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Supporting Online Material for

PTPσ Is a Receptor for Chondroitin Sulfate Proteoglycan, an Inhibitor of Neural Regeneration

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SUPPLEMENTARY ONLINE MATERIALS FOR SHEN ET AL.

Materials and Methods

Statistics

Error bars show SEM; p values were calculated by Student's unpaired t test.

DNA constructs, fusion proteins, mice, and cultured cells

The Ncn-AP fusion protein construct was generated by PCR amplification of nucleotide 1-2889 of a full-length mouse neurocan clone (Accession: BC065118, IMAGE:6853253) and subcloning the neurocan fragment into the NheI and HindIII sites of the APtag5 vector (*1*). The resulting fusion protein includes neurocan amino acids 1-963 (N-terminal Ig domain, tandem link domains and central chondroitin sulfate attachment domain) fused to placental alkaline phosphatase. PTPσ–Fc and PTPσ-AP fusion protein constructs were generated by PCR amplification of nucleotide 1-2538 of a full-length mouse PTPσ clone (Accession BC052462, IMAGE 6834684), encoding the short isoform of PTPσ, which differs from the long isoform by alternative splicing to give 4 or 8 fibronectin domains (*2, 3*). The PTPσ fragment was subcloned into the NheI and HindIII sites of the pSectagIg and APtag5 vectors to give Fc and AP fusions respectively. To generate the PTPσ ΔLys constructs, lysine residues K68, K69, K71 and K72 in the N-terminal Ig domain (*4*) were simultaneously changed to alanines using PCR.

Fusion proteins were produced in transiently transfected 293T cells. For purification, proteins were produced in Opti-MEM plus ITS-A (Invitrogen), and purified with protein A-Sepharose beads (4 Fast Flow, Amersham). The AP activity of the fusion proteins was determined by measuring substrate turnover on a microplate reader as described (*1, 5*).

PTPσ –/– mice (*6*) were kindly provided by Dr. Michel Tremblay. Mouse C8-D1A cerebellar astrocytes were from the ATCC and were maintained in DMEM containing 10% bovine calf serum (Invitrogen).

Chondroitinase digestion

For cell free binding assays, chondroitinase ABC (Sigma) was reconstituted to 0.5 mg/ml in 0.01% BSA and added at a final concentration of 0.05 mg/ml (5 units/ml) to 100 µl of 30 nM Ncn-AP. For treatment of astrocytes, cultures were gently rinsed 3 times in culture dishes with warm DMEM, and incubated at 37°C with chondroitinase ABC (1 unit/ml) in enzyme buffer (100 mM Tris, pH=8.0, 100 mM sodium acetate and 0.02% bovine serum albumin). Cells were then rinsed with HBAH before binding assays with AP fusion proteins. To test the effect on DRG neuron culture, for each culture well, chondroitinase ABC (0.2 unit) was mixed with or without CSPGs (25 μg; Millipore) in enzyme buffer in a total volume of 100 μl. The mixtures were incubated at 37°C for 3 hours and then added to the culture medium for outgrowth assays. Spinal cord cryosections were rinsed with Hanks Buffered Saline Solution (HBSS) and incubated at 37°C for 3 hours with chondroitinase ABC (1 unit/ml) in enzyme buffer, then were rinsed with HBSS before fusion protein binding assays.

Cell-free binding assays

To test fusion proteins labeled with an AP tag for binding to fusion proteins labeled with an Fc tag, binding assays were performed using 96-well Reactibind Protein A-coated plates (Pierce) as described (*7*). Wells were washed with HBSS containing 20mM Hepes pH 7.0 and incubated with PTPσ–Fc or control Fc conditioned media (4 µg/ml) for 2 hours. Wells were blocked in HBAH (HBSS with 0.5 mg/ml BSA and 0.1% NaN_3) for 2 hours and incubated with Ncn-AP or control AP for 1.5 hours. Unless otherwise stated, all AP fusion proteins were

normalized for equal AP activity. Wells were washed 5 times with HBAH and assayed for bound AP activity as described (*1, 5*). For experiments involving treatment with chondroitinase ABC (Sigma), chondoitinase-treated or mock-treated Ncn-AP were incubated at 37°C for 2 hours, added to PTPσ–Fc coated Reactibind Protein A plate wells and assayed for binding.

