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Supplemental Information

SorCS2 Regulates Dopaminergic Wiring and Is Processed into an Apoptotic Two-Chain Receptor in Peripheral Glia

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Supplemental Experimental Procedures

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SUPPLEMENTAL INFORMATION

EXTENDED EXPERIMENTAL PROCEDURES

Cell lines and transfection

All cell lines were cultured at 37 °C in a humidified atmosphere containing 5 % CO₂. Human embryonic kidney HEK293 cells and rat Schwannoma RN22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Bio-Whittaker) supplemented with 10 % fetal calf serum (FCS). Chinese hamster ovary (CHO-K1) cells were cultured in serum-free HyQ-CCM5 medium (HyClone). All culture media were supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml) (Gibco-BRL). Transfection of the cells was carried out using FUGENE 6 (Roche) as transfection agent. 48-72 hrs post transfection, stable transfectants were selected at 300 µg/ml zeocin and/or 400 µg/ml G418 (Gibco-BRL) and identified by Western blotting and immunocytochemistry.

Primary cultures

Dorsal root ganglion (DRG) and superior cervical ganglion (SCG) cultures were prepared from neonate mice by digesting the isolated ganglia in trypsin (0.125%) and collagenase (1 mg/ml), followed by seeding on poly-L-lysine (Sigma)/laminin (Invitrogen) in DMEM supplemented with 10% FCS, Primocin (Lonza), 20 µM 5-Fluoro-2'-deoxyuridine, 20 µM Uridine and 2 nM nerve growth factor (NGF). Cerebellar granule neurons (CGN) were prepared from P6 pups, digested in trypsin and seeded on poly-L-lysine/Laminin in neurobasal media (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), Glutamax and Primocin. Separation of DRG cultures into purified neuron and glia cultures was achieved by preplating the DRG culture for 2-3 hrs on an uncoated cell culture dish. Attached cells (glia) were maintained in DMEM/FCS, which was replaced regularly over the next days with ice cold DMEM/FCS. Unattached cells (mostly neurons) were plated as described above in neuron media with NGF and mitotic inhibitor. The purity of the cultures were confirmed by visual inspection and after 10 DIV the cultures were >99% pure. Cultures of hippocampal neurons were derived from P0 neonate hippocampus that were dissected in cold PBS and placed in ice-cold L-15 medium (Invitrogen). Papain (Worthington) was diluted in L-15 (20 units/ml, EDTA 2 mM) and activated at 37°C for 20 min. Tissues were digested in the papain solution for 30 min at 37°C and the enzyme

reaction quenched with DMEM + 10% FCS. After a brief spin down, the tissue pellet was resuspended by DMEM (10% FCS and DNaseI 10 units/ml) and triturated with fire-polished plastic pipettes. Dissociated cells were spun down again and resuspended in Neurobasal (Invitrogen) supplemented with B27 (Invitrogen), Primocin (Lonza) and 5-fluoro-2'-deoxyuridine (Sigma) for plating. Hippocampal cells were cultured in 12-well dishes (approx. 1.5 mio cells per well) or 12mm coverslips (5000 cells per coverslip) coated with poly-L-lysine and laminin. Half of the medium was replaced twice a week and the neurons were lysed for Western blot analysis at DIV12. Hippocampal neurons were co-transfected with constructs encoding SorCS2 variants and GFP using Lipofectamine 2000 (for growth cone analysis) or by nucleofection using the Amaxa system (for Western blot analysis). Cortical glial cells were prepared as described in (Burke et al., 1998). Embryonic dopaminergic neurons were prepared by isolation of the ventral mesencephalon from E14.5 fetuses, as described by (Pardo et al., 1997). Dissociated cells were seeded on poly-L-Lysine coated coverslips in DMEM + 15% FCS, and the media changed to Neurobasal/B27 the following day. Explants of E14.5 ventral midbrain was prepared by isolation of a 1.5x1.5 mm area of the ventral mesencephalon (described by (Pardo et al., 1997)). Each piece were further divided into 12-16 pieces and mounted into a platform of collagen (3 mg/ml). The explants were maintained in DMEM/10% FCS the first day and the media changed the following day to neurobasal/B27. Explants (DIV2), hippocampal and cerebellar granule neurons (DIV3) were stimulated with proBDNF for 20 min, fixed in 4% PFA and fluorescently labeled against actin (Phalloidin-rhodamine) and TH. Growth cone morphology was subsequently evaluated by an investigator blinded to the experiment. Significance was assessed using two-tailed t-test.

Embryonic schwann cells were prepared from isolated DRGs from E12.5-E13.5 fetuses as described by (Kim et al., 1997; Kim et al., 1995). DRGs were dissociated in 0.25% trypsin in Hanks Balanced Salt solution, and hereafter seeded out in DMEM/10% FBS with NGF (Harlan Bioproducts, BT-5025, 50 ng/ml) on uncoated plastic culture dishes. After 18 hours, the medium was changed to N2/NGF medium (DMEM:F12 1:1, N2 supplement (Gibco 17502-048), NGF (50 ng/ml). After 96 hours the extensive neuronal network including Schwann cells were harvested, and thus isolated from fibroblasts, and were digested in trypsin (0.25%) and collagenase type I (0.1%). After digest, the remaining fibroblasts were killed using anti-Thy1.2 (Serotec, MCA1474T) and rabbit complement.

Schwann cells were seeded on poly-L-coated plastic dishes in DMEM/10%FBS. After 24 hours the medium was changed to DMEM/N2 supplement, with Neuregulin- β 1 EGF domain (R&D, 396-HB-050, 10ng/ml) and Forskolin (Sigma, F-6886, 2.5 μ M).

