Recovery of forearm and fine digit function after chronic spinal cord injury by simultaneous blockade of inhibitory matrix CSPG production and the receptor PTPσ.

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Abstract

Spinal cord injuries, for which there are limited effective clinical treatments, result in enduring paralysis and hypoesthesia due, in part, to the inhibitory microenvironment that develops and limits regeneration/sprouting, especially during chronic stages. Recently, we discovered that targeted enzymatic modulation of the potently inhibitory chondroitin sulfate proteoglycan (CSPG) component of the extracellular and perineuronal net (PNN) matrix via Chondroitinase ABC (ChABC) can rapidly restore robust respiratory function to the previously paralyzed hemi-diaphragm after remarkably long times post-injury (up to 1.5 years) following a cervical level 2 lateral hemi-transection. Importantly, ChABC treatment at cervical level 4 in this chronic model also elicited rapid, albeit modest, improvements in upper arm function. In the present study, we sought to further optimize and elucidate the capacity for nerve sprouting and/or regeneration to restore gross as well as fine motor control of the forearm and digits at lengthy chronic stages post injury. However, instead of using ChABC, we utilized a novel and more clinically relevant systemic, non-invasive combinatorial treatment strategy designed to both reduce and overcome inhibitory CSPGs simultaneously and spatially extensively. Following a three-month upper cervical spinal hemi-lesion, we show that the combined treatment has a profound effect on functional recovery of the chronically paralyzed forelimb and paw, specifically during walking as well as precision movements of the digits. Our exciting pre-clinical findings will begin to dramatically enhance our understanding of the basic mechanisms underlying functionally beneficial regenerative events occurring at chronic injury stages for clinically relevant translational benefits.

Introduction and Background

Each year, between 250,000 and 500,000 people worldwide suffer a debilitating spinal cord injury (SCI) with over half occurring at the cervical level, leading to millions chronically paralyzed as reported by the World Health Organization[1]. Damage to the high cervical spinal cord can result in paralysis of much of the body, with devastating impacts on controlled, voluntary arm, hand, and digit movements leading to a severe decrease in individual autonomy and overall quality of life[2,3]. The last several decades of extensive research have demonstrated the crucial role of the cell and axon growth inhibitory properties of chondroitin sulfate proteoglycans (CSPGs) during normal neural

development as well as their inhibition of re-growth of cells or their processes after injury[4-9,12,]. In particular, we have focused on studying the role of the CSPG family of extracellular matrix molecules in the glial scar and the perineuronal net (PNN) as critical inhibitors of functional regeneration and sprouting after SCI[10-14].

Within a day after injury, CSPGs increase within and adjacent to the forming glial scar[15-18]. This upregulation also occurs in the PNN in areas rostral and caudal to the injury site in denervated distal targets[10-14,73]. Thus, inhibitory CSPGs up-regulate expansively along the neuraxis within, proximal and distal to the lesion epicenter, leading to limited regeneration through the lesion or back into target nuclei and curtailing potential sprouting/plasticity over large distances that could occur from remaining or spared fiber systems either ipsilateral or contralateral to the lesion[10-16,19,20,75]. The regulatory effects of CSPGs on both myelin and axon regeneration as well as neuronal plasticity after an acute [9,21-26] or sub-chronic [5,17,21,24] SCI, and in the context of many other conditions [8,12,84] have been well documented.

The glycosaminoglycan (GAG) side chains, especially those containing CS-E, are known to bind with highest affinity to their receptor PTPσ [32] and provide much of the inhibitory properties of CSPGs. Their effects can be greatly decreased by enzymatic digestion using the bacterial enzyme, Chondroitinase ABC (ChABC) [13,27-33,74-77]. ChABC is routinely used for degrading the chondroitin sulfated GAG-chains away from their resident core proteins and has been shown in various models of SCI to have pre-clinical benefits with improvements in both locomotor ability and increased axonal regeneration/sprouting toward and into previously denervated spinal sensory or motor centers [10,13,36,37,73,74]. There has only been limited success when ChABC was used alone or when coupled with other strategies, such as rehabilitative training, in sub-chronic SCI models. Somewhat improved forepaw function has been reported when ChABC was combined with pellet reaching training when the treatment was given at 4 weeks post dorsal hemisection injury[36]. In another study, ChABC combined with lengthy treadmill training beginning 6 weeks after severe contusion cord lesion modestly improved locomotor behavior[37]. When ChABC was locally delivered over an extended period and combined with neural precursor cells or trophic factors, very limited improvements in locomotor function have been reported in clip compression lesioned SCI animals with a 6-week delay prior to treatment[38]. Unlike acute SCI, in the chronic state, dense glial scarring has formed around the lesion epicenter and increases in CSPGs in the PNNs has maximized within deafferented spinal levels[7,10,15,39]. Furthermore, the inflammatory response has largely died down and the opportunity for neuroprotection has long past[12].

We had been focusing on the return of diaphragm function at much longer chronic stages after lateral C2 hemi-section. Recently, we documented some rather remarkable results showing complete and persistent return of diaphragm function at greatly protracted chronic stages (up to 1.5 years post lesion) upon local matrix and PNN degradation with ChABC placed directly in the vicinity of the denervated phrenic motor pool[40]. The robust recovery of breathing, which occurs rapidly after a near lifetime of paralysis was surprisingly superior to anything achieved at acute or sub-chronic stages after injury using similar techniques. The ability to stimulate recovery of such strong, rapid hemi-diaphragm function after enzyme mediated matrix degradation at C4 takes about **3 months** post

injury to manifest initially after hemi-lesion at C2 and this repair potential chronically, continues to improve even more over time[40]. It seems likely that, in partial injury models, improvements via enzyme administration in past studies may have been limited, at least in part, because not enough time had been allowed to pass prior to treatment to enable the slow process of potentially meaningful spontaneous plasticity to reach sufficient levels. While able to breathe with the intact hemi-diaphragm, ambulate, feed, and drink, high cervical hemisected animals also exhibit long-lasting deficits in forelimb behavior, including reduced gross and fine movements of the ipsi-lesional forelimb and the paw. Our previous ChABC enzyme applications at C4 to the very long chronically C2 injured animal, in order to restore diaphragm function, were not optimized for effects upon the forelimb, which is innervated from spinal levels around C5-C8[40,41]. However, the strategy, none-the-less, elicited rapid albeit relatively small improvements in upper arm function, likely mediated via enzyme diffusion caudally towards the cervical enlargement[40].