For experiments to test binding of aggrecan or CS to PTPσ-Fc, purified aggrecan (bovine; Sigma) was dissolved at 2 mg/ml in HBSS, and was biotinylated by mixing 20 µl aggrecan with 12 µl of 10 mM EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce) in PBS in a total volume of 200 µl, and incubating at 37˚C for 3 hours. Purified chondroitin sulfate (Sigma #C4384) was dissolved at 2 mg/ml in water, and was similarly biotinylated by mixing 200 µl CS with 28ul of 10mM Sulfo-NHS-LC-LC-Biotin. Unincorporated biotin was removed using a Zeba Desalt Spin Column (Pierce). 100 µl of PTPσ-Fc or control Fc conditioned media (4 µg/ml) was added to Reactibind Protein A-coated microtitre plate wells (Pierce) and incubated at room temperature for 1 hour. Following five HBAH washes, biotinylated aggrecan or CS was added at the indicated concentrations and the plates incubated at room temperature for 1 hour. Wells were washed 10 times with HBAH and incubated with alkaline phosphatase-conjugated streptavidin (Pierce) at room temperature for 1 hour. Wells were then washed 10 times with HBAH and assayed for bound AP activity as described (*1, 5*).

PTPσ fusion protein binding to cells or tissue

Astrocyte cultures were rinsed with PBS and pre-incubated with blocking buffer (10% normal goat serum and 0.1% NaN_3 in PBS) at room temperature for 1 hour without or with 20 µg/ml anti-CS antibody (CS56, Sigma), or a control antibody matched for the IgM isotype (anti-His, Santa Cruz). Cells were then rinsed with HBAH and incubated for 1.5 hours with 20 nM PTPσ–AP or AP control. Cell cultures were rinsed 5 times with HBAH, lysed, and bound AP activity was measured in microplates with a spectrophotometer as described (*1, 5*).

For binding experiments with lesioned spinal cord cryosections, mice were anesthetized with mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), and a T8 laminectomy was performed. To produce a dorsal hemisection injury, the dorsal spinal cord was first cut with a pair of microscissors and then a fine microknife was drawn bilaterally across the dorsal aspect of the spinal cord. After hemostasis was achieved, the muscle layers and the skin were sutured. Seven days post-lesion, mice were perfused with 4% paraformaldehyde and post-fixed over-night, then the spinal cords were cryo-proteced in 30% sucrose, and 20 µm sagittal cryosections were cut. Spinal cord cryosections were then probed with AP fusion proteins as previously described (*1, 5*) or by immunofluorescence as described below.

Immunolocalization

Astrocytes were cultured on coverslips and fixed with 4% paraformaldehyde at room temperature for 15 min. Blocking was with 10% normal goat serum in PBS without detergent, without or with addition of CS56 antibody (20 μg/ml; Sigma). Cells were then incubated at room temperature with purified PTPσ-Fc (20 μg/ml) in blocking buffer for 1.5 hours and goat anti-human IgG conjugated with Alexa Fluor 488 (1 μg/ml; Molecular Probes) for an additional 1.5 hours. After brief treatment with DAPI nuclear counterstain and 3 rinses with PBS, samples were mounted with Fluoromount (SouthernBiotech) before imaging.

Cultured DRG neurons were fixed with 4% paraformaldehyde at room temperature for 30 min, rinsed with PBS and pre-incubated for 1 hour in blocking buffer (10% normal donkey serum and 0.4% Triton-X100 in PBS). Cells were then incubated in blocking buffer with anti-GAP43 antibody (2.5 μg/ml; Novus) and subsequently donkey anti-rabbit antibody conjugated with Alexa Fluor 488 (1 μg/ml; Molecular Probes), each overnight at 4°C. After brief treatment with

DAPI nuclear counterstain and 3 rinses with PBS, samples were mounted with Fluoromount (SouthernBiotech) before imaging.

Spinal cord cryosections were rinsed with HBSS and blocked for 1 hour in 10% normal goat serum in PBS without detergent. Primary antibodies (anti-neurocan or CS56, Sigma; each 20 μg/ml) were mixed with either Fc or PTPσ-Fc (each 20 μg/ml) in blocking buffer and incubated with cryosections for 1.5 hours at room temperature. Sections were gently rinsed and incubated with secondary antibodies in blocking buffer for 1.5 hours at room temperature (goat anti-mouse antibody conjugated with Alexa Fluor 594 or goat anti-human IgG antibody conjugated with Alexa Fluor 488, Molecular Probes, each 1 μg/ml). Sections were then rinsed 3 times in PBS and mounted with Fluoromount (SouthernBiotech) before imaging.

