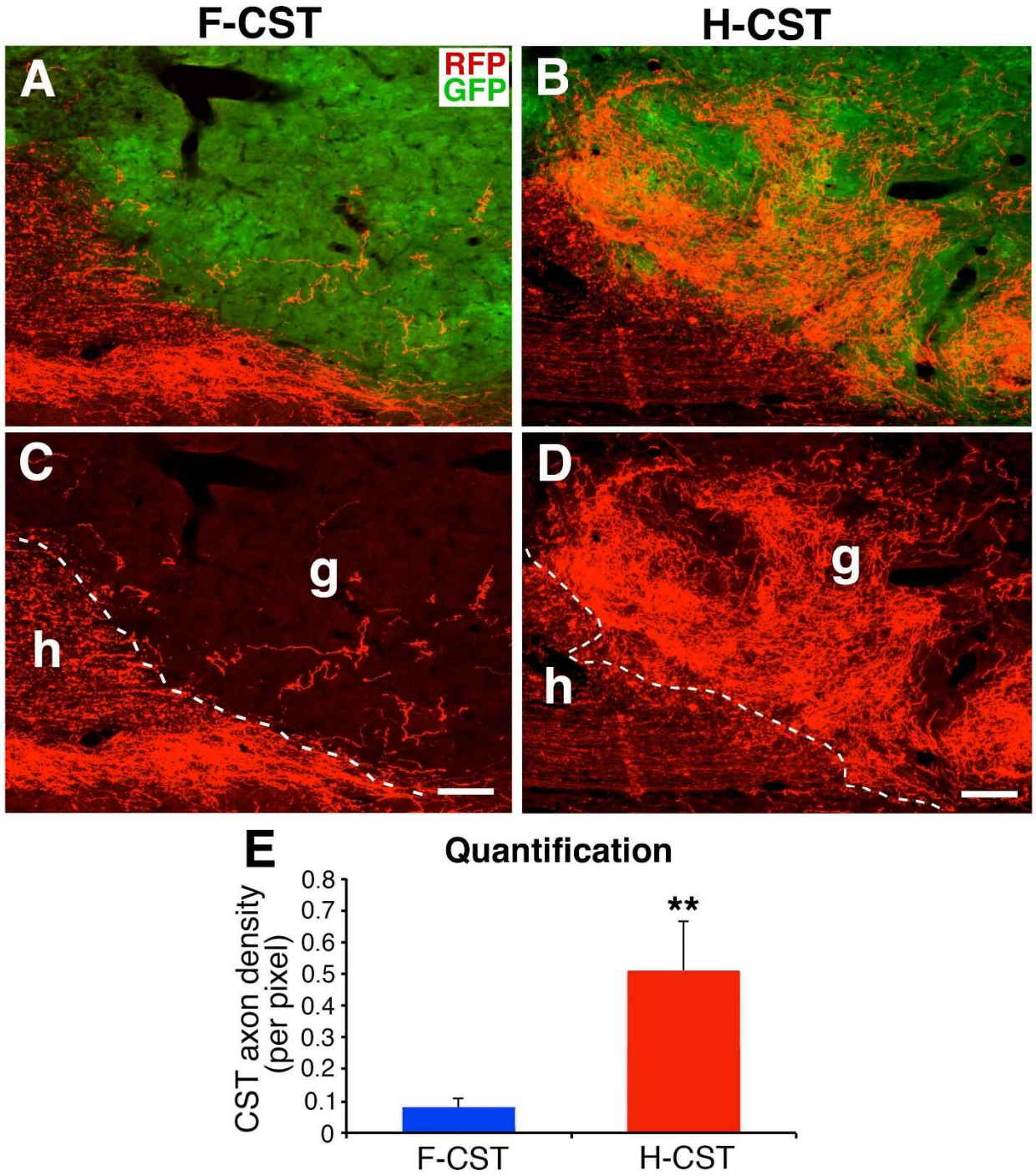

Figure 2: Preferential H-CST regeneration into cervical NPC graft. (**A, C**) Rare regeneration of RFP labeled F-CST axons into GFP labeled NPC graft in a sagittal section view. Dashed lines indicate host (h)-graft(g) interface. (**B, D**) Robust regeneration of H-CST into NPC graft. Scale = 120 µm. (**E**) Quantification of regenerated CST in NPC graft (normalized with CST density at C1 cross section, **p<0.01, student T-test, N = 7/group). (Adapted from Lu, P. Unpublished data).

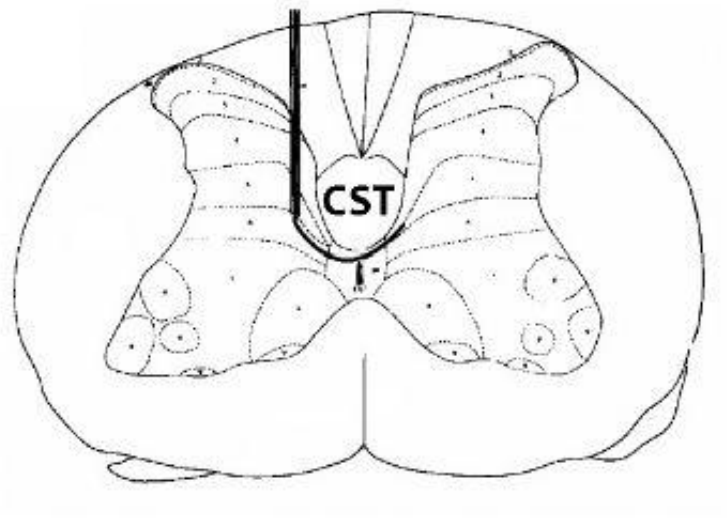

Figure 3: Wireknife hook in spinal cord lesion model. Shows a wireknife hook that can transect the main corticospinal tract in the dorsal column of a cervical spinal cord injury model. Adapted from Weidner et al., 1999.