Cloning, mutagenesis, and recombinant proteins

The human SorCS2 full length receptor (accession number NM_020777) and mutants were expressed with an artificial signal peptide derived from osteonectin (accession number NP_003109). SorCS2 encompassing its native signal peptide was also cloned. No difference in processing and cellular trafficking was observed between the two SorCS2 signal peptide variants (data not shown). SorCS2 amino acids 51 to 385 was derived, as a PCR fragment, from a EST clone (accession number R61161) and amino acids 386 to 1159 was excised from clone KIAA1329 (accession number AB037750) (Nagase et al., 2000). The 3'-UTR of KIAA1329 was shortened by digestion and self-ligation prior to ligation with the signal peptide and the EST fragments. The SorCS2 cDNA was expressed using the pcDNA3.1/zeo vector (Invitrogen). Insertion of a BlnI/NotI linker into the full length SorCS2 construct generated a cDNA fragment encoding the extracellular amino acids 51-1079. The soluble SorCS2 variant (SorCS2-sol) was expressed as a myc-his tagged peptide using the pcDNA4/*myhis* vector (Invitrogen). Site-directed mutagenesis was performed using QuickChange Multi kit (Stratagene) using SorCS2 variants as templates. Full length rat p75^{NTR} inserted into pcDNA3.1 was kindly provided by Professor Moses Chao, Skirbal Institute, New York. Purified Fc-tagged p75^{NTR} extracellular domain was from R&D Systems. Soluble SorCS2 variants were purified from the culture medium of stably transfected CHO cells using TALON-matrix affinity chromatography (Invitrogen). Recombinant mouse proNGF was from Chemicon, Mouse NGF was from Austral Biologicals. Neurotrophic factor propeptides were expressed in *E.coli* using the Glutathione S-Transferase Gene Fusion System (GE Healthcare) as previously described (Munck Petersen et al., 1999; Westergaard et al., 2005). The cDNA sequence encoding the last 28 aa of the cytoplasmic tail of murine SorCS2 was PCR-amplified using primers that generate BamHI- and XhoI-sites as described above and cloned into the pGEX4T-1 vector, expressed as a GST-fusion protein, and purified.

Deglycosylation

SorCS2 variants were immunoprecipitated from lysates of transfected cells and heated in 10 μ l 1% SDS (95°C, 5 min). Precipitates were then cooled before the addition of 90 μ l of 20 mM NaH₂PO₄, 10 mM EDTA, 0.5 % Triton X-100, pH 7.2, and 0.5 U PNGase F (Roche) and incubated at 30 °C for 18 h. Reactions were stopped by addition of reducing SDS sample buffer, and subsequently analyzed by Western blotting. Treatment with neuromidase or O-glycosidase was performed in a similar manner.

Metabolic labeling

Transfected HEK293 cells were labelled using ~200 μ Ci/ml L-[³⁵S]-cysteine and L-[³⁵S]-methionine (Pro-mixTM; GE Healthcare) in medium without methionine or cysteine in the presence of Brefeldin A (Pierce). After 4 h, the cells were washed and changed to normal culture medium, and newly synthesized SorCS2 variants were chased for specific time periods. SorCS2 was subsequently immunoprecipitated from cell lysates using α ECD, α CT, or α PRO, separated by SDS-PAGE, and visualized using phosphoimaging.

Immunofluorescence

HEK293 cells stably transfected with SorCS2 variants were cultured on poly-L-lysine coated cover slips and incubated with cold medium containing proBDNF or proNGF for 2 h on ice. The cells were fixed in 4% paraformaldehyde (PFA), pH 7.4, and bound proNT was subsequently visualized by incubation with anti-NGF mAb (Promega) or anti-BDNF mAb (a generous gift from Professor Yves Barde) followed by secondary Alexa 546-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse secondary antibodies (Invitrogen), respectively. Images were obtained by confocal microscopy (LSM 780, Carl Zeiss, Germany). Primary cultures were stained for SorCS2 using α ECD (10 μ g/ml) and Alexa 488-conjugated goat anti-rabbit secondary antibodies. Cryosections (14 μ M) of mouse embryos (sagittal sections), brain (coronal sections), DRG, and sciatic nerves were stained using a similar protocol including a blocking step with goat anti-mouse Fc Fab fragments (Jackson Labs). Sections were stained using the following antibodies. Rabbit anti-proBDNF and rabbit anti-proNGF were from Alomone (Both used 1:200). Rabbit anti-NF200 (1:1000) was from Abcam. Rabbit anti-peripherin (1:8000) was from Abcam. Rabbit anti-TrkA (1:2500) was a generous gift from Professor Lou Reichardt, UCSF. Goat anti-TrkB (1:400) was from R&D Systems. FITC-conjugated IB4 (10 μ g/ml) was from Sigma.

Sheep anti-SorCS2 (1:50) was from R&D Systems. Rabbit anti-p75^{NTR} 9651 (1:1500) was a generous gift from Professor Moses Chao. Goat anti-p75^{NTR} (1:50) was from R&D Systems. Goat anti-Sortilin (1:50) was from R&D Systems. Rabbit anti-cleaved Caspase-3 (1:200) was from Cell Signalling. Rabbit anti-GFAP (1:500) was from Dako. Mouse anti-TH (1:2000) was from BD laboratories. For further information on our use of primary antibodies, please see www.pabmabs.com.

In situ hybridization

The *in situ* hybridization was conducted by Prof. Gregor Eichele, Max Planck Institute, Hannover, as part of an integrated project founded by the European Community (Framework programme 6; www.eurogene.org), and the data is extracted from the Genepaint database (www.genepaint.org).