Immnuoprecipitation and Western blot

Astrocyte cell lysates were grown in 10 cm culture dishes, rinsed with cold PBS and lysed for 30 min on ice in buffer containing 100 mM Tris, pH 8.0, 1 mM EDTA, 1% NP40, 1% Octyl glucoside and protease inhibitors (Roche). Lysates were cleared by microcentrifugation. 1 mg of total protein was mixed with 10 μg of Fc fusion proteins and 30 μl protein A sepharose beads (50% slurry; Amersham) in lysis buffer in a total volume of 1 ml, and gently rotated overnight at 4°C. Beads were washed 5 times with cold lysis buffer and incubated with sample loading buffer at room temperature for 1 hour before electrophoresis. Western blot analysis was performed with anti-neurocan (0.2 μg/ml, Sigma) as previously described (*7*).

DRG neurite outgrowth assay

8-well culture slides (BD Biosciences) were briefly coated with nitrocellulose methanol solution, dried and subsequently coated with proteins at 37°C for 2 hours. In experiments with soluble CSPGs, slides were coated with a 100 μl mixture of poly-D-lysine (200 μg/ml; Sigma) and

laminin (5 μg/ml; BD Biosciences). In experiments with MAG, the slides were coated with a 100 μl mixture of poly-D-lysine (200 μg/ml), laminin (5 μg/ml) and either Fc or MAG-Fc (each 50 μg/ml; R&D). In experiments with neurocan, the slides were coated with a 100 μl mixture of poly-D-lysine (200 μg/ml), laminin (10 μg/ml) and neurocan (5 μg/ml; Millipore or US Biologicals). Coated slides were rinsed with PBS immediately before use, taking care to avoid drying. For experiments with NGF, culture slides precoated with poly-D-lysine were purchased from BD Biosciences.

DRGs from P8-12 PTPs +/+ or –/– mice were cut off from all roots. Neurons were dissociated as described (*8*) with modification. DRGs were rinsed in PBS with 1 mM EDTA and allowed to settle without centrifugation. Enzyme digestion was carried out sequentially with collagenase II (4 mg/ml, Worthington) and trypsin (0.25%, Gibco) each for 15 min at 37°C. DRGs were then gently rinsed with DMEM containing 10% BCS and then with culture medium (Neurobasal medium supplemented with 2% B27, 20 nm L-glutamine and 1% penicillin/streptomycin, all from Invitrogen). Dissociated cells were filtered using a cell strainer (BD Biosciences). Approximately 2000 cells suspended in 500 ml medium were seeded into each well of the culture slides.

For experiments with CSPG mixture, cells were first allowed to attach and start growing for 18 hours, then replenished with fresh medium with or without treatment reagents, and cultured for an additional 24 hours. The CSPG mixture contains large, extracellular CSPGs isolated from embryonic chicken brain; the major components are neurocan, phosphacan, versican, and aggrecan (Millipore cat. no. CC117). Unless otherwise stated, cultures contained no neurotrophins. For experiments with NGF, the culture medium was supplemented with 50 ng/ml NGF (Sigma) 30 minutes after seeding, then cells were analyzed 24 hours later. For experiments with MAG or neurocan, cell were grown on substrates coated with the protein of

interest for 48 hours. After culturing, DRG neurons were fixed and immunostained for GAP43 positive neurons, and imaged by scanning the entire wells using a Nikon Ti inverted fluorescence microscope. The total length of neurites and total neuron number for each well was measured using MetaMorph Neurite Outgrowth software, and used to calculate average outgrowth length per neuron.

Adult DRG neuron spot assay

For the spot assay (*9*), glass coverslips were coated with nitrocellulose and poly-L-lysine, and spotted with a 2 µl solution of aggrecan (0.6 mg/ml; Sigma) and laminin (5 µg/ml; Invitrogen) in calcium- and magnesium-free HBSS (CMF-HBSS, Invitrogen). These spots were allowed to dry completely and covered with a laminin bath (5 µg/ml in CMF-HBSS) at 37°C until immediately before cell plating (approximately 5 hours).

Dorsal root ganglion neurons were dissociated from adult (>P21) wild type or PTPσ –/– mice as described previously (*10*), with modifications. Briefly, DRGs were removed and their central and peripheral roots were cut off. Ganglia were incubated in collagenase II (200 U/ml; Worthington) and dispase II (2.5 U/ml; Roche) in HBSS. DRGs were rinsed and triturated in CMF-HBSS three times followed by low-speed centrifugation and removal of the supernatant. Dissociated DRGs were resuspended in Neurobasal-A media supplemented with B-27, Glutamax and penicillin/streptomycin (all from Invitrogen), counted and plated at a density of 2500 cells per coverslip. After 5 days *in vitro* cultures were fixed in 4% paraformaldehyde in PBS for 30 minutes, washed 3 times in PBS, and incubated in blocking solution for 2 hours at room temperature. Coverslips were incubated in primary antibody overnight. Primary antibodies used were anti-CS (CS56) and anti-β-tubulin III (both 1:500; Sigma) and were followed by appropriate secondary antibodies (Molecular Probes). The average number of β-