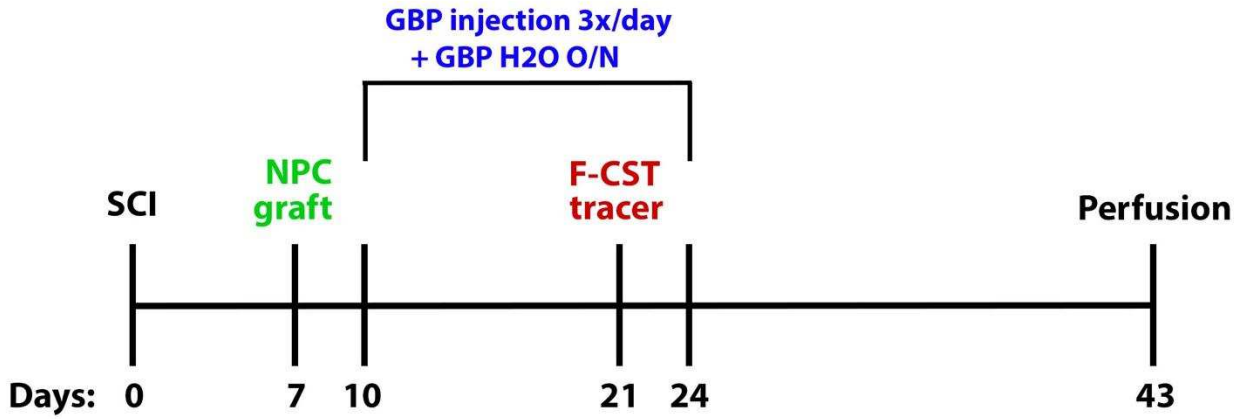

Figure 4: Experimental timeline. Shows day 0 as the day of SCI, 1 week later as NPC grafting, 3 days after NPC grafting experimental animals began receiving gabapentin for 2 weeks, 2 weeks after NPC grafting all animals received cortical F-CST tracer injections and survived additional 3 weeks when all animals were sacrificed.

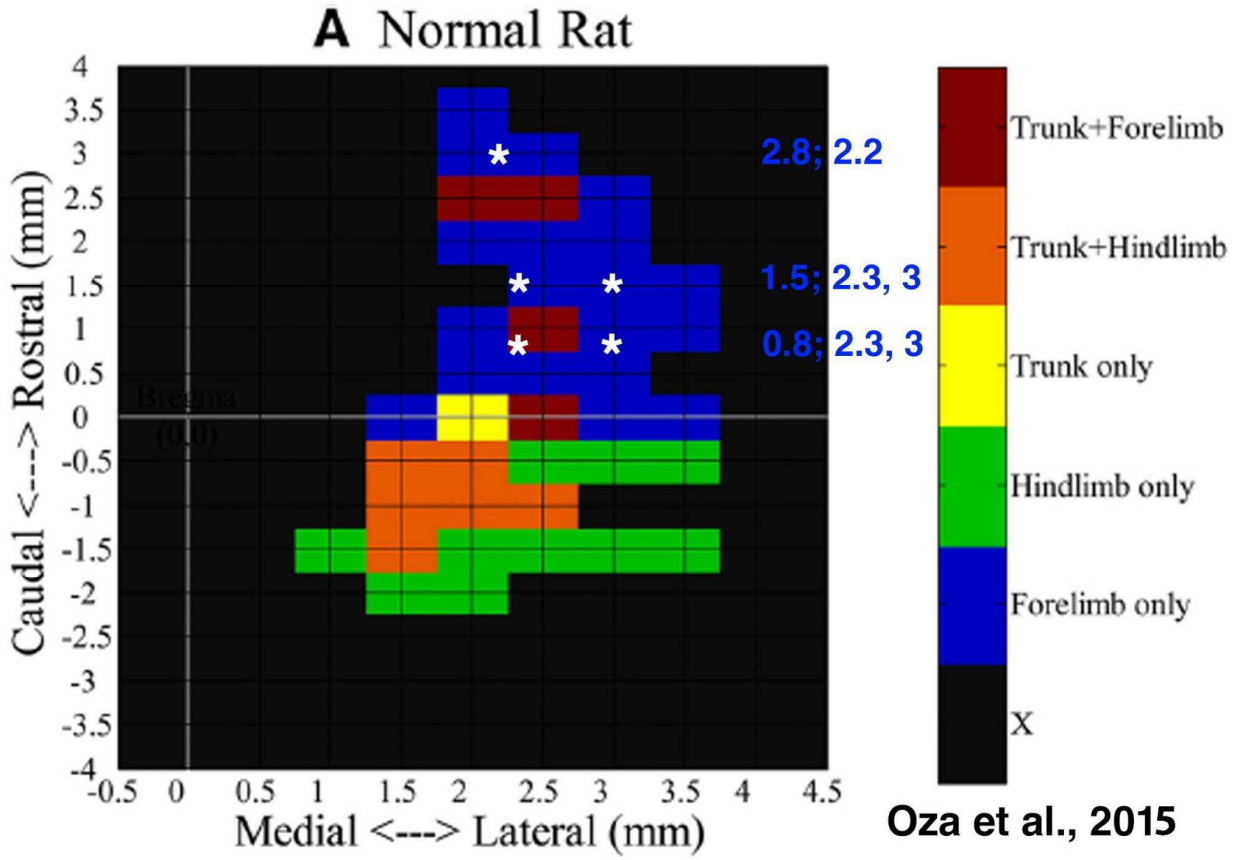

Figure 5: Cortical Map for F-CST 5-point injection in one hemisphere. Shows the coordinates were as follows: anterior-posterior (mm)/medial-lateral (mm) (2.8/±2.2; 1.5/±2.3, ±3; 0.8/±2.3, ±3) for the left (+mm) and right (-mm) hemispheres, respectfully. Adapted from Oza and Giszter, 2015.

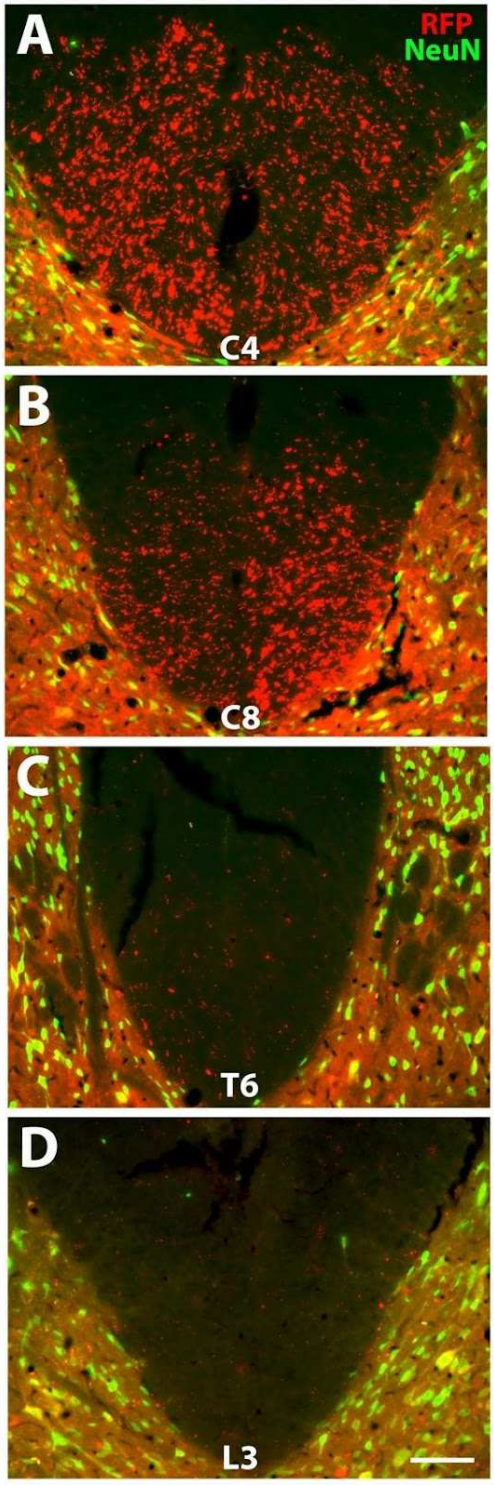

Figure 6: Expression of F-CST tracer in cervical spinal cord. (**A-B**) shows strong RFP-expressing F-CST in the cervical level (C-4 and C-8) of the spinal cord. (**C**) shows only a few RFP-expressing F-CST in the mid-thoracic level (T-6) of the spinal cord. (**D**) shows no RFP-expressing F-CST in the mid-lumbar (L-3) level of the spinal cord. Scale bar = 60 μm.