Surface plasmon resonance

Surface plasmon resonance measurements were performed on a Biacore 3000 Instrument (Biacore, Uppsala, Sweden) equipped with CM5 sensor chips activated as described previously (Munck Petersen et al., 1999). Soluble SorCS2 variants were immobilized to densities 74-83 fmol/mm² in 10 mM sodium acetate, pH 4.0, and remaining coupling sites were blocked with 1 M ethanolamine. Samples were injected at 5 µl/min at 25°C in 10mM HEPES, 150 mM NaCl, 1.5 mM CaCl₂, 1 mM EGTA, and 0.005% Tween 20, pH 7.4 (CaHBS). Binding was expressed in relative response units (RU); the difference in response between the immobilized protein flow cell and the corresponding control flow cell. Kinetic parameters were determined using BIAevaluation 4.1.

Tissue preparation

Mice were killed by cervical dislocation. Dissected tissue was lysed in 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 140 mM NaCl, pH 7.8, and 1% Triton X-100 containing protease inhibitors (Complete, Roche) using a pellet pestle and centrifuged at 10.000 x g for 10 min at 4°C. Samples were kept on ice at all times and protein concentration was determined using the Bicinchoninic Acid kit from Sigma.

Western blotting

Protein samples were separated by SDS-PAGE and electro-blotted for 2 hours onto polyvinylidene difluoride (PVDF) filters (Amersham) in 192 mM glycine, 25 mM Tris-HCl, pH 8.0. Membranes were subsequently blocked in 0.05 M Tris-base, 0.5 M NaCl, supplemented with 2% skimmed milk powder and 0.5% Tween-20 (TST). After blocking, membranes were washed in 10 mM HEPES pH 7.8, 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.2% skimmed milk powder, 0.05% Tween-20, and incubated with primary antibodies diluted in washing buffer as follows, rabbit anti-p75^{NTR} (1:750, Abcam), rabbit anti-SorCS2 serum (1:2000), anti-SorCS2 mAb S21 (5 µg/ml). For further information on our use of primary antibodies, please see www.pabmabs.com. Following another washing step, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit anti-mouse, swine anti-rabbit, or rabbit anti-goat (Dako)) diluted 1:1000 in washing buffer. Following a final washing step, blots were visualized with the ECL plus Western Blotting detection system (Amersham) and Fuji film LAS1000.

Co-immunoprecipitation

Stably transfected HEK293 cells were treated with (in the case of SorCS2/p75^{NTR}) or without (when studying the interaction between the SorCS2 N-terminal and C-terminal fragments) 2 nM reducible protein cross-linker dithiobis-(succinimidylpropionate) (DSP) (Pierce) according to manufacturer's instructions. After washing, cells were lysed on ice for 10 minutes in TNE buffer supplemented with protease inhibitors. Samples were immunoprecipitated overnight at 4°C by use of Gammabind G-Sepharose beads (GE Healthcare) coupled with antibodies against either SorCS2 or p75^{NTR}. Unspecific binding was removed by washing 5 times in TBS containing 0.05% Tween-20, and proteins eluted by boiling samples in reducing sample buffer (20 mM dithioerythritol (DTE), 2.5% SDS).

Cell death assay

Schwannoma cells stably transfected with SorCS2 variants were seeded into poly-lysine coated black 96-well tissue culture plates (PerkinElmer) at a density of 10,000 cells/well. After 24 hours, cells were washed three times in DMEM without phenol red, supplemented with antibiotics, 1% glutamax (Gibco), 1.5 µg/mL insulin, 50 µg/mL transferrin, 30 nM selenium, and 30 nM triiodothyronine (all from Sigma), and incubated with or without proNGF. Following 72 hours of incubation, the number of dead and live cells was scored

with the fluorescent-based MultiTox-Fluor Multiplex cytotoxicity assay (Promega) on a Victor³ 1420 multilabel counter. Only the live cell component of the assay was used as this was found to depend linearly on the actual number of live cells per well. The relative cell death was then expressed as the reduction in the live cell signal per cent. At least two different polyclonal stable cell lines for each construct were analyzed with similar results. Mock transfected cells served as control. Significance was assessed using two-tailed t-test.

Sciatic nerve morphology

Sciatic nerve segments from adult mice were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 6 hours in 4° C. Samples were postfixed in 2% osmium tetroxide for 2 hours and dehydrated in ethanol (30% to 100% gradient). After two passages in propylene oxide and o/n in a 1:1 mixture of propylene oxide and Glauerts' mixture of resins, specimens were embedded in Glauerts' mixture of resins (equal parts of Araldite M and the Araldite Harter, HY 964, subsequently added 0.5% of the plasticizer dibutylphthalate). 2% of accelerator 964 was finally added to the resin to promote polymerization. From each nerve, 2.5µm thick series of semi-thin transverse sections were cut (ultramicrotome) and stained with 1% Toluidine blue for high resolution light microscopy examination and design-based stereology (see below). For electron microscopy, ultra-thin sections (70nm thick) were cut from the same samples using the same ultramicrotome and stained with saturated aqueous solution of uranyl acetate and lead citrate. The total cross-sectional area of the whole nerve was measured by light microscopy and 12–16 sampling fields were selected using a systematic random sampling protocol (Geuna, 2000; Geuna et al., 2000; Larsen, 1998). Mean fiber density was calculated by dividing the total number of nerve fibers within the sampling field by its area (N/mm²). Total fiber number (N) was finally estimated by multiplying the mean fiber density by the total cross-sectional area of the nerve. Fiber and axon areas were measured and the diameter of fiber (D) and axon (d) were calculated and subsequently used to calculate myelin thickness $[(D-d)/2]$, myelin thickness/axon diameter ratio $[(D-d)/2d]$, and axon/fiber diameter ratio, the g-ratio (D/d).