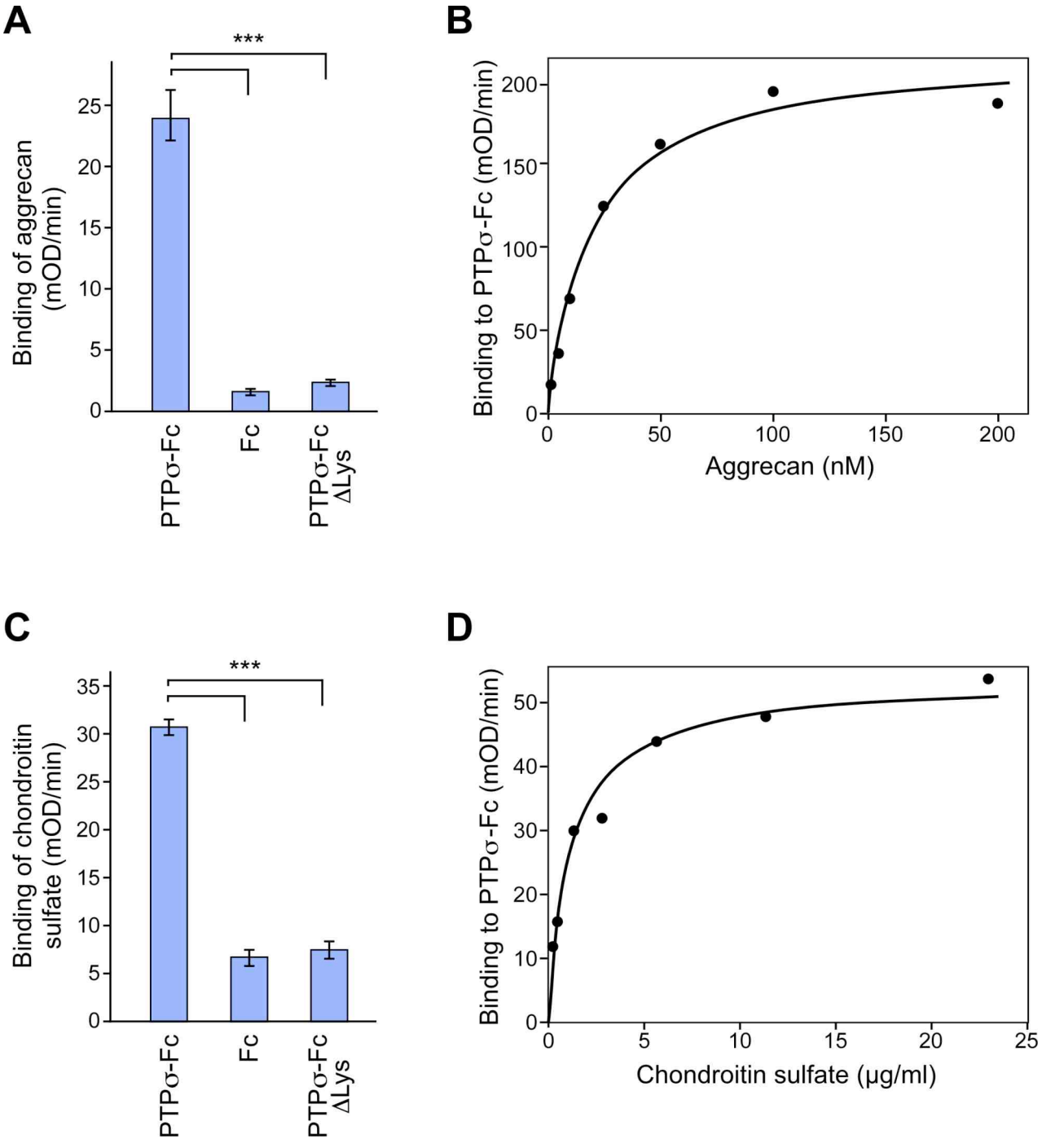
tubulin expressing neurites growing from the interior of the spot and crossing the outermost rim (visualized by CS56) were counted.