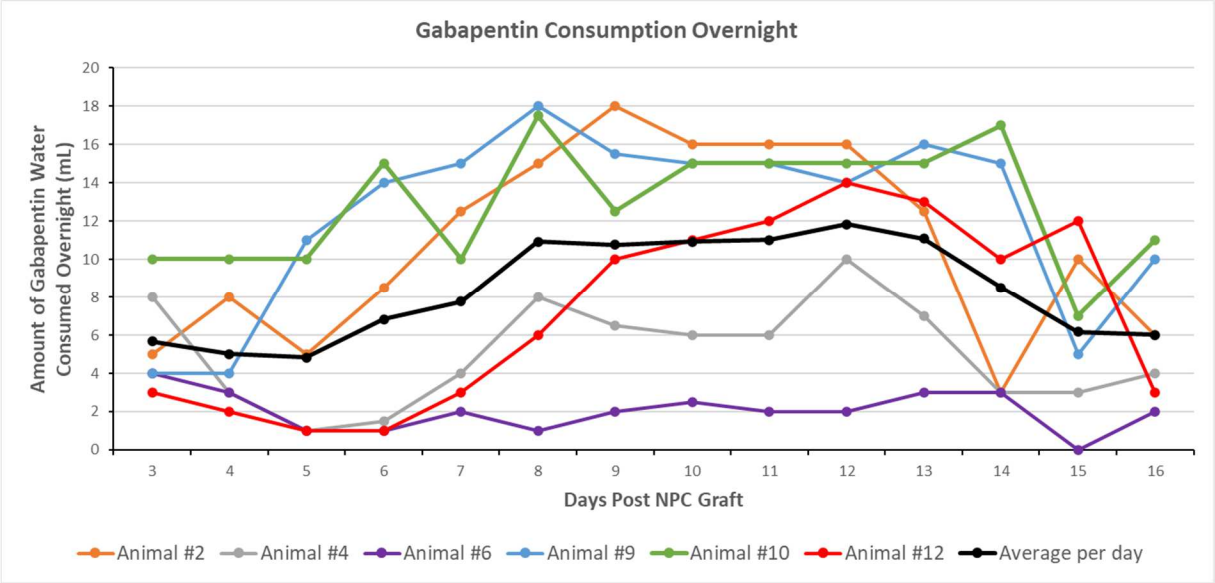

Figure 7: Gabapentin administration in the experimental rat group. Beginning 3 days after neural progenitor cell (NPC) grafting, 6 experimental rats were given gabapentin water ad libitum overnight from 5pm-9am for 14 days in addition to injections during day-time. Gabapentin water [5 mg/mL] was given in conical tubes, and consumption was measured for each rat for 14 days. Consumption of Gabapentin water over one night for one experimental rat is represented by each colored line. The black dotted line represents the average amount of Gabapentin water consumed each night across all 6 experimental animals. Lines connecting the data help visualize the trend of the consumption of Gabapentin water among the experimental rats.