Anatomical analysis of the adult dopaminergic system

6 WT and 6 SorCS2 knockout mice (14 weeks old), were sacrificed with transcardial perfusion with phosphatebuffered 4% paraformaldehyde pH=7.4. The brains were removed and placed in the fixative for 1 day until coronal (n=2), sagittal (n=2), or horizontal (n=8) vibratome sectioning into 50 μ m thick sections. The sections were stored in a cryoprotective buffer, and then subsequently stained with a Nissl stain, and immunohistochemically by staining for TH. The latter were performed on free floating sections rinsed initially in Tris buffered saline (TBS) + 1% Triton for 3 x 5 min. The sections were then incubated for 20 min. with avidin 0,1%, rinsed with TBS for 2 min. and then incubated for 20 min with Biotin 0,01%. After a rinse with TBS for 2 min. the sections were preincubated with 1% Triton X-100 and 0,2% milk in TBS for 30 min. followed by incubation with the primary antibody (polyclonal rabbit anti-tyrosine hydroxylase; P 40101-0, Pel Freez, Arkansas, USA) diluted 1:600 for 72 hrs at 4° C. The sections were then rinsed with TBS in 1% Triton for 3 x 15 min before incubation with the secondary ab (anti-rabbit IgG, biotin labeled, abcam, ab6720) diluted 1:400 in TBS 1% Triton + 0,2% milk for 1 hr at room temp. The sections were then rinsed in TBS, followed by blockade of endogenous tissue peroxidase in all glasses with 3% H₂O₂ and 10% methanol in TBS for 15 min. The sections were then rinsed with TBS + 1% Triton for 3 x 15 min, before avidin-peroxidase (Sigma: A 3151) diluted 1:200 (5 μ g/ml) were applied to all glasses for 1 hr at room temp. After a new rinse with TBS + 1% triton for 3 x 15 min. we visualized the avidin-bound peroxidase by incubating with 0,1 % DAB-solution including 0,3 % H₂O₂ for 5 min. Then a brief wash, before dehydration in graded alcohol and xylene followed by mounting and coverslipping with DePX. The sections were analyzed on a Leica DM5000B with attached camera.

Quantitative image analysis of TH+ innervation of the infralimbic cortex

Wt mice, p75^{NTR} knockout mice, and SorCS2 knockout mice (14 weeks old, n=4 per genotype) were perfused and the brains vibratome sectioned as described in the previous paragraph. The 50 μ m thick coronal sections were after an initial rinse in TBS + 1% Triton for 3x5 min, then transferred to 80 °C preheated sodium citrate buffer for 30 min for heat induced epitope retrieval, before the sections were TH-stained as described above under anatomical analysis.

The infralimbic cortex corresponding to Figure 6B and S7C-D were identified and one section from each of the twelve animals selected for subsequent staining quantification. This procedure was based on the measurement of the area covered by staining (area fraction, or % of area covered by DAB staining), because the direct measurement of the DAB staining intensity is not valid as DAB staining does not meet the Beer-Lambert criteria for photometric material – e.g. there is no linear association between the staining intensity and the DAB concentration.

All sections were photographed using the same microscope setting for illumination intensity, field aperture etc., as well as the same setting of the camera (exposure time, levels, and white balance).

First, 24 bits color photographs were taken with a Leica DFC 480 camera mounted on a Leica DM5000B microscope with the picture size 2560 × 1920 pixels. A 20x objective was used. The pixels on the resulting image represent an area with a size of 0.167 μm × 0.167 μm for the 20x objective. Six photographs were taken, from randomly chosen (systematic random sampling) fragments of the analyzed area (right infralimbic cortex).

To allow measurement, photographed sections were converted into 8 bit image followed by segmentation (thresholding). Because the DAB gives a brown color which is difficult for analysis (it is a mixture of all RGB colors), segmentation were performed using the blue channel from RGB images (Brey et al., 2003). The pictures were analyzed with the use of autothreshold. The area covered by the staining were estimated in the Fiji program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, 160 Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997–2005). The mean area fraction was calculated per brain and then data were presented on charts and analyzed using Man-Whitney U test.

Dopamine levels

To determine the concentrations of 3,4-dihydroxyphenylalanine (DOPA), dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT), the striatal and frontal cortical samples were thawed and homogenized with a Teflon pestle adding 0.2 ml ice-cold 0.5 M/l perchloric acid containing 0.05% w/v sodium thiosulfate (Pharmacy, Aarhus University Hospital). The homogenate was centrifuged for 5 min at 13.200 rpm. The supernatant was analyzed by HPLC using Perkin-Elmer Series 200 HPLC pump (Titan) connected in series with a Rheodyne 7025 injector (20 μl loop), a

Phenomenex Sphereclone 5 μ ODS (2) (250 x 4.6 mm, Phenomenex), a Coulochem III (ESA) electrochemical detector in amperometry mode (400mV). For data acquisition Dionex Chromeleon Software 6.8 was used. The mobile phase was a mixture of 90% buffer containing 37 mM NaH₂PO₄ x H₂O (4.55 g/l), 1 mM octanesulphonic acid sodium salt (195 mg/l), 0.04 mM EDTA (14 mg/l), pH 3.3 adjusted with acetic acid and 10% ethanol. At a flow rate of 1 ml/min DOPA eluted around 4 min, DOPAC around 8 min, dopamine around 10 min, HVA around 15 min and 3-MT around 20 min. Metabolite concentrations were determined by comparison the signals with those of the respective compound, concentration 1 μ g/ml each. For details, see (Gamache et al., 1993; Mikkelsen et al., 1999). Significance was assessed using two-tailed t-test.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Processing sites in SorCS2. Additional results related to Figure 2.