Spinal cord dorsal column crush lesion model

For *in vivo* experiments to examine the effect of a lesion on sensory fibers, adult female mice were anesthetized with inhaled isofluorane gas (2%) for all surgical procedures. All procedures were performed in a blinded fashion. A dorsal column crush spinal cord injury was performed as described previously (*10*), with modifications. Briefly, a T1 laminectomy was performed to expose the C8 spinal cord segment. Small holes were made in the dura 0.5 mm lateral to the midline with a 30 gauge needle and a dorsal column crush lesion was made by inserting Dumont # 3 jeweler's forceps approximately 0.5 mm into the dorsal spinal cord and squeezing, holding pressure for 10 seconds and repeating two additional times. The holes in the dura were covered with gel film, and the muscle layers were closed with sutures and the skin with surgical staples. The animals received Marcaine (1.0 mg/kg) subcutaneously along the incision as well as buprenorphine (0.1 mg/kg) intramuscularly. The dorsal columns were labeled unilaterally with Texas Red-conjugated dextran 3000 MW (DexTR, Invitrogen) 2 days prior to perfusion. The sciatic nerve of the right hindlimb was exposed and crushed with forceps, and a total of 1 µl of DexTR was injected via a Hamilton syringe into the sciatic nerve at the crush site. Animals were killed at 14 days post spinal cord injury with an overdose of isofluorane and perfused intracardially with PBS followed by 4% paraformaldehyde. Tissue was harvested and postfixed in 4% paraformaldehyde overnight, cryoprotected in 30% sucrose, frozen in OCT mounting media and cut into 20 µm thick longitudinal sections using a cryostat. Tissue was stained with anti-GFAP antibody (Accurate Chemical & Scientific) and CS56 antibody (Sigma) and incubated with Alexa Fluor 488 and Alexa Fluor 647 secondary antibodies (Invitrogen) respectively. Sections were imaged on a Zeiss Axiovert 510 laser scanning confocal microscope. All measurements were made in a blinded fashion. The lesion

center was determined by the characteristic GFAP staining profile. Two separate quantification paradigms were employed to determine the average position of injured fibers. First, for the quantitation shown in Fig. 4F, the distances between the endings of the 5 labeled axons closest to the center of the lesion were measured using Zeiss LSM 5 Image Browser software. The ventral-most sections from each animal were used in this quantification paradigm because the deepest part of the lesion tends to be most anatomically consistent between animals. Second, for the quantitation in fig. S4, the "fiber front" was defined as the point closest to the center of the lesion at which the labeled axons formed a fascicle of closely apposed fibers approximately 30 μm wide. The distance from this “fiber front” to the center of the lesion was then measured. The measurements were taken from every section containing a fasicle of at least 30 μm in every animal using Zeiss LSM 5 Image Browser software.

Figure S1


A
***
Binding of aggrecan (mOD/min)
0
5
10
15
20
25
PTPσ-Fc
Fc
PTPσ-Fc ΔLys
B
Binding to PTPσ-Fc (mOD/min)
0
50
100
150
200
Aggrecan (nM)
C
***
Binding of chondroitin sulfate (mOD/min)
0
5
10
15
20
25
30
35
PTPσ-Fc
Fc
PTPσ-Fc ΔLys
D
Binding to PTPσ-Fc (mOD/min)
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10
20
30
40
50
Chondroitin sulfate (μg/ml)