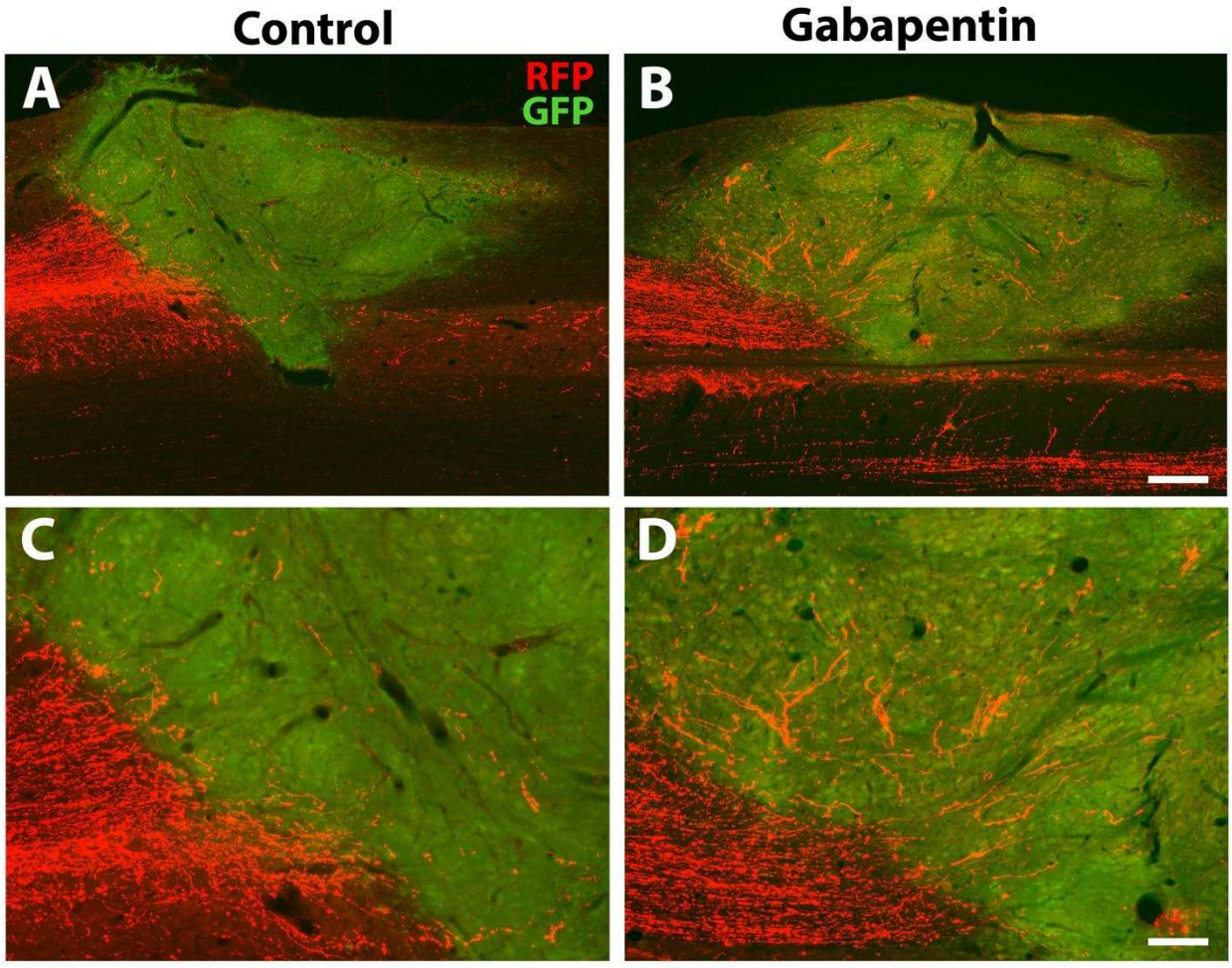

Figure 8: Moderate regeneration of the F-CST into NPC Graft
RFP and GFP double immunolabeling shows (**A, C**) rare regeneration of RFP-labeled F-CST into GFP-expressing NPC graft placed in a C4 dorsal column lesion site in a control subject and (**B, D**) moderate regeneration of F-CST into NPC graft in an experimental subject that received Gabapentin for two weeks. Scale bar = 3 mm (**A-B**); 60 μm (**C-D**).

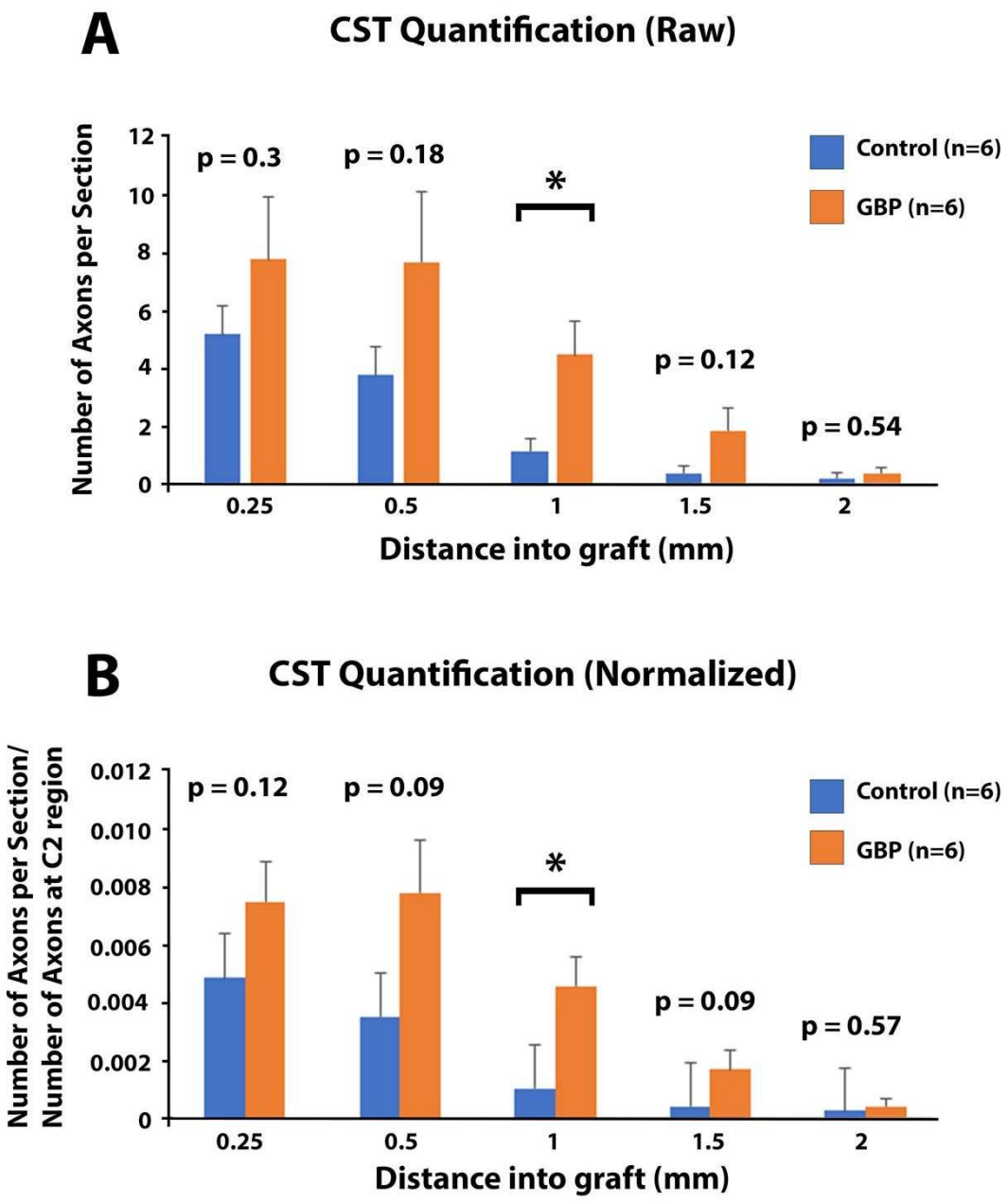

Figure 9: Quantification of CST axons regeneration. (**A**) Comparison of the raw average number of axon profiles crossing a virtual line per section at distances of 0.25 mm, 0.5 mm, 1 mm, 1.5 mm, and 2 mm into the graft for both control (n=6) and experimental groups (n=6). There is a statistically significant difference between the control and experimental group at a distance of 1 mm. (**B**) Comparison of the normalized average of number of axons per section measured at distances of 0.25 mm, 0.5 mm, 1 mm, 1.5 mm, and 2 mm into the graft divided by the number of axons in the main tract of CST at C2 level. There is a statistically significant difference between the control and experimental group at a distance of 1 mm into the graft.