(A) The domain structure of SorCS2. The signal peptide (SP) is followed by the propeptide (PRO), the Vps10p-domain (Vps10p-D), the polycystic kidney disease domain (PKD), the leucine-rich region (LRR), a transmembrane region (TM), and a C-terminal cytoplasmic tail (CT).

(B) The SorCS2 N-terminal starting at Arg51 after removal of the signal peptide. The amino acid numbering refers to human SorCS2. Putative furin cleavage sites in the propeptide are in bold (Site 1-3).

(C) Alignment of the residues surrounding the identified two-chain cleavage site (Site 4) of SorCS2 from different species. Strictly conserved residues are indicated by shading.

(D) The heterodimerization of the SorCS2 two-chain receptors does not depend on disulfide bond formation as the band pattern is unchanged by the presence or absence of reductant (DTT) as shown by WB using α ECD.

(E) Co-localization of the extracellular domain (ECD) with the cytoplasmic tail (CT) of the SorCS2-two variant expressed in HEK293 cells. Cells were stained with a rabbit polyclonal antibody raised against the cytoplasmic tail (anti-CT) and a mouse mAb raised against the extracellular domain of SorCS2 (anti-ECD mAb).

(F) Relative plasma membrane expression of SorCS2 variants as determined by surface biotinylation followed by precipitation with Streptavidin beads and immunodetection of SorCS2 in the precipitate (surface exposed) and supernatant (intracellularly), respectively. Western blotting for the insulin receptor and beta-actin was performed as quality control for the surface and intracellular preparations.

(D) Internalization of SorCS2. Transfected HEK293 cells expressing SorCS2-wt, SorCS2-one, or SorCS2-two were incubated for 2 hrs on ice with α ECD. Cells were either fixed immediately thereafter (0 min) or chased for 45 min at 37 °C prior to fixation. SorCS2 antibodies (*green*) are targeted to a Vit1b-positive Golgi-compartment (*red*) independent of the SorCS2 variant present.

Figure S2. Generation of the *Sorcs2* $-/-$ mouse. Additional results related to Figure 4.

(A) Gene targeting strategy for inactivation of *Sorcs2* by homologous recombination. For further details, see Experimental Procedures.

(B-D) Characterization of SorCS2 knockout mouse tissue by Southern blotting, Western blotting of the hippocampus (upper panel) and DRG and SCG homogenates (lower panel), and by immunofluorescence staining of hippocampus (CA1) and DRG (α ECD).

Figure S3. p75^{NTR} subcellular localization is unaltered in SorCS2 KO neurons. Additional results related to Figure 4.

(A-C) Examples of SorCS2 and p75^{NTR} expression and localization in DIV7 hippocampal neurons derived from P0 Wt, SorCS2 KO, and p75^{NTR} KO mice.

Figure S4. In situ hybridization and immunohistochemistry showing SorCS2 expression during embryonic development. Additional results related to Figure 5.

(A-H) In situ hybridization for SorCS2 in E14.5 mouse embryos (sagittal sections). (D-H) Zoom views of the areas indicated in panels a-c. Cb, cerebellum; Cx, cortex; Hi, hippocampus; He, heart; Ht, hypothalamus; Ki, kidney; Li, liver; Lg, lung; Me, medulla oblongata; Mb, midbrain; P, pons; Sc, spinal cord; St, striatum; T, thalamus.

(I) Control for the specificity of the hybridization on lung tissue using anti-sense (SorCS2 as, left panel) or sense (SorCS2 s, right panel) probes.

(J) No expression of SorCS2 (green) in cerebellum, cortex, and dorsal hippocampus at E15.5.

(K) No expression of sortilin (green) in the embryonic dopaminergic system (E15.5). In contrast, high sortilin levels are found in postnatal dopaminergic neurons (P5).

Sagittal sections are shown. Higher magnifications are shown to the right. Dopaminergic neurons were identified by staining for TH (red) and nuclei are stained using Hoechst.

Figure S5. Increased dopaminergic innervation of frontal cortex in SorCS2 knockouts. Additional results related to Figure 6.

(A-B) TH-stained sagittal sections through the Wt brain (A) and SorCS2 KO brain (B) illustrating the nigrostriatal system in its full extent. Note that both groups of animals displayed a similar innervation pattern. Compass to the right indicates A, anterior, P, posterior, D, dorsal, and V, ventral directions.

(C-D) An increased innervation density was however noticed in the infralimbic/cingulate cortex of the SorCS2 KO animals as illustrated in horizontal TH-stained sections from Wt (C) and SorCS2 KO (D) animals corresponding to the position marked by line CD on image A. Note that the both animals display a similar innervation pattern but the SorCS2 KO animal is much denser innervated in cingulum, indusium griseum and the supragranular layers of the infralimbic cortex. Compass to the right indicates A, anterior, P, posterior, L, dorsal, and M, ventral directions.

Figure S6. SorCS2 KO sciatic nerve is protected from injury. Additional results related to Figure 7.

(A) Two-chain SorCS2 is upregulated following sciatic nerve lesion (24 hours post injury). Two-chain SorCS2 levels are increased both proximal (P) and distal (D) to the lesion (ipsilateral side) as determined by WB analysis (755 ± 243 % and 2018 ± 401 %, respectively, compared to unlesioned nerve, $n=3$, 50 μ g tissue homogenate per lane, 12-16 weeks old mice were used).

(B) Sciatic nerve injury in perinatal (P2) Wt and SorCS2 KO mice analyzed by SorCS2 staining 24 hours post injury. Sections from the contralateral side and the ipsilateral side distal to the injury are shown.

(C-D) Sciatic nerve injury in adult Wt and SorCS2 KO mice analyzed by TUNEL staining. Data obtained from sections distal to the injury are shown ($n=3$).

(E) Sciatic nerve injury in perinatal (P2) Wt and SorCS2 KO mice analyzed by TUNEL staining. Data obtained from sections distal to the injury are shown ($n=3$).

Error bars indicate SEM. * $p < 0.05$.

Figure S7. Characterization of the peripheral nervous system in uninjured SorCS2 KO mice. Additional results related to Figure 7.

(A) The neuronal subtype composition of lumbar DRGs from wt is not statistically different from that of SorCS2 KO mice as estimated from quantifications of TrkA-positive neurons (wt = $23.2 \pm 2.63\%$ and KO = $26.7 \pm 2.92\%$, $p=0.60$), TrkC-positive neurons (wt = $12.2 \pm 0.19\%$ and KO = $14.3 \pm 1.39\%$, $p=0.20$), IB4-positive neurons (wt = $30.6 \pm 4.88\%$ and KO = $43.1 \pm 5.03\%$, $p=0.15$), NF200-positive neurons (wt = $36.4 \pm 2.88\%$ and KO = $34.9 \pm 4.52\%$, $p=0.80$), p75^{NTR}-positive neurons (wt = $33.7 \pm 1.13\%$ and KO = $35.9 \pm 3.42\%$, $p=0.57$), and peripherin-positive neurons (wt = $42.2 \pm 1.94\%$ and KO = $44.9 \pm 3.10\%$, $p=0.49$).

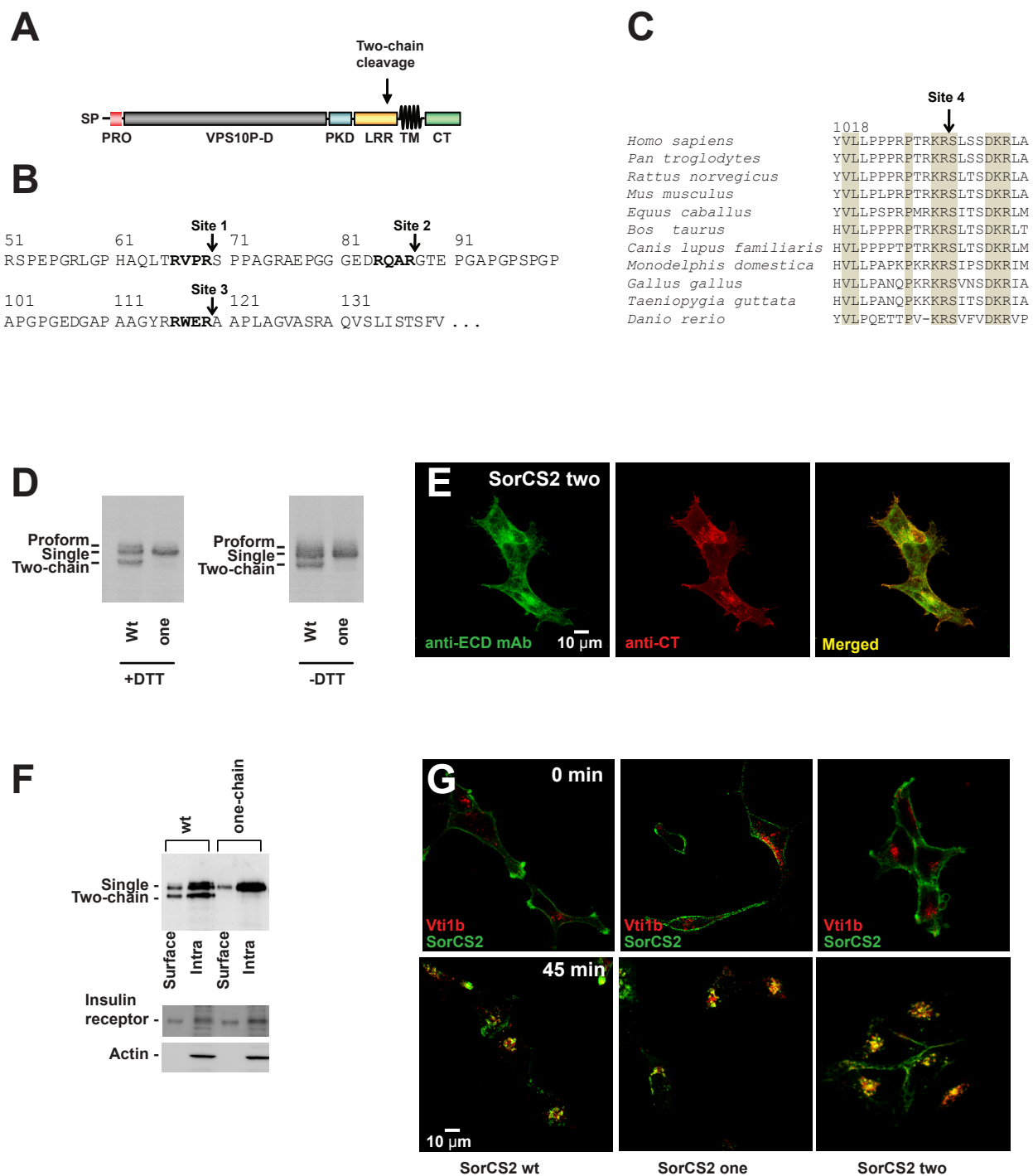
Representative images of whole DRG cross sections generated from confocal tile scans are shown (n=3 of 12 weeks old mice).

(B) Sciatic nerve morphology was not affected by loss of SorCS2 as assessed by histological analysis of sciatic cross sections using light (lower left panel) and electron microscopy (lower right panel) (n=7 of each genotype, 12 weeks old mice). Furthermore, quantifications of S100-positive cells/area in horizontal sciatic sections suggested that the number of Schwann cells were similar in wt and KO mice (0.021 ± 0.0046 cells/ μm^2 in wt versus 0.016 ± 0.0010 cells/ μm^2 in KO mice, $p=0.34$). Six sections through each nerve from each animal (n=3) were stained for S100 and analyzed by confocal microscopy and ImageJ software.

(C-E) Nociception, tactile sensation and motor function were identical in naïve wildtype and SorCS2 knockout animals as determined using hotplate ($p=0.35$), Von Frey ($p=0.36$), and rotarod ($p=0.79$) tests.

Error bars indicate SEM. Significance was evaluated using two-tailed t-test.

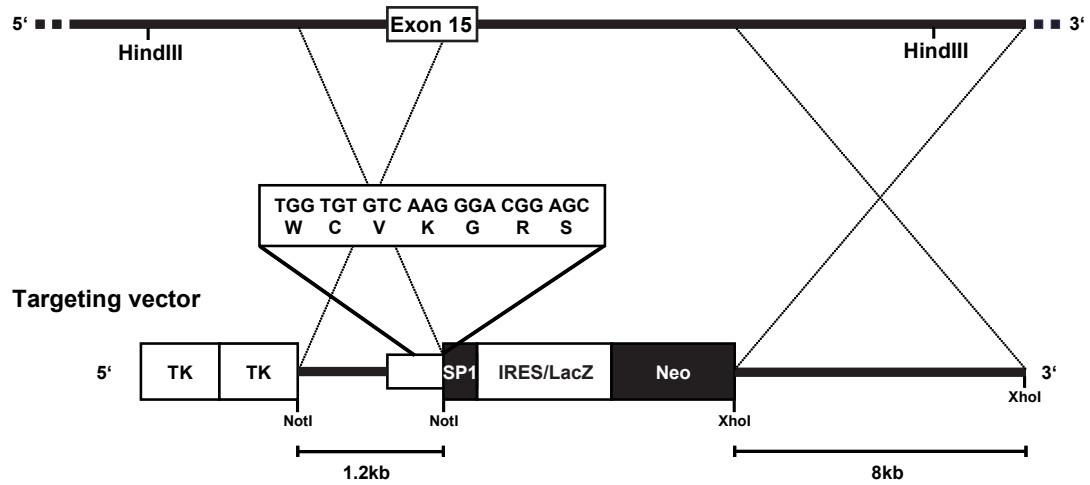
Supplementary figure 1



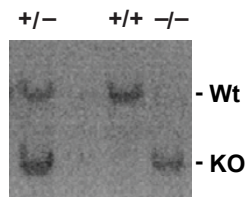
Supplementary figure 2

A

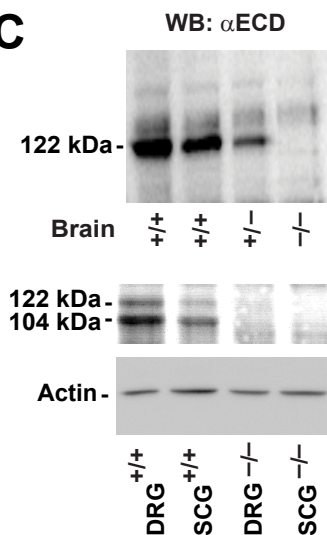
Wild-type allele



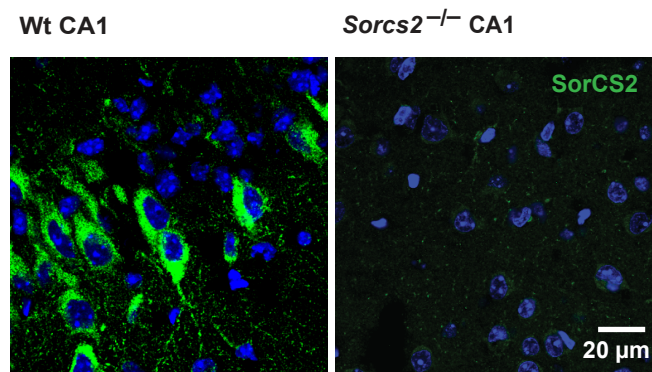
B



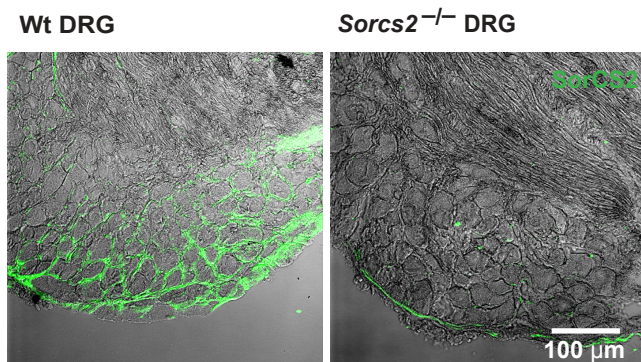
C



D

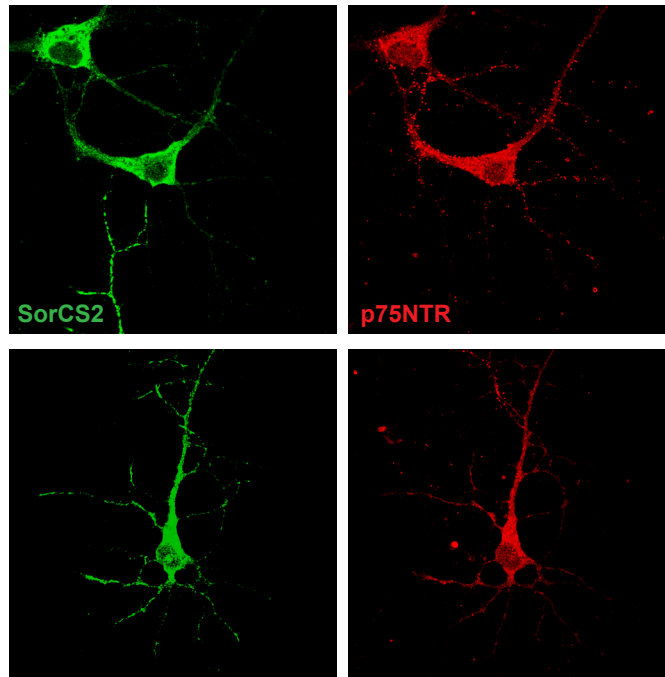


E



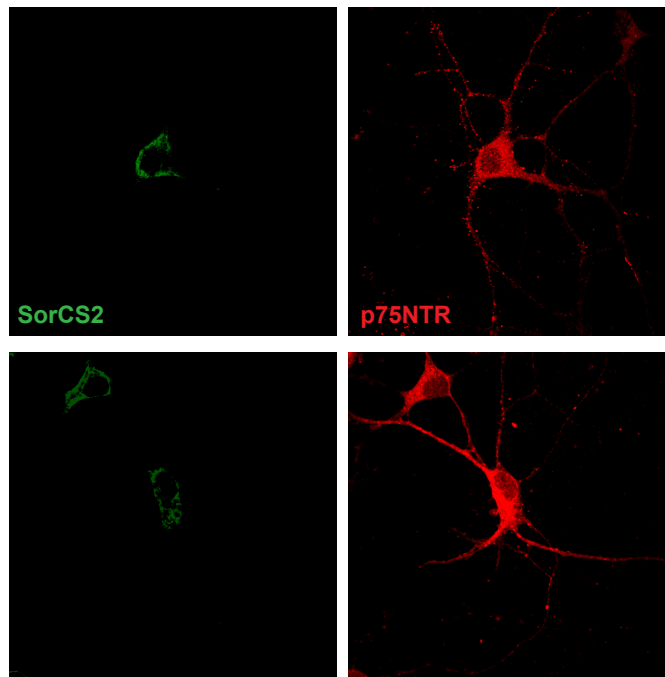
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Wt



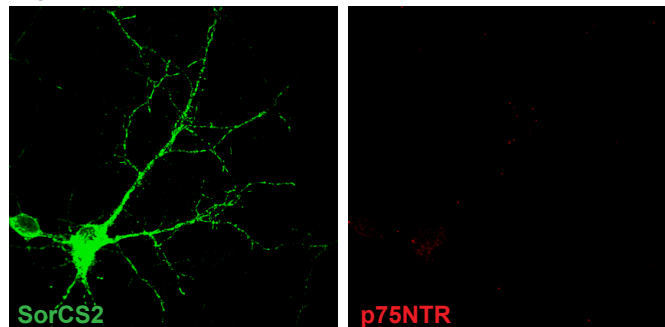
B

Sorcs2^{-/-}

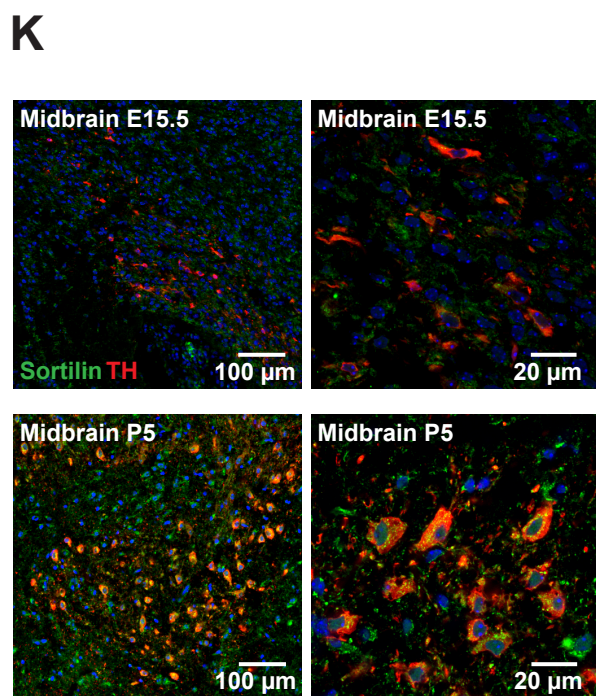
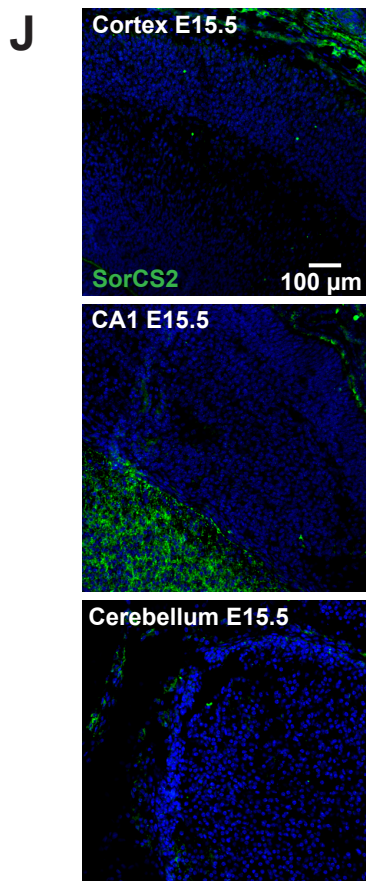