Figure S2

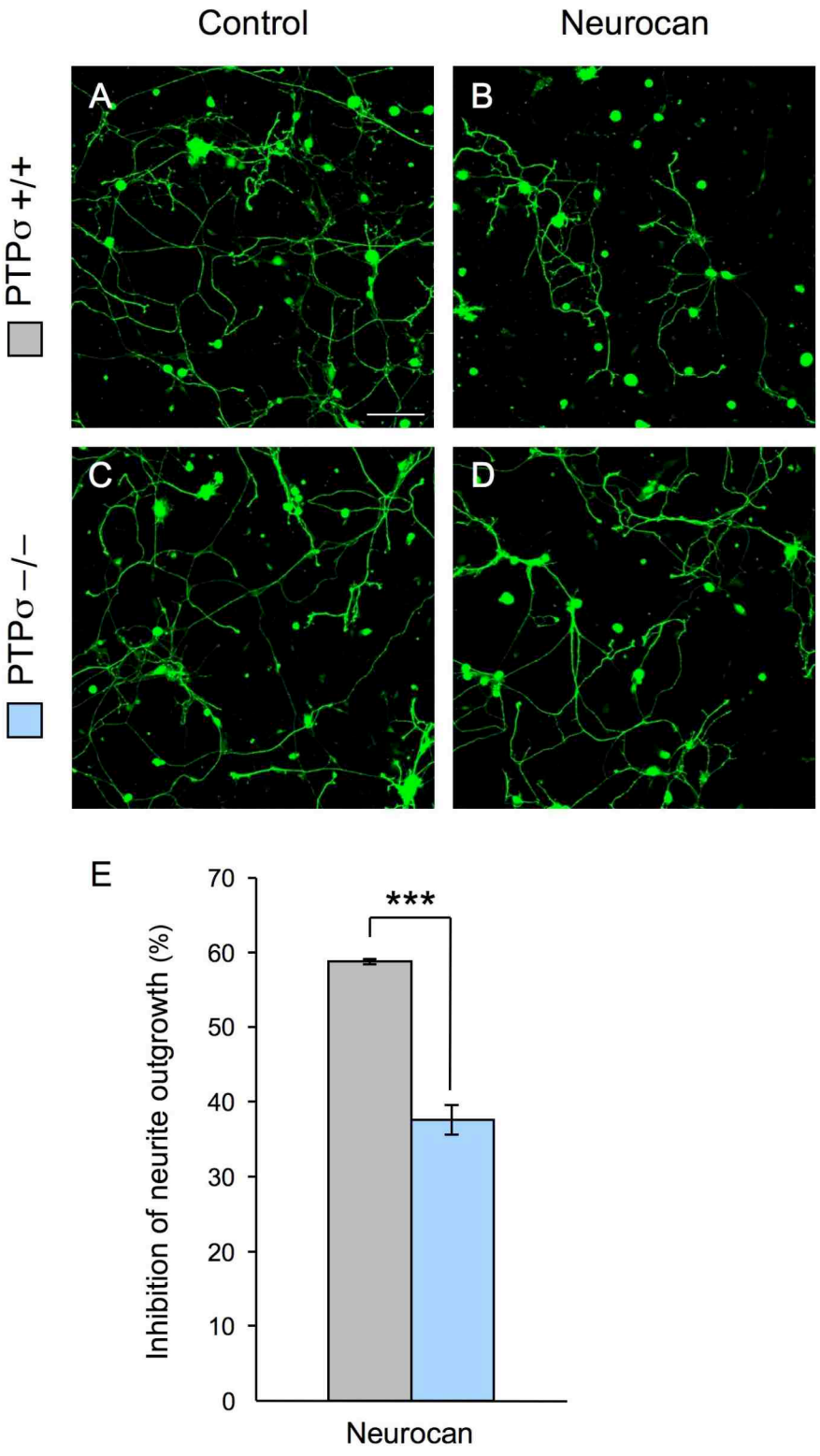

Control
Neurocan
A
B
C
D
PTPσ +/+
PTPσ −/−
E
Inhibition of neurite outgrowth (%)
70
60
50
40
30
20
10
0
***
Neurocan