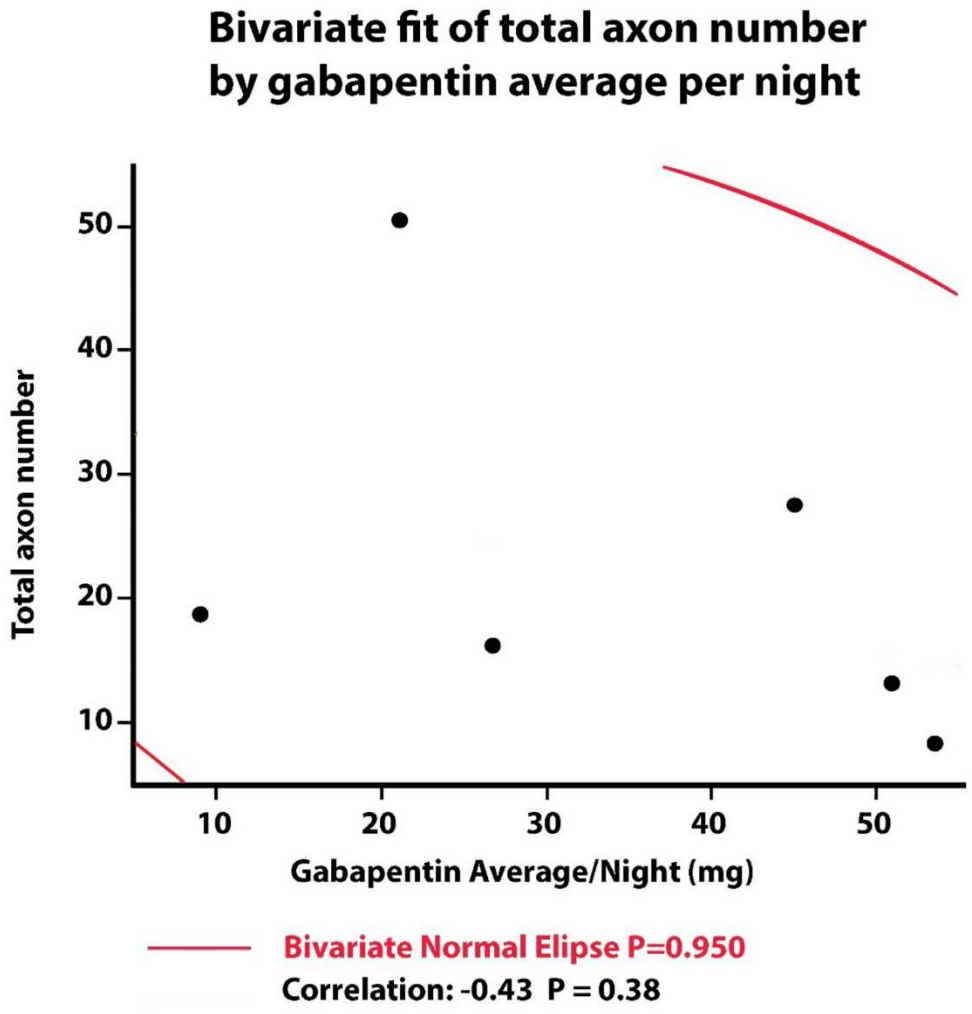

Figure 10: Bivariate fit of total axon number by gabapentin average per night. Shows there is no strong correlation between number of axons of the F-CST that regenerated into the neural progenitor cell graft and the amount of Gabapentin water consumed by each experimental rat during the night.

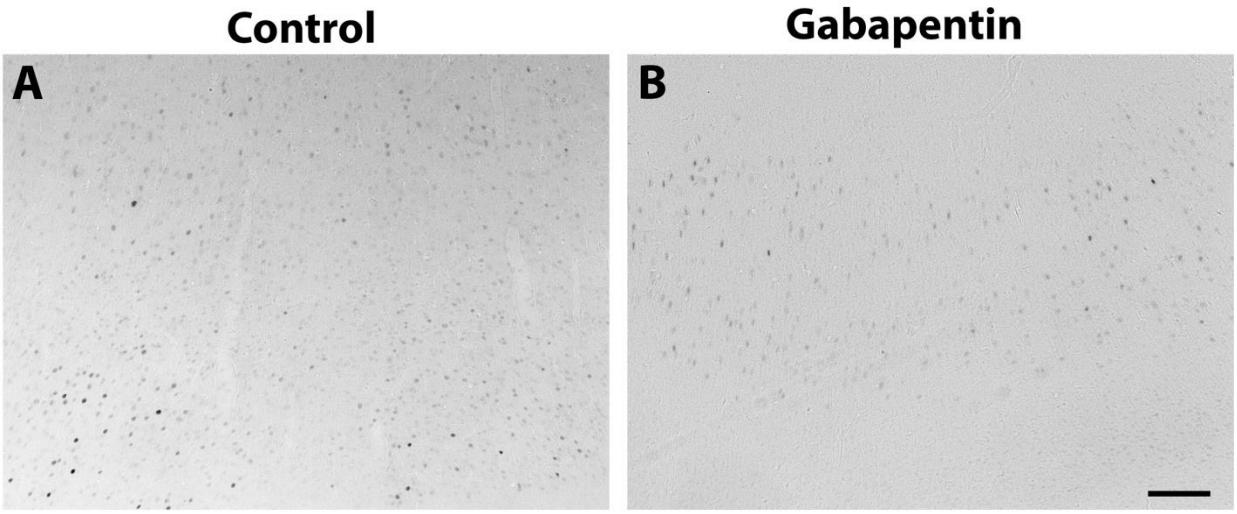

Figure 11: C-Fos staining of glutamatergic neurons in the motor cortex. (**A**) Shows C-Fos immunolabeling in a coronal brain section in a control rat. (**B**) C-Fos immunolabeling in a coronal brain section in an experimental subject 1 hour following the final gabapentin subcutaneous injection. Scale bar = 60 μm.

DISCUSSION

Summary

Gabapentin administration did not promote robust regeneration of the forelimb corticospinal tract (F-CST) but trended toward enhanced regeneration following mid-cervical spinal cord injury (SCI) and neural progenitor cell (NPC) grafting comparing to control group (**Figure 9**). Gabapentin subcutaneous injections were consistent and may have sufficiently suppressed F-CST neuronal synaptic activity temporally during the day. Gabapentin water consumption orally, however, was highly variable and overnight suppression of F-CST synaptic activity may not have been as successful as it could have been (**Figure 11**). Therefore, it is unclear whether Gabapentin consistently and continuously suppressed synaptic activity of F-CST to promote their regeneration. It is possible that we could further increase the regeneration of the F-CST with consistent and continuous inhibition of their remaining synaptic activity. This could be done with stronger pharmacological agents that are more continuously metabolized throughout the day and night.

Problems

Gabapentin blocks A2D2:

Mechanistically, Gabapentin targets the alpha2delta2 (A2D2) subunit of the Alpha2delta protein which is responsible for regulating neuronal synaptic activity (Sun et al. 2020). Our work proposes that Gabapentin administration silences the neuronal synaptic activity regulated by the A2D2 subunit, which could release synaptic suppression of axon regeneration and promote F-CST regeneration. It is possible that Gabapentin alone is not enough to silence F-CST neuronal synapses. Recent data suggest that Gabapentin can only inhibit the A2D2 subunit of the Alpha2delta protein that has multiple redundant subunits, such as alpha2delta1 and alpha2delta3

that are not functionally impaired (Schöpf, et al., 2021). Knock down all three subunits with shRNA approach could completely silence synaptic activity of F-CST to promote their regeneration after SCI (Schöpf, et al., 2021).