The use of ChABC has not been applied clinically due to a variety of potential complications such as heat lability of a bacterial enzyme which, in addition, has to be administered intraparenchymally into the injured spinal cord, which can further traumatize an already damaged CNS structure. Further, the injection field is limited to a discrete area, so positive behavioral improvements are dependent upon the precision of locally restricted alterations in the matrix. Several labs have engineered stable, longer lived chondroitinase or more widespread viral vector release strategies although the direct delivery issue still remains with these approaches[79-83]. In order to target CSPG mediated inhibition more globally, we have focused our attention on the possibility of using systemic agents that could potentially diminish the abundance of CSPGs or disrupt the association with their major receptor without directly touching the spinal cord in the presence of any evolving lesion. To accomplish this, we utilized a high dose of a subcutaneously delivered protein tyrosine phosphatase receptor (PTPσ) inhibitor, intracellular sigma peptide (ISP). This TAT-wedge domain peptide has been shown to work acutely in vitro and in vivo to prevent the conversion of growth cones into a dystrophic state through the excessively tight substrate adhesion mediated via the PTPσ receptor and the CSPGs[42-47]. In addition, we investigated the therapeutic effectiveness of a clinically approved, orally administered small molecule perineuronal net inhibitor, 4-methylumbelliferone (we call it PNNi for use in the CNS), which serves to limit a major transmembrane scaffold for PNN assembly resulting in the PNN unable to be stably formed or maintained[50]. Thus, in addition to the use of the receptor disrupting wedge peptide (ISP), we employed a novel strategy in combination which can simultaneously and expansively reduce the matrix PNN CSPGs[50].

Our chronic LC2H SCI model allows us to test the role of PNN associated CSPGs in curtailing potential functional sprouting from the intact side of the cord at the level of the cervical enlargement or likely elsewhere within the CNS related to forearm and paw function. By strongly interrupting the PTPσ-CSPG interaction we were able to significantly restore functional movements of the impaired forelimb during over ground locomotor behavior as well as cereal manipulations by the fingers during eating. The synergistic effect of ISP and PNNi further supports the crucial role of CSPG mediated inhibition via the PTPσ receptor in curtailing functional synaptic plasticity at lengthy chronic time-points following SCI.

Methods

Ethical declaration and animal husbandry

All experiments were approved by the Institutional Care and Use Committee at Case Western Reserve University (CWRU), Cleveland. Animals were housed in groups of two or three, exposed to a normal dark–light cycle with free access to food, water, and environmental enrichment ad libitum. The health and welfare of the animals was monitored daily by the study investigators and veterinary staff at Case Western Reserve University.

Cervical level two hemitransection (LC2H) injury and systemic treatment(s)

Spinal cord injury surgeries were performed as previously described[39]. Adult female Sprague Dawley rats (280 ± 20 g; Harlan Laboratories Inc., Indianapolis, IN, USA) were anaesthetized with an intraperitoneal injection of ketamine+xylazine cocktail (70 mg kg^{-1}/7 mg kg^{-1}). The dorsal neck-shoulder area was shaved, cleaned, and sanitized using Betadine and 70% ethyl alcohol, and analgesics were administered through subcutaneous injection of meloxicam (1 mg kg^{-1}). Body temperature was maintained and monitored throughout the surgery at 37 ± 1 °C. A dorsal midline incision ~3 cm in length was made over the cervical region. After the skin and paravertebral muscles were retracted, a laminectomy was performed over C2 and C3 and the rostral spinal cord was exposed. A 21G syringe needle was positioned at the midline of the spinal cord, and a left lateral durotomy and hemisection were performed caudal to the C2 dorsal roots making certain that the needle tip extended to and was dragged along the ventral boney lamina surface. This process was repeated five times and ranged from the midline to the most lateral extent of the spinal cord. The muscle layers were sutured together with 3-0 Vicryl and the skin closed using wound clips. The animals were given meloxicam (1 mg kg^{-1}) and sterile saline subcutaneously for up to 5 days post-surgery along with nutritional support should the animals' weight have dropped more than 5% of that pre-injury. The anatomical completeness of the injury was confirmed through microscopy and behavioral assessment. While animals were able to survive immediately post-injury and exhibited no signs of malnutrition or infection, animals that exhibited persistent autophagy as a result of the injury before or during the systemic treatment phase were excluded from the study. Prior to the initiation of the systemic treatment(s), all animals were confirmed to achieve a performance score no better than 4.5 ± 1 out of 17 at three days post-injury as described in the locomotor assessment, and 8 ± 1 twelve weeks post-injury (Fig. 1; see *Methods- Forelimb Locomotor Scale[49])*. For cereal eating assessments, all animals were confirmed to receive a performance score no better than 0 ± 1 out of 9 at three days post-injury, and 2 ± 1 twelve weeks post-injury (Fig. 3; see *Methods- Irvine, Beattie, and Bresnahan Forelimb Recovery Scale[50])*.

Three months after LC2H, the rats began a systemic treatment protocol that included a daily 0.5 mL subcutaneous injection of 500 ug of intracellular sigma peptide (ISP),

combined with oral feeding of Nutella mixed with the small molecule perineuronal net CSPG inhibitor, PNNi. Control animals were injected with saline and fed Nutella not containing PNNi. Two separate cohorts received ISP or PNNi alone. These systemic treatments were administered with the goal to simultaneously disrupt the interaction between would-be sprouting axons and the PTPσ receptor as well as to reduce the density of inhibitory extracellular perineuronal net components. Each cohort (n = 8-10) received the treatments daily for 60 days and was tested blindly and weekly for arm and hand function for the duration of treatment and continuing four weeks following the termination of treatments. Our assessments (see below for details) of forelimb/paw function were conducted using the forelimb locomotor scale (FLS)[49] and the IBB forelimb recovery scale[50]. Both are rating scales similar to the well-known BBB scoring method for overground walking[51] but are focused on the forelimb and digits.