Figure S3

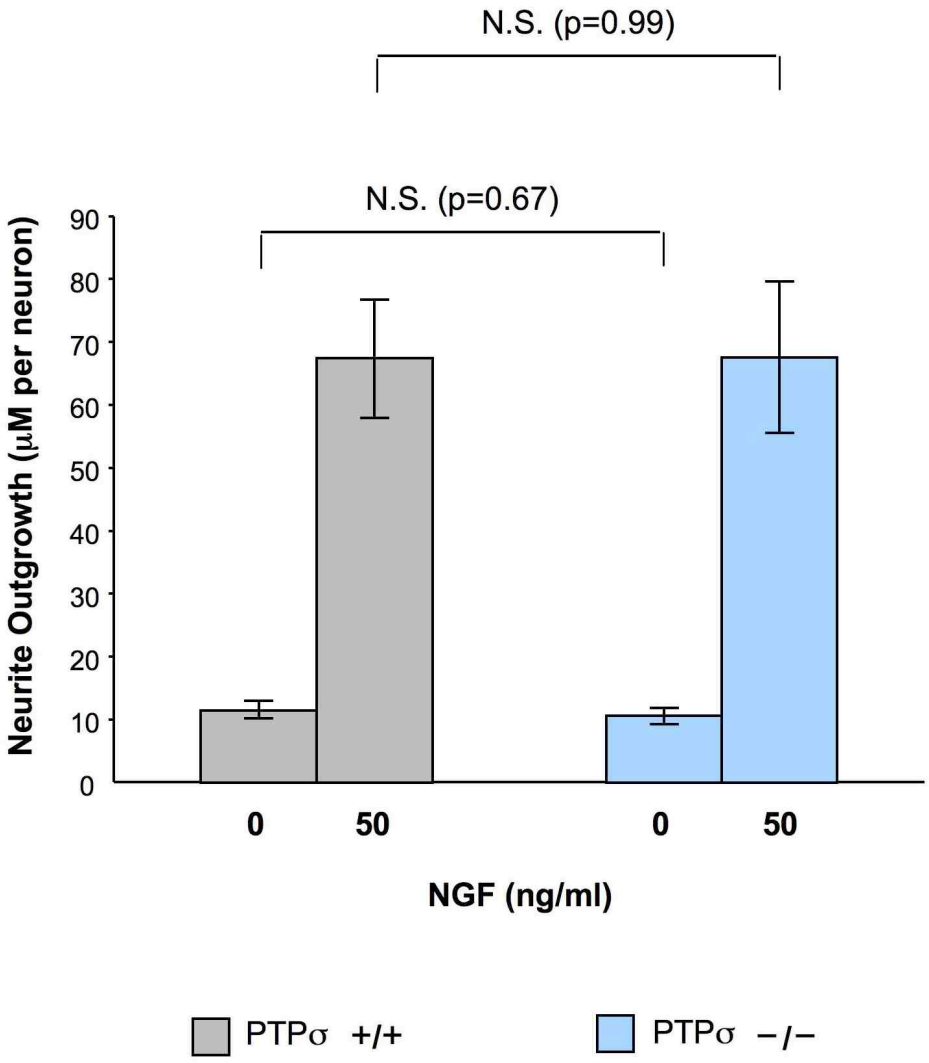
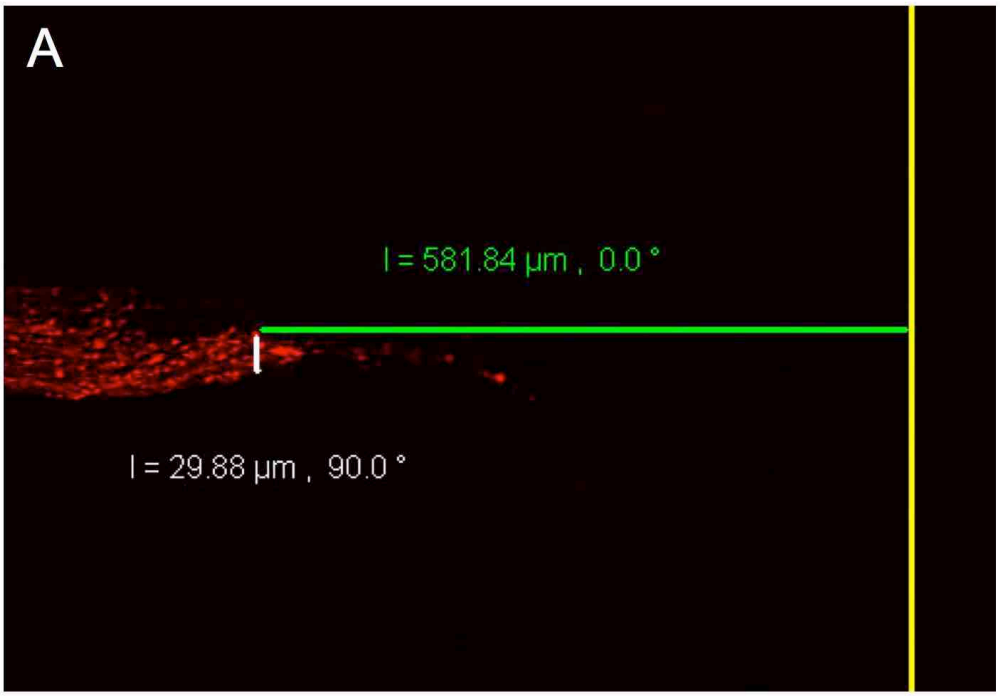
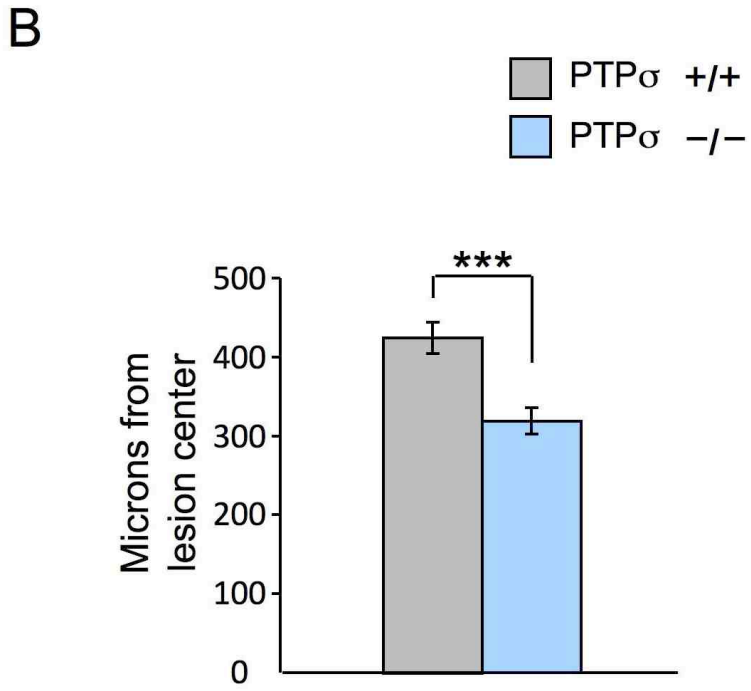