Gabapentin concentration fluctuation:

One factor which may explain the results we observed is the fluctuation in gabapentin concentration throughout the subjects' bodies. It is possible that the neuronal synapses of F-CST in the rats were not being continually silenced throughout the day nor night due to the unpredictable pharmacokinetics of gabapentin metabolism (Bockbrader, et al., 2010). During the daytime, the rats received a subcutaneous injection of 133 mg/kg of Gabapentin every 4 hours from 9am to 5pm, but based on pharmacokinetics, it is likely that the plasma concentration of gabapentin peaked approximately 1-2 hours after each injection (Mills et al., 2020). This could have led to periods of low concentration of Gabapentin which could not sustain synaptic inhibition, and may therefore have a less pronounced effect on our results.

Furthermore, the overnight period was even more variable in gabapentin water consumption. We could not ensure that the rats were drinking a minimum amount of gabapentin water every night from 5pm to 9am, nor could we control when they were drinking this water. It is likely that the plasma concentration of gabapentin peaked around 3 hours post oral dose, and eventually fell again due to the slow rate of absorption (Bockbrader et al., 2010). It is possible that if a subject only consumed 3 mL of gabapentin water at 5pm, then they had a subclinical dose of gabapentin for most of the night-time.

How specific is Gabapentin to F-CST:

Given our selected methods of gabapentin administration, both subcutaneously and orally, we cannot be certain that the mechanistic actions of gabapentin as an inhibitor of

excitatory synapses were specific to neurons of the F-CST. While our method of drug delivery was clinically relevant (Usach et al., 2019), it was not necessarily specific to the neurons of the forelimb region of brain and corticospinal tract. In the future, we could quantify the concentration of gabapentin in the forelimb region of the brain via an alpha2delta2 ELISA assay. This could help us analyze whether the amount of gabapentin circulating in the forelimb region of the brain is at a therapeutic dose to silence neuronal synapses. If it is not, then we could potentially follow up with another experiment that targets the F-CST more specifically, such as intracranial delivery using an osmotic pump.

Side effects:

One important side effect to note from gabapentin is its ability to cause drowsiness. By administering the gabapentin subcutaneously three times a day, it is possible that the subjects could have been mildly sedated by the medication. The drowsiness the subjects experienced could have had an effect on their normal behavior and even contributed to the lesser overnight water consumption.

Sample size:

An important caveat to consider with our experiment is the small sample size. Only 12 animals in total were included in our analysis, and 6 animals per group is trivial for determining statistical significance. While we can conclude that there was a trend toward greater regeneration of the F-CST with the experimental group receiving gabapentin, we cannot draw any conclusions. Given that the sample size was not very large, we would need to repeat this experiment in order to draw any further conclusions.

New Strategies

TTX:

One future direction that we are currently pursuing is silencing F-CST neuronal synapses with a stronger pharmacological drug, tetrodotoxin (TTX). TTX has been shown to transiently silence neuronal synapses in the brain following models of stroke (Lysko et al., 1994). Following a period of brain hyperactivity like stroke or ischemia, TTX administration was noted in some studies to elicit functional recovery (Graber and Prince, 2004; Bucciarelli et al., 2021). TTX may be a more potent and therefore more effective inhibitor of F-CST neuronal synapses. In our case, this could mean that TTX could prevent synaptic activity signals from sustaining collaterals. By better inhibiting these signals, it is possible that we could enhance the regeneration of the F-CST. Furthermore, implanting a mini-osmotic pump filled with TTX directly into the forelimb region of the brain could provide more specific targeting of F-CST neurons.

Inhibitory DREADDs:

Another strategy to silence F-CST neuronal synapses is the use of a highly specific inhibitory designer receptor exclusively activated by designer drug (DREADD) receptor hM4Di. A recent study from our lab has demonstrated the ability of hM4Di to silence neuronal activity in the F-CST in behavioral and ex vivo electrophysiological testing (Biane et al., 2019). This result indicates the potential of hM4Di to be highly specific and transient in silencing neuronal synapses. This leaves another option available to pursue future studies in silencing sustaining collaterals of the F-CST.

Botox:

One more strategy that can be employed to silence neuronal synapses in the F-CST region of the brain is botulinum toxin (Botox). A recent study demonstrated that botulinum

neurotoxin serotype A could silence neurons responsible for relaying pain from the spinal cord to the brain (Maiaru, et al. 2018). The advantage of Botox is its long-lasting effect and it is reversible. A single injection of Botox is highly specific and transient (weeks to months), a key aspect that we could utilize in silencing F-CST neurons.

Alternative approach PTEN/SOCS3 and delivery of reprogramming genes:

There is an alternative approach to enhancing F-CST regeneration that should be considered if the synaptic suppression of regeneration hypothesis is incorrect. Knocking down PTEN/SOCS3 is still a viable option that targets the intrinsic issue of the environment for CNS regeneration. Liu et al., 2010 showed increased CST axon sprouting and regeneration in a model of spinal cord injury with PTEN deletion and mTOR enhancement. These results suggest that adult CST neurons can regain some regenerative capabilities after deletion of PTEN and upregulation of mTOR as immature CST neurons. Sun et al., 2011 demonstrated the synergistic effect of double deletion of PTEN/SOCS3 in a model of optic nerve injury on axonal regeneration. Their data indicate that the two pathways affected by PTEN/SOCS3 deletion which resulted in enhanced axonal regeneration could provide a solution to enhance F-CST regeneration after SCI. We could also try a new approach from a recent study (Lu et al., 2020) by ectopic expression of 3 out 4 reprogramming genes for generation of induced pluripotent stem cells (iPSCs): Oct4, Sox2 and Klf4 that restores youthful DNA methylation patterns and transcriptomes, to promote F-CST regeneration after cervical SCI.

REFERENCES

Afshari FT, Kappagantula S, Fawcett JW. Extrinsic and intrinsic factors controlling axonal regeneration after spinal cord injury. Expert Rev Mol Med. 2009 Dec 8;11:e37.

Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms. Front Neurol. 2019 Mar 22;10:282.

Anderson KD. Targeting recovery: priorities of the spinal cord-injured population. J Neurotrauma. 2004 Oct;21(10):1371-83.

Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, Coppola G, Khakh BS, Deming TJ, Sofroniew MV. Astrocyte scar formation aids central nervous system axon regeneration. Nature. 2016 Apr 14;532(7598):195-200.

Bennett J, M Das J, Emmady PD. Spinal Cord Injuries. 2022 May 11. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan–.

Biane JS, Takashima Y, Scanziani M, Conner JM, Tuszynski MH. Reorganization of Recurrent Layer 5 Corticospinal Networks Following Adult Motor Training. J Neurosci. 2019 Jun 12;39(24):4684-4693.