Behavioral training and assessments

Animals were acclimated to the laboratory room testing environment and cereals in the home cage for five consecutive days. The animals were also handled by the researchers twice a day to minimize any stress/anxiety prior to exposure to the testing apparatuses measured by the animal's reliability to perform the assessment. The following week, the animals were acclimated to the various testing apparatuses which included a ten-minute session each day in the glass cylinder used in the cereal eating assessment. The animals were trained to eat at least 3 of each cereal type (sphere-shaped i.e. Cocoa Puffs, or donut-shaped i.e. Fruit Loops) within the ten-minute session. For acclimation to the FLS testing platform, rats were placed on top of an open field platform approximately 4' in diameter and allowed to explore for 5 minutes. FLS and IBB pre-training were conducted for five consecutive days. Baseline behavior performance was acquired following training and before LC2H (Fig. 1A).

Forelimb function assessments

Forelimb function was determined through assessments which involved monitoring behavior that the animals performed naturally. This included the forelimb locomotor assessment scale (FLS)[49] and the Irvine, Beattie and Breshnahan (IBB) forelimb recovery scale[50]. Baseline values were taken prior to injury and at 3 months after injury just prior to daily subcutaneous injection of high dose ISP or saline alone or combined with Nutella ± PNNi administration (for 60 days) and then weekly following treatment application (Fig. 1A). Statistical comparisons were made between treatment groups and within behavioral measurements using two-way, repeated-measures ANOVA with post-hoc Bonferroni Correction (Graphpad Prism).

Forelimb Locomotor Scale (FLS): These testing methods were established previously[49]. Briefly, rats are encouraged to continuously walk on top of an elevated circular platform approximately 4' in diameter. Rats that remained stationary for longer than 10-15 sec were enticed to move by having them follow a pencil or a piece of paper, or by lightly tapping or scratching on the side of the open field. If the animal failed to respond to these

stimuli, it was picked up by the forequarters and placed in the center of the open field or opposite its previous position, which usually caused it to move. Rats were recorded during locomotion using a high-speed (60 FPS) camera for offline scoring to assess forelimb walking functionality. Videos were scored in a blinded fashion in slow-motion (at least 50%) playback using the Lightworks video editing software. Recorded videos containing 3-4 minutes of the animal freely walking on the elevated platform were measured using an 18-point scale (0-17). Animal locomotor behaviors were assessed for impairments and assigned a score reflecting the ability of the animal to perform steps that were consistently plantar, parallel, and weight-bearing[49].

The Irvine, Beattie, and Bresnahan (IBB) Forelimb Recovery Scale: These testing methods were established previously[50]. Briefly, animals were placed in the 10" H x 8" W clear glass cylinder that was used for previous acclimation and training. Each rat was given one sphere-shaped (Cocoa Puff) and one donut-shaped (Fruit Loop) cereal to eat. Their manipulations of the cereals were recorded using a high-speed (60 FPS) camera for offline scoring to measure forelimb and digit abilities during eating. Videos were scored blinded in slow-motion (at least 50%) playback using the Lightworks video editing software. Recorded videos containing the animal eating one sphere-shaped cereal (i.e. Cocoa Puff) and one donut-shaped cereal (Fruit Loop) were measured using a 10-point scale (0-9)[50]. The animal's ability to dexterously manipulate the cereals was assessed for impairments and assigned a score reflecting the ability of the animal to grasp the cereal with subtle adjustments made using the second, third and fourth digits and wrist movements[50].

Stimulation of forelimb utilization

All animals were placed in a pool (4 feet diameter) filled to a depth of ~18 inches at a temperature of 35-38 degrees Celsius to match the rat's natural internal temperature. Animals were placed in the pool for 1 minute. 2-4 rats were placed in the pool at one time. After swimming, rats were partially dried with a cotton towel and then placed in empty cages lined with paper towels and allowed to further dry themselves and groom uninterrupted for 1 hour at room temperature. The rats were then replaced back into their home cages. Swimming took place 5 days a week beginning two weeks after treatment application began, excluding the day prior to and the day of behavioral acquisition using the FLS and IBB assessments described above (Fig. 1A).

Histology for serotonergic fibers

At 20 WPI, animals were anesthetized with a cocktail of ketamine and xylazine prior to cardiac perfusion with 4% paraformaldehyde dissolved in PBS. Spinal tissue containing regions of interest were dissected and post-fixed in 4% paraformaldehyde overnight. Prior to sectioning, the tissue was cryoprotected with sequential treatments first in a solution of 30% sucrose in PBS and then in a 1:1 mixture of 30% sucrose in PBS + OCT. After embedded in OCT, the tissue was sectioned to 30 µm coronal samples and free-floating sections were prepared for subsequent immunofluorescence analysis using

standard protocols. Primary antibodies targeting SERT (1/1000; EnCor Biotechnology; Cat No. RPCA-SERT) and WFA-Lectin (1/1000; Vector Laboratories; Cat No. B-1355) were coupled to Alexa Fluor 488 and Alexa Flour 555 conjugated secondary antibodies (1/500), respectively. Nuclei were visualized using Hoechst 33342 (1/3000; Life Technologies; Cat No. C10338). All stained sections were mounted onto slides, coverslipped using an antifadent mounting medium, and examined using an inverted Leica SP8 confocal microscope.

Statistical assessment

For statistical analysis, divergences where p was < 0.05 were considered significant. Power analysis was conducted prior to all experiments to ensure numbers (n) of animals per group were sufficient. The data are presented as mean ± SEM. Data were subjected to the Shapiro–Wilk test for normalcy prior to analysis to ensure a normal distribution. At the time of processing and analyses of all experiments and assessments, investigators were blinded as to the treatment group of each animal. The functional and behavioral recordings from every animal in each group were analyzed without exclusion based on the outcome.

Results

All treatments improve gross function following chronic cervical SCI

All rats displayed a baseline pre-injury FLS performance level at a score of 17, or consistent plantar stepping of the forepaw, predominant parallel placement and continuous toe clearing[49] (Fig. 1B and Fig. 2). At 3 days and at 1 week post-injury (WPI), all rats exhibited an FLS score early on of ~4 and a bit later on improving slightly to ~7, respectively (Fig. 1B {3 days: control = 4.6 ± 0.9; ISP = 5.1 ± 0.7; PNNi = 3.9 ± 0.9; ISP+PNNi = 3.5 ± 0.7} {1 WPI: control = 6.5 ± 0.6; ISP = 6.9 ± 0.4; PNNi = 7.2 ± 0.3; ISP+PNNi = 7.1 ± 0.6}). These findings demonstrate the consistency of the targeted removal of descending supraspinal motor inputs upon inducing the lateral transection across all of the treatment groups immediately following SCI. Unsurprisingly, by 12 WPI, all rats had reached a pre-treatment baseline FLS score of ~8 indicating that the animals displayed some spontaneous recovery of function that was consistent prior to treatment and not statistically different between groups (Fig. 1B-C and Fig. 3 {12 WPI: control = 8.2 ± 0.4; ISP = 8.6 ± 0.2; PNNi = 9.2 ± 0.2; ISP+PNNi = 8.9 ± 0.3}). An FLS score of 8 denotes the animals' ability to perform partial weight-supported steps but only on the dorsal surface of the impaired limb/paw (Fig. 3)[49].

Interestingly, the control group never gained further FLS assayed function beyond that demonstrated in the pre-treatment assessment, and therefore, any recovery observed is likely due to the administered treatment (Fig. 1B-C and Fig.3 {12 WPI: 8.2 ± 0.4 v 24 WPI: 8.1 ± 0.4}). The various treatments caused a fairly rapid increase in performance as early as the first week after their onset (Fig. 1B {13 WPI: control = 8.1 ± 0.43; ISP = 9.7 ± 0.55; PNNi = 10.4 ± 0.77; ISP+PNNi = 10 ± 0.5} {ANOVA, ISP p = 0.678, F = 1.627; PNNi p = 0.132, F = 2.355; ISP+PNNi p = 0.4712, F = 1.9}). There was a significant difference

between the FLS score at 14-WPI in the PNNi alone treated animals (Fig. 1B {ANOVA, p = 0.037, F = 2.818}), while the combination treatment group revealed a significance in recovery beginning at 16 WPI which then improved slowly during the drug administration phase (Fig. 1B {ANOVA, p = 0.0279, F = 2.982}). At 24 WPI, the control rats maintained an average FLS score of ~8, while rats that received ISP alone, PNNi alone, or in combination displayed significant improvements in forelimb recovery during walking compared to the control group (Fig. 1B-C and Fig 3. {ANOVA, ISP p = 0.0089, F = 3.639; PNNi p = 0.0261, F = 3.264; ISP+PNNi p = 0.0007, F = 4.189). There was evidence of synergy between the 2 treatments. Thus, behavioral recovery occurred especially in rats that received the combined treatment of ISP and PNNi. At 24 WPI, ISP alone and PNNi alone treated animals reached an average FLS score of ~11, which functionally translates to continuous plantar stepping but with poor wrist control and no toe clearance (ISP = 11.7 ± 0.86; PNNi = 11.3 ± 1.37). The combination treated rats received an average final score of ~12, indicating continuous weight bearing and plantar stepping that is predominantly parallel but without toe clearance. However, the best responding animals in the group receiving both PNNi and ISP reached strikingly high performance levels (Fig 1B-C and Fig. 3 {highest 24 WPI FLS: ISP+PNNi = 16}).

The combination treatment best improves skilled forelimb and digit function following a partial chronic cervical SCI

When assessing for cereal eating ability, all rats displayed a baseline pre-injury performance level at a score of 9 when both the soon to be injured (left) and uninjured (right) were measured. Normal performance is described as almost always maintaining properly shaped paws (conforming to that of the cereal) during grasping of the cereal with fully flexed elbow, extensive distal limb movements, subtle cereal adjustments and volar supported manipulations[50]. At all weekly behavioral testing time-points before and during systemic treatment administration, the uninjured forelimb displayed consistent normal function during cereal eating. At 3 days (DPI) and at 1 week post-injury (WPI), all rats exhibited an IBB score of ~0.5 and ~2, respectively (Fig. 3A and 2B {average IBB score 3 DPI: control = 0.3 ± 0.1; ISP = 0.6 ± 0.3; PNNi = 0.4 ± 0.2; ISP+PNNi = 0.6 ± 0.3} {average IBB score 1 WPI: control = 1.9 ± 0.8; ISP = 1.0 ± 0.3; PNNi = 1.6 ± 0.5; ISP+PNNi = 2.1 ± 0.8}). By 12 WPI, all rats reached a pre-treatment baseline IBB score of ~2, which translates to cereal eating behavior that uses the non-volar surface of the injured forelimb, with a predominant fixed forepaw position that is clubbed into a fist-like flexed state, while some animals display an extended forepaw phenotype[50] (Fig. 3A and 3B, Fig. 4). Unlike the FLS data detailed above, the IBB assessment did not reveal a significant effect of treatment until week four of treatment administration. Furthermore, the significant behavioral recovery observed was only demonstrated in the animals that received both PNNi and ISP in combination (Fig. 3A, Fig. 4; average IBB score 17 WPI: control = 2.5 ± 0.6; ISP+PNNi = 4.8 ± 0.5, {ANOVA, p = 0.014, F = 2.30}).

Our behavioral results using the IBB assessment to measure forelimb and paw cereal manipulations further demonstrated that the combination treatment group eventually developed the ability to use their once paralyzed paw/fingers remarkably better than controls. The control animals recovered paw/finger function only minimally over time

(compared to normal animals) to an IBB score between 2 and 3, where the affected paw may remain partially clubbed and mostly rests on the ground and where, at best, some crude, exaggerated cereal adjustments are made[50] (Fig. 3C and Fig. 4, average IBB score 24 WPI: control = 2.3 ± 0.5). The paw does not exhibit adaptability and fails to conform to the shape of the piece of cereal. While animals receiving either ISP or PNNi alone did not show a similar effect of meaningful forelimb functional recovery as we showed in the FLS assessment, these animals did show a strong trend towards significance by the end of the study compared to the control group (Fig. 3A-C and Fig. 4; {average IBB score 24 WPI: ISP = 3.5 ± 0.5; PNNi = 4.3 ± 0.7} {ANOVA, ISP $p = 0.5$, $F = 1.20$; PNNi $p = 0.07$, $F = 1.99$}). On the contrary, the combination of ISP and PNNi led to clear improvements in paw/finger function during cereal eating[50]. On average, at 24 weeks post injury and with the combinatorial treatment, the once paralyzed animals recovered to an IBB score of between 5 and 6 with extensive contact manipulatory movements[50] (Fig. 3A-C and Fig. 4; average IBB score 24 WPI: ISP+PNNi = 5.3 ± 0.6; {ANOVA, $p = 0.02$, $F = 2.94$}). Our most encouraging finding was that the best responding animals, especially when eating a donut-shaped cereal such as a Fruit Loop, could achieve a remarkable score of 7 (9 is the highest possible score)[50] where sometimes normal, shape adapting grasping can occur. In summary, these behavioral data confirm the hypothesis that modulating CSPGs in the scar and especially in the PNN as well as their receptor simultaneously is central to the recovery of precision digit function long after SCI.

Histological analyses reveal dense sprouting of 5-HT axons in the spinal cord of combination treated animals

In our previous studies showing the therapeutic efficacy of ISP **acutely** after severe contusive spinal cord injury[14], we demonstrated extensive sprouting of serotonergic axons just at and below the lesion. The sprouting occurred in unusually shaped, dense clusters. Upon examination of 5-HT axons just caudal to the chronic injury site in our combination treated animals, we have again observed this 5-HT axonal sprouting phenomenon (Fig. 5).

Discussion

In our present study, we assessed functional recovery of forelimb behavior and have revealed, for the first time, that treatments with PNNi and ISP alone but especially in combination stimulate remarkable recovery of forearm and paw function in a severe model of clearly chronic cervical SCI. Furthermore, these functional improvements in forelimb movement persisted for at least 4 weeks after the daily treatment(s) had ended. When given for two months following a chronic LC2H, ISP or PNNi administered alone were able to significantly improve proximal and distal forelimb behavior during more crude locomotion. Moreover, we assessed fine motor behaviors during cereal eating and showed exceedingly improved digit function when we administered our systemic treatments together.

It is quite interesting that recovery of function begins to occur rapidly following systemic treatment in our chronic injury model. Surprisingly, behavioral improvements occur much more quickly than they do after acute injury[14]. This functional recovery mimics that which we have shown previously in our ChABC treated chronic LC2H SCI respiratory model suggesting, again, that sprouting likely had already occurred but is somehow being masked by the PNN[40]. Thus, in our 12-week cervical hemi-lesion model, certain sub-populations of the contralateral descending supraspinal tracts with small numbers of axons that re-decussate within the spinal cord caudal to the level of injury, or intervening interneurons with similar properties of plasticity, may be very slowly sprouting new processes that re-cross the midline to the denervated hemisphere ipsilateral to the lesion even in the presence of the enhanced PNN[52-58]. Various motor systems underlying forelimb and paw movement, including the serotonergic, propriospinal, and cortico-spinal neurons have an innate propensity to sprout on their own, although at a very slow rate, over time[29, 56-61]. These new collaterals could serve as latent, or inactive synapses which over time could be beneficial, harmless or possibly even disadvantageous to recovery[40,62]. Our newly reported results demonstrate that the potential activity of such sprouting can be remarkably supportive to functional recovery but is largely kept dormant by CSPGs within the PNN in the vicinity of the relevant motor pools.

The PNN and its associated CSPGs may encroach into the synaptic cleft and block transmitter passage across the synapse of sprouted axons

What is the mechanism by which the PNN may curtail synaptic activity and perhaps help to create silent or latent synapses? PNN holes get smaller in Alzheimer's disease models so this phenomenon is known to occur although the invasion of the net in terms of its function smothering effect on the synapse has not been elucidated[65]. We are planning to use high resolution microscopic techniques to further investigate this possible activity silencing mechanism on denervated phrenic as well as cervical enlargement resident motor neurons. Latent synapses are known to occur throughout the CNS[78.] One of the most interesting and well-known latent descending motor projections is the crossed phrenic pathway[55,64-66]. It is already present but can be re-awakened in the adult in seconds after an LC2H injury by creating anoxic stress via lesion of the contralateral phrenic nerve[40,55]. Perhaps, in addition to other proposed mechanisms that can lead to synaptic latency, synapses of sprouted axons in the cervical enlargement could be made inactive via this same type of matrix encroaching phenomenon[64-69].

The therapeutics of high dose ISP

Recently, an independent study confirmed the functional benefits of subcutaneous peptide treatment showing that 500 ug/day of ISP given acutely after contusive injury enhanced hindlimb locomotor, sensory and bladder recovery to highly improved levels[70]. Indeed, with the use of the well characterized BBB locomotor rating scale, their contusion-injured animals (that stabilized at a control baseline score of 6) on average improved by a remarkable 9 full points to a level of 15[70]. Importantly, while bladder function showed a dose response when given at 44 ug/day[14], the much higher dose of 500 ug/day experiment provides the first evidence for a dose response by ISP in promoting

enhancements in locomotor behavior, at least after acute SCI. Similarly, ISP has some therapeutic effects after acute T8 hemisection in the adult rat using an automated gait analyses system[44,45]. This high dose 500 ug/day of ISP given subcutaneously is that which we used in our current study alone or in combination with an orally administered small molecule, PNNi.

The therapeutics of PNNi

Hyaluronan (HA) is the major scaffold for PNN assembly and if HA synthesis is blocked then the PNN is not able to structure itself properly[48,70,72]. In addition to the use of the wedge peptide ISP, we employed a novel strategy in combination which can substantially reduce the PNN CSPGs within the CNS via systemic delivery of an already clinically approved small molecule proteoglycan synthesis inhibitor called 4-Methylumbelliferone (4MU) or hymecromone[48,71,72]. Referred to as PNNi in our study, we repurposed this small molecule in our model of chronic SCI. One of the major challenges for translation of SCI regenerative strategies from rodent to human is the enormous relative increase in size of the human which necessitates careful considerations of dosages, toxicity and routes of delivery to optimize the amount and time of residence of the therapeutic drug within the CNS. Oral delivery of a small molecule inhibitor that is already clinically approved is a preferred strategy for drug delivery. A novel therapeutic approach to overcome the inhibitory effects of the PNN involves curtailing the production of HA. PNNi is able to specifically inhibit HA synthesis[72]. PNNi acts as a competitive substrate for hyaluronan synthase, an enzyme critical for HA production[72]. Preliminary findings have shown that inhibiting HA synthesis with PNNi in vitro and in vivo resulted in significant decreases in the amount of CSPGs present. We therefore hypothesized that PNNi, and especially when administered together with ISP, could be an obvious dual therapy since the combined strategy would decrease the ligand as well as block the receptor globally and could be an effective, clinically viable treatment method following chronic partial cervical lesion. PNNi as well as ISP are non-invasive treatments with low toxicity that can be stopped at any time to reverse the receptor blockade and HA-synthesis inhibiting effects of the combination and allow for the PNN to be re-established. This can help avoid potential long term adverse side effects and help stabilize newly formed synapses.

Our behavioral findings demonstrate a robust return of forelimb function months after a partial spinal transection during gross and fine voluntary motor behaviors that are innate and ecologically relevant to our model organism. The ability to recover these behaviors is more than likely owed to sprouting of their underlying neural pathways, particularly serotonergic, corticospinal or propriospinal tracts descending contralateral to the SCI and forming latent, re-decussated terminals within the spinal cord cervical enlargement containing forelimb muscle motor neurons. Thus, we were able to induce plasticity of the spinal tissue by manipulating the presence and effect of the PNN on spared, sprouting descending axons using treatments that either inhibited the PNN-binding neuronal receptor RPTPσ (via ISP), or impaired the PNN to be synthesized and maintained. These results suggest that the systemic targeting of the PNN and PTPσ receptor at chronic SCI periods is a viable therapeutic target for treatment that can be extended or ended at any

time or combined with other beneficial interventions such as rehabilitative training or neuromodulation, with limited adverse side-effects.

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Figure 1 A-D. (A) Timeline of the experimental protocol. (B-D) The Forelimb Locomotor Score is significantly improved following systemic treatment to remove CSPGs in the PNN and/or modulate receptor PTPσ

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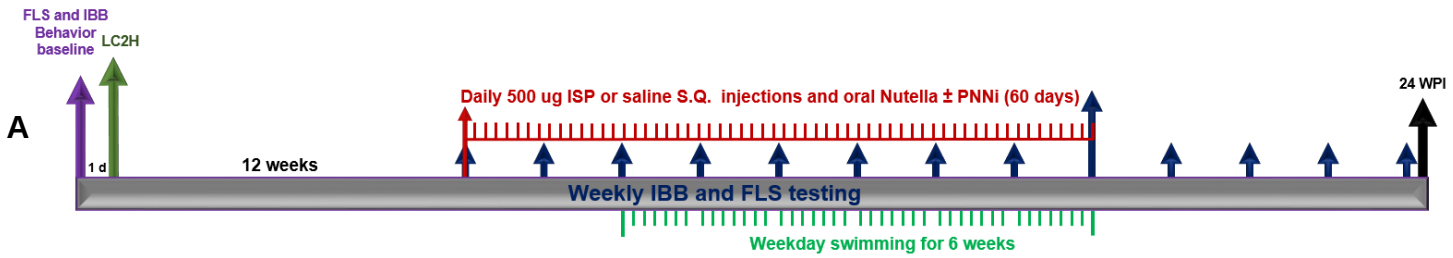
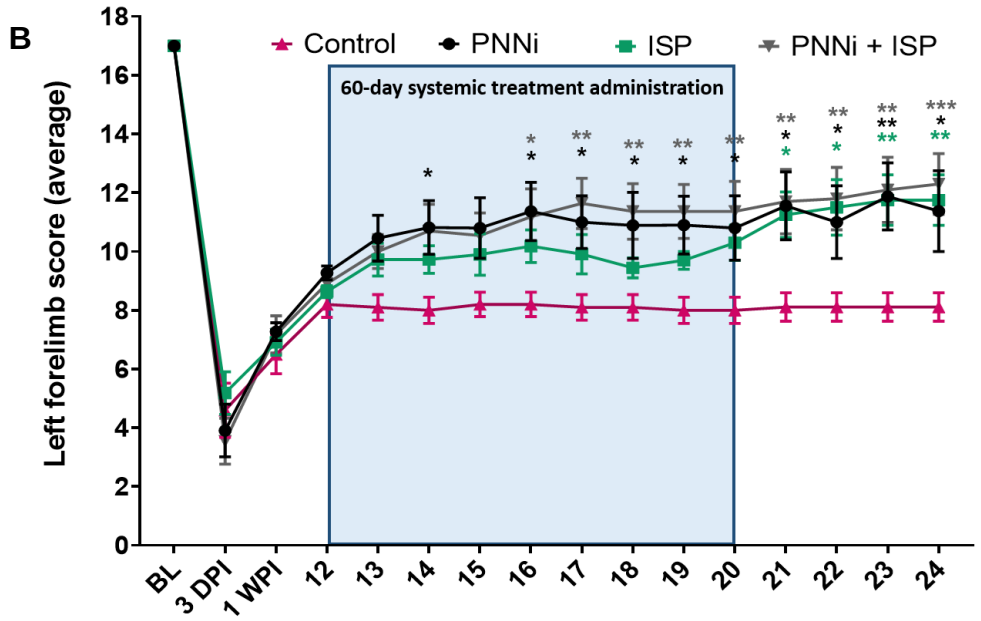
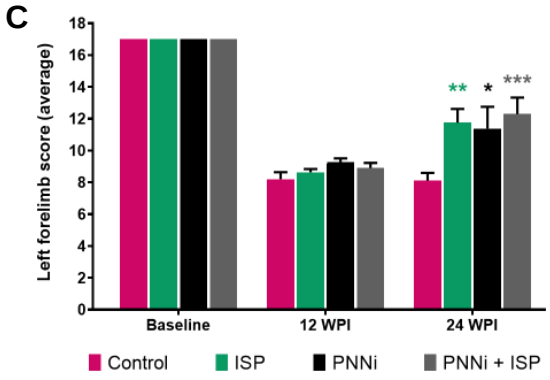


Chronic injury, treatment, and testing timeline





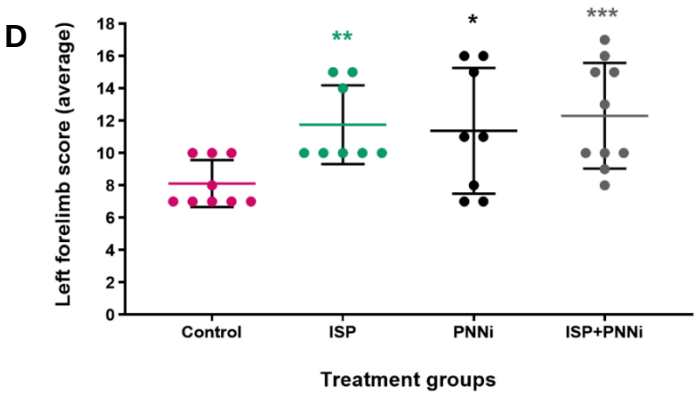


Figure 2. Example still-frame images from actively walking rats at baseline, 12WPI, and 24 WPI (four weeks after systemic treatment ended) during FLS analysis (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

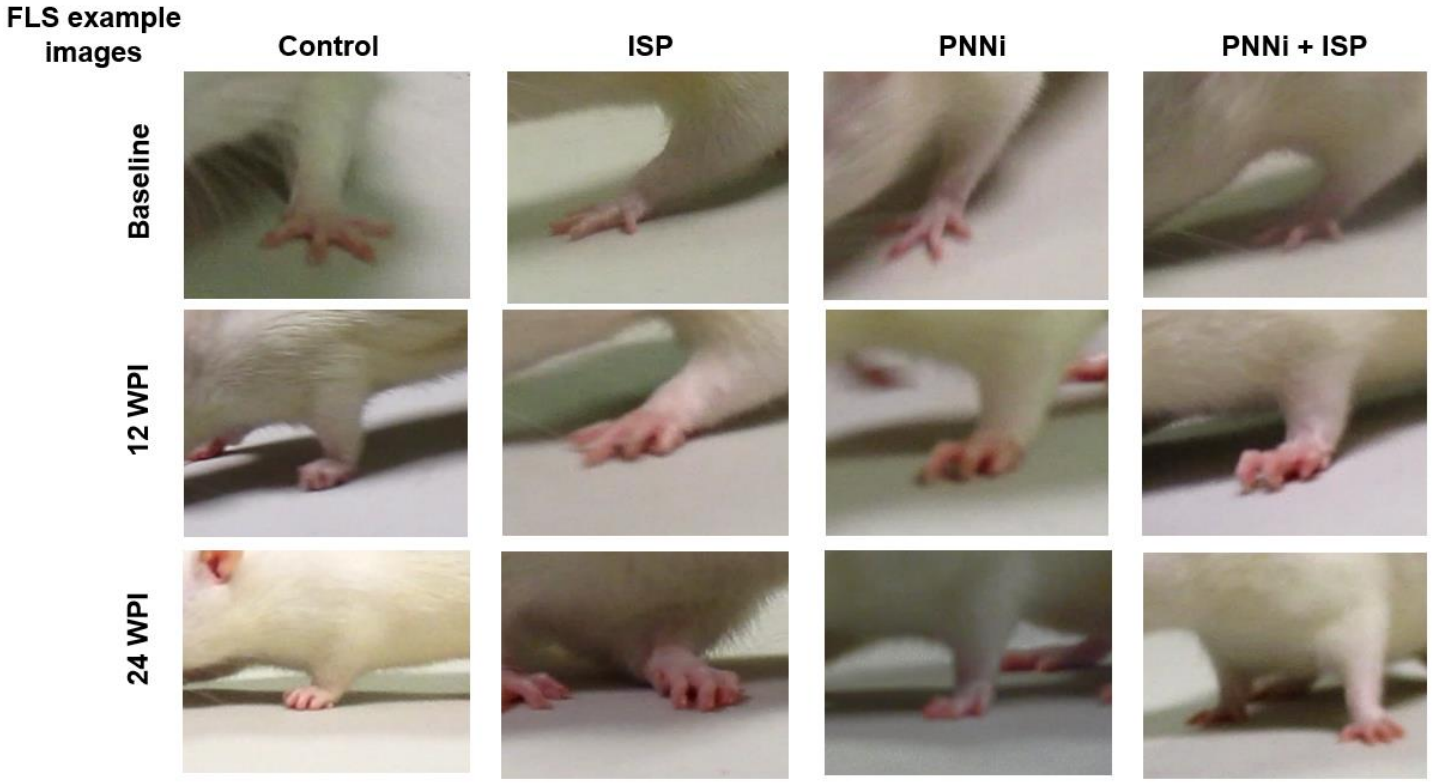


Figure 3 A-C. Cereal eating ability assessed using the IBB-rating scale is significantly improved especially following combined systemic treatment to both remove CSPGs in the PNN and modulate receptor PTPσ.

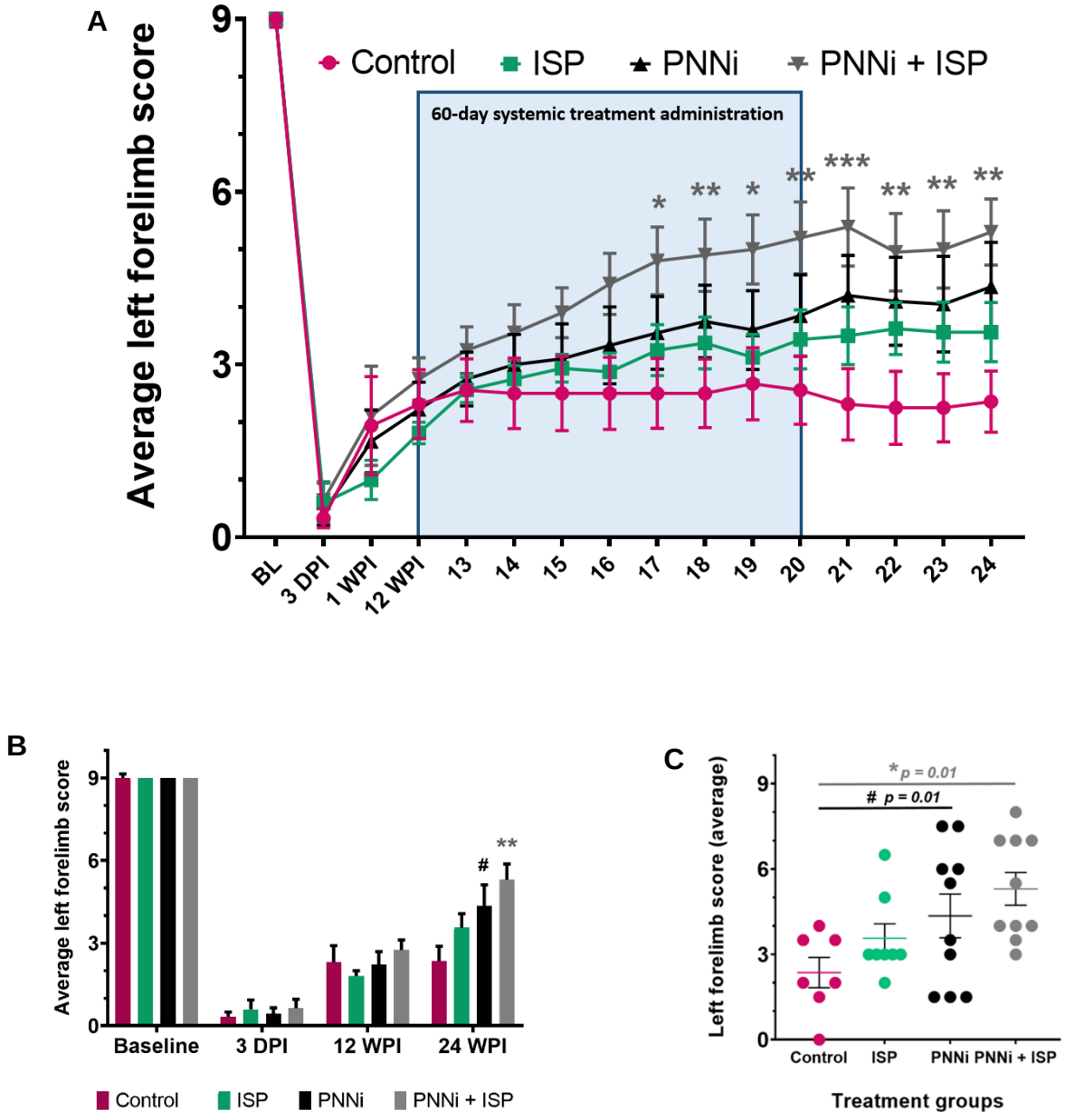
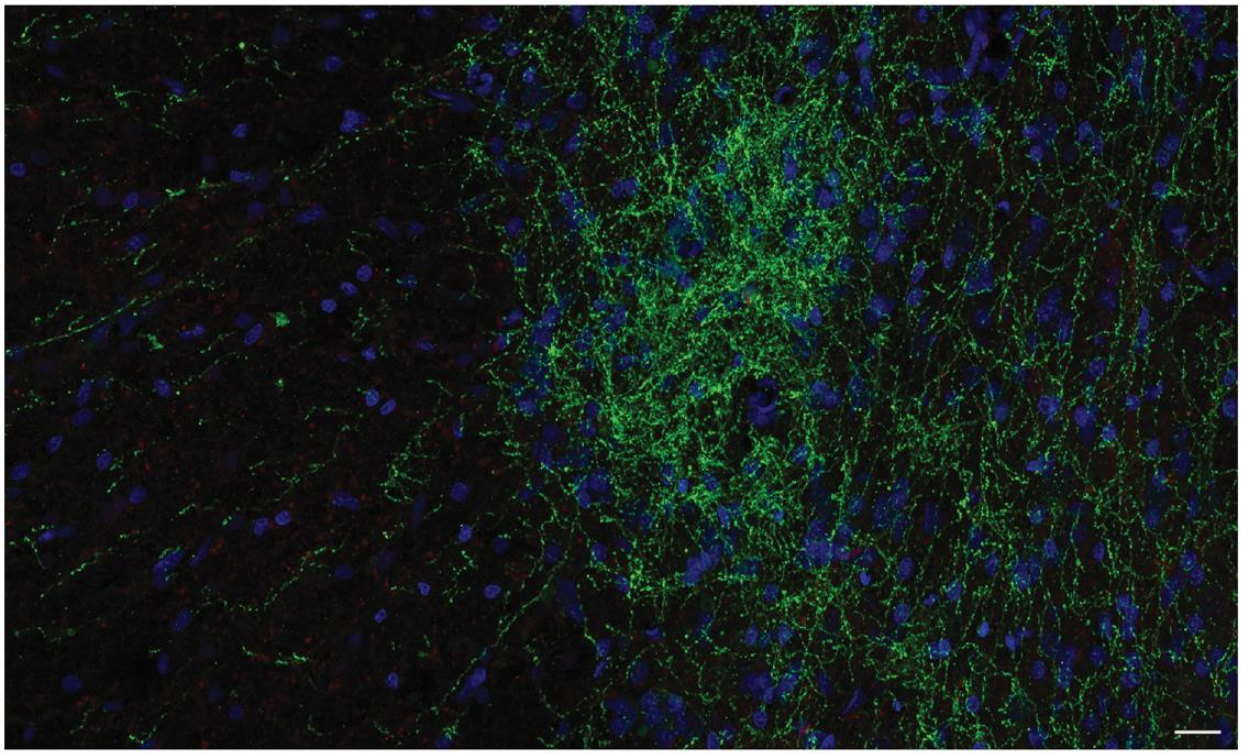


Figure 4. Example still-frame images of animals eating cereal at baseline, 12WPI, and 24 WPI (four weeks after systemic treatment ended) during **IBB** analysis (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

Figure 5. Example immunofluorescent image of densely sprouted ventral horn serotonergic fibers (green) immediately caudal to the LC2H in an animal treated with combined ISP and PNNi.



SERT WFA-Lectin Nuclei