N.S. (p=0.99)
N.S. (p=0.67)
Neurite Outgrowth (μM per neuron)
90
80
70
60
50
40
30
20
10
0
0
50
0
50
NGF (ng/ml)
PTPσ +/+
PTPσ −/−

Figure S4


A
l = 581.84 µm , 0.0 °
l = 29.88 µm , 90.0 °


B
PTPσ +/+
PTPσ −/−
***
Microns from lesion center
500
400
300
200
100
0

Supplementary Figure legends

Figure S1. Binding of aggrecan or CS to the PTPσ ectodomain. Aggrecan or CS were biotinylated, and were detected with streptavidin-AP after binding to substrate-anchored PTPσ-Fc. (A) Aggrecan bound to PTPσ-Fc at levels higher than to Fc control. The PTPσ ΔLys mutant did not show binding significantly above control level. (B) Aggrecan binding to PTPσ-Fc was saturable. A hyperbolic curve fit produced a K_D of 19 nM. (C) Isolated CS bound to PTPσ-Fc at levels higher than to Fc control. The PTPσ ΔLys mutant did not show binding significantly above control level. (D) CS binding to PTPσ-Fc was saturable. Because the CS preparation is heterogeneous in molecular weight, its molarity and therefore the K_D cannot be calculated precisely in this experimental format. However, the data fit well to a hyperbolic binding curve (R^2=0.95) and based on a typical 10-50 kDa size range of CS preparations, the K_D is likely to be in the nanomolar range. Considering that binding energy is related to the logarithm of K_D ($\Delta G = -RT.\ln K$) these data suggest it is likely that most or all of the binding energy for the interaction of PTPσ with CSPGs comes from binding to the CS chains. However, we cannot rule out the possibility that PTPσ could also interact with the core protein of neurocan, aggrecan or other CSPGs. Error bars, SEM. ***p<0.001, Student's t test.

Figure S2. PTPσ –/– neurons showed less inhibition by neurocan than wild type neurons. DRG neurons from postnatal day 8-12 wild type or PTPσ –/– mice were dissociated and plated on slides coated with or without neurocan. (A, C) control, without neurocan; (B, D) 5 μg/ml neurocan. Cells were grown for 48 hours before immunostaining, and total length of GAP-43 positive neurites was quantified with Metamorph software. (E) Effect of neurocan, expressed as percent inhibition of outgrowth. Error bars, SEM. n=3 mice for each genotype. ***p<0.001, Student's t test. Scale bar, 100 μm.

Figure S3. PTPσ –/– and wild type neurons did not show significantly different outgrowth with NGF stimulation. DRG neurons from postnatal day 8-12 wild type or PTPσ –/– mice were dissociated and plated on slides coated with poly-D-lysine. 30 minutes after seeding, cells were treated with or without 50 ng/ml NGF, and were cultured for an additional 24 hours before immunostaining. Total length of GAP-43 positive neurites was quantified with Metamorph software. Error bars, SEM. n=3 mice for each genotype. Without NGF, p=0.67; with NGF, p=0.99; Student's t test.

Figure S4. Adult PTPσ –/– sensory neurons can extend further into a spinal cord lesion: additional quantitation method. Animals received a dorsal column crush injury, followed by axon tracing with DexTR, as described in Fig. 4. (A) Here, the extent of the axons is assessed by measuring to a "fiber front" which is defined as the point at which there is a fasicle of contiguous fibers approximately 30 μm wide or more. Yellow line shows location of the lesion center; green line shows distance to fiber front. (B) Quantitation of the distance of wild type or PTPσ –/– axon fiber front from the lesion center. Wild type n=80, PTPσ –/– n=53 sections; error bars, SEM; ***p<0.0001, Student's t test.

Supplementary references

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