Blackmore MG, Wang Z, Lerch JK, Motti D, Zhang YP, Shields CB, Lee JK, Goldberg JL, Lemmon VP, Bixby JL. Krüppel-like Factor 7 engineered for transcriptional activation promotes axon regeneration in the adult corticospinal tract. Proc Natl Acad Sci U S A. 2012 May 8;109(19):7517-22.

Bockbrader HN, Wesche D, Miller R, Chapel S, Janiczek N, Burger P. A comparison of the pharmacokinetics and pharmacodynamics of pregabalin and gabapentin. Clin Pharmacokinet. 2010 Oct;49(10):661-9.

Bucciarelli GM, Lechner M, Fontes A, Kats LB, Eisthen HL, Shaffer HB. From Poison to Promise: The Evolution of Tetrodotoxin and Its Potential as a Therapeutic. Toxins (Basel). 2021 Jul 24;13(8):517.

Ceto S, Sekiguchi KJ, Takashima Y, Nimmerjahn A, Tuszynski MH. Neural Stem Cell Grafts Form Extensive Synaptic Networks that Integrate with Host Circuits after Spinal Cord Injury. Cell Stem Cell. 2020 Sep 3;27(3):430-440.e5.

Dulin JN, Adler AF, Kumamaru H, Poplawski GHD, Lee-Kubli C, Strobl H, Gibbs D, Kadoya K, Fawcett JW, Lu P, Tuszynski MH. Injured adult motor and sensory axons regenerate into appropriate organotypic domains of neural progenitor grafts. Nat Commun. 2018 Jan 8;9(1):84.

Escartin C, Galea E, Lakatos A, O'Callaghan JP, Petzold GC, Serrano-Pozo A, Steinhäuser C, Volterra A, Carmignoto G, Agarwal A, Allen NJ, Araque A, Barbeito L, Barzilai A, Bergles DE, Bonvento G, Butt AM, Chen WT, Cohen-Salmon M, Cunningham C, Deneen B, De Strooper B,

Díaz-Castro B, Farina C, Freeman M, Gallo V, Goldman JE, Goldman SA, Götz M, Gutiérrez A, Haydon PG, Heiland DH, Hol EM, Holt MG, Iino M, Kastanenka KV, Kettenmann H, Khakh BS, Koizumi S, Lee CJ, Liddelow SA, MacVicar BA, Magistretti P, Messing A, Mishra A, Molofsky AV, Murai KK, Norris CM, Okada S, Oliet SHR, Oliveira JF, Panatier A, Parpura V, Pekna M, Pekny M, Pellerin L, Perea G, Pérez-Nievas BG, Pfrieger FW, Poskanzer KE, Quintana FJ, Ransohoff RM, Riquelme-Perez M, Robel S, Rose CR, Rothstein JD, Rouach N, Rowitch DH, Semyanov A, Sirko S, Sontheimer H, Swanson RA, Vitorica J, Wanner IB, Wood LB, Wu J, Zheng B, Zimmer ER, Zorec R, Sofroniew MV, Verkhratsky A. Reactive astrocyte nomenclature, definitions, and future directions. Nat Neurosci. 2021 Mar;24(3):312-325.

Geoffroy CG, Lorenzana AO, Kwan JP, Lin K, Ghassemi O, Ma A, Xu N, Creger D, Liu K, He Z, Zheng B. Effects of PTEN and Nogo Codeletion on Corticospinal Axon Sprouting and Regeneration in Mice. J Neurosci. 2015 Apr 22;35(16):6413-28.

Graber KD, Prince DA. A critical period for prevention of posttraumatic neocortical hyperexcitability in rats. Ann Neurol. 2004 Jun;55(6):860-70.

He Z, Jin Y. Intrinsic Control of Axon Regeneration. Neuron. 2016 May 4;90(3):437-51.

Hilton BJ, Husch A, Schaffran B, Lin TC, Burnside ER, Dupraz S, Schelski M, Kim J, Müller JA, Schoch S, Imig C, Brose N, Bradke F. An active vesicle priming machinery suppresses axon regeneration upon adult CNS injury. Neuron. 2022 Jan 5;110(1):51-69.e7.

Jin D, Liu Y, Sun F, Wang X, Liu X, He Z. Restoration of skilled locomotion by sprouting corticospinal axons induced by co-deletion of PTEN and SOCS3. Nat Commun. 2015 Nov 24;6:8074.

Kadoya K, Lu P, Nguyen K, Lee-Kubli C, Kumamaru H, Yao L, Knackert J, Poplawski G, Dulin JN, Strobl H, Takashima Y, Biane J, Conner J, Zhang SC, Tuszynski MH. Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. Nat Med. 2016 May;22(5):479-87.

Kumamaru H, Lu P, Rosenzweig ES, Kadoya K, Tuszynski MH. Regenerating Corticospinal Axons Innervate Phenotypically Appropriate Neurons within Neural Stem Cell Grafts. Cell Rep. 2019 Feb 26;26(9):2329-2339.e4.

Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, Tedeschi A, Park KK, Jin D, Cai B, Xu B, Connolly L, Steward O, Zheng B, He Z. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. Nat Neurosci. 2010 Sep;13(9):1075-81.

Lorenzana AO, Lee JK, Mui M, Chang A, Zheng B. A surviving intact branch stabilizes remaining axon architecture after injury as revealed by in vivo imaging in the mouse spinal cord. Neuron. 2015 May 20;86(4):947-954.

Lu P, Graham L, Wang Y, Wu D, Tuszynski M. Promotion of survival and differentiation of neural stem cells with fibrin and growth factor cocktails after severe spinal cord injury. J Vis Exp. 2014 Jul 27;(89):e50641.

Lu P, Tuszynski MH. Introduction to neuroscience letters special issue: "Plasticity and regeneration after spinal cord injury". Neurosci Lett. 2017 Jun 23;652:1-2.

Lu P, Wang Y, Graham L, McHale K, Gao M, Wu D, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B, Conner JM, Marsala M, Tuszynski MH. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell. 2012 Sep 14;150(6):1264-73.

Lu Y, Brommer B, Tian X, Krishnan A, Meer M, Wang C, Vera DL, Zeng Q, Yu D, Bonkowski MS, Yang JH, Zhou S, Hoffmann EM, Karg MM, Schultz MB, Kane AE, Davidsohn N, Korobkina E, Chwalek K, Rajman LA, Church GM, Hochedlinger K, Gladyshev VN, Horvath S, Levine ME, Gregory-Ksander MS, Ksander BR, He Z, Sinclair DA. Reprogramming to recover youthful epigenetic information and restore vision. Nature. 2020 Dec;588(7836):124-129.

Lysko PG, Webb CL, Yue TL, Gu JL, Feuerstein G. Neuroprotective effects of tetrodotoxin as a Na+ channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbil global brain ischemia. Stroke. 1994 Dec;25(12):2476-82.

Maiarù M, Leese C, Certo M, Echeverria-Altuna I, Mangione AS, Arsenault J, Davletov B, Hunt SP. Selective neuronal silencing using synthetic botulinum molecules alleviates chronic pain in mice. Sci Transl Med. 2018 Jul 18;10(450):eaar7384.

Meves JM, Zheng B. Synaptic Suppression of Axon Regeneration. Neuron. 2016 Oct 19;92(2):267-269.

Mills PO, Tansey CO, Genzer SC, Mauldin MR, Howard RA, Kling CA, Jackson FR, Matheny AM, Boothe DM, Lathrop GW, Powell N, Gallardo-Romero N. Pharmacokinetic Profiles of Gabapentin after Oral and Subcutaneous Administration in Black-tailed Prairie Dogs (Cynomys ludovicianus). J Am Assoc Lab Anim Sci. 2020 May 1;59(3):305-309.

Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016 Mar;7(2):27-31.

National Spinal Cord Injury Statistical Center (NSCISC). Spinal cord injury facts and figures at a glance. 2020. Birmingham, AL: University of Alabama at Birmingham.

Oza CS, Giszter SF. Trunk robot rehabilitation training with active stepping reorganizes and enriches trunk motor cortex representations in spinal transected rats. J Neurosci. 2015 May 6;35(18):7174-89.

Park KW, Lin CY, Li K, Lee YS. Effects of Reducing Suppressors of Cytokine Signaling-3 (SOCS3) Expression on Dendritic Outgrowth and Demyelination after Spinal Cord Injury. PLoS One. 2015 Sep 18;10(9):e0138301.

Perrin-Terrin AS, Jeton F, Pichon A, Frugière A, Richalet JP, Bodineau L, Voituron N. The c-FOS Protein Immunohistological Detection: A Useful Tool As a Marker of Central Pathways Involved in Specific Physiological Responses In Vivo and Ex Vivo. J Vis Exp. 2016 Apr 25;(110):53613.

Polizzotto MN, Bartlett PF, Turnley AM. Expression of "suppressor of cytokine signalling" (SOCS) genes in the developing and adult mouse nervous system. J Comp Neurol. 2000 Jul 24;423(2):348-58.

Poplawski GHD, Kawaguchi R, Van Niekerk E, Lu P, Mehta N, Canete P, Lie R, Dragatsis I, Meves JM, Zheng B, Coppola G, Tuszynski MH. Injured adult neurons regress to an embryonic transcriptional growth state. Nature. 2020 May;581(7806):77-82.

Quintero GC. Review about gabapentin misuse, interactions, contraindications and side effects. J Exp Pharmacol. 2017 Feb 9;9:13-21.

Rosenzweig ES, Salegio EA, Liang JJ, Weber JL, Weinholtz CA, Brock JH, Moseanko R, Hawbecker S, Pender R, Cruzen CL, Iaci JF, Caggiano AO, Blight AR, Haenzi B, Huie JR, Havton LA, Nout-Lomas YS, Fawcett JW, Ferguson AR, Beattie MS, Bresnahan JC, Tuszynski MH. Chondroitinase improves anatomical and functional outcomes after primate spinal cord injury. Nat Neurosci. 2019 Aug;22(8):1269-1275.

Schöpf CL, Ablinger C, Geisler SM, Stanika RI, Campiglio M, Kaufmann WA, Nimmervoll B, Schlick B, Brockhaus J, Missler M, Shigemoto R, Obermair GJ. Presynaptic α2δ subunits are key organizers of glutamatergic synapses. Proc Natl Acad Sci U S A. 2021 Apr 6;118(14):e1920827118.

Schwab ME, Strittmatter SM. Nogo limits neural plasticity and recovery from injury. Curr Opin Neurobiol. 2014 Aug;27:53-60.

Schwab ME. Myelin-associated inhibitors of neurite growth and regeneration in the CNS. Trends Neurosci. 1990 Nov;13(11):452-6.

Schwab ME. Nogo and axon regeneration. Curr Opin Neurobiol. 2004 Feb;14(1):118-24.

Silver J, Miller JH. Regeneration beyond the glial scar. Nat Rev Neurosci. 2004 Feb;5(2):146-56.

Sofroniew MV. Dissecting spinal cord regeneration. Nature. 2018 May;557(7705):343-350.

Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, Zhang K, Yeung C, Feng G, Yankner BA, He Z. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature. 2011 Nov 6;480(7377):372-5.

Sun T, Duan L, Li J, Guo H, Xiong M. Gypenoside XVII protects against spinal cord injury in mice by regulating the microRNA□21□mediated PTEN/AKT/mTOR pathway. Int J Mol Med. 2021 Aug;48(2):146.

Sun W, Larson MJ, Kiyoshi CM, Annett AJ, Stalker WA, Peng J, Tedeschi A. Gabapentinoid treatment promotes corticospinal plasticity and regeneration following murine spinal cord injury. J Clin Invest. 2020 Jan 2;130(1):345-358.

Tedeschi A, Dupraz S, Laskowski CJ, Xue J, Ulas T, Beyer M, Schultze JL, Bradke F. The Calcium Channel Subunit Alpha2delta2 Suppresses Axon Regeneration in the Adult CNS. Neuron. 2016 Oct 19;92(2):419-434.

Tran AP, Warren PM, Silver J. New insights into glial scar formation after spinal cord injury. Cell Tissue Res. 2022 Mar;387(3):319-336.

Tran AP, Warren PM, Silver J. The Biology of Regeneration Failure and Success After Spinal Cord Injury. Physiol Rev. 2018 Apr 1;98(2):881-917.

Usach I, Martinez R, Festini T, Peris JE. Subcutaneous Injection of Drugs: Literature Review of Factors Influencing Pain Sensation at the Injection Site. Adv Ther. 2019 Nov;36(11):2986-2996.

Warner FM, Cragg JJ, Jutzeler CR, Grassner L, Mach O, Maier DD, Mach B, Schwab JM, Kopp MA, Kramer JLK. Association of timing of gabapentinoid use with motor recovery after spinal cord injury. Neurology. 2020 Dec 15;95(24):e3412-e3419.

Weidner N, Grill RJ, Tuszynski MH. Elimination of basal lamina and the collagen "scar" after spinal cord injury fails to augment corticospinal tract regeneration. Exp Neurol. 1999 Nov;160(1):40-50.

Wiegert JS, Mahn M, Prigge M, Printz Y, Yizhar O. Silencing Neurons: Tools, Applications, and Experimental Constraints. Neuron. 2017 Aug 2;95(3):504-529.

Zhu Y, Soderblom C, Trojanowsky M, Lee DH, Lee JK. Fibronectin Matrix Assembly after Spinal Cord Injury. J Neurotrauma. 2015 Aug 1;32(15):1158-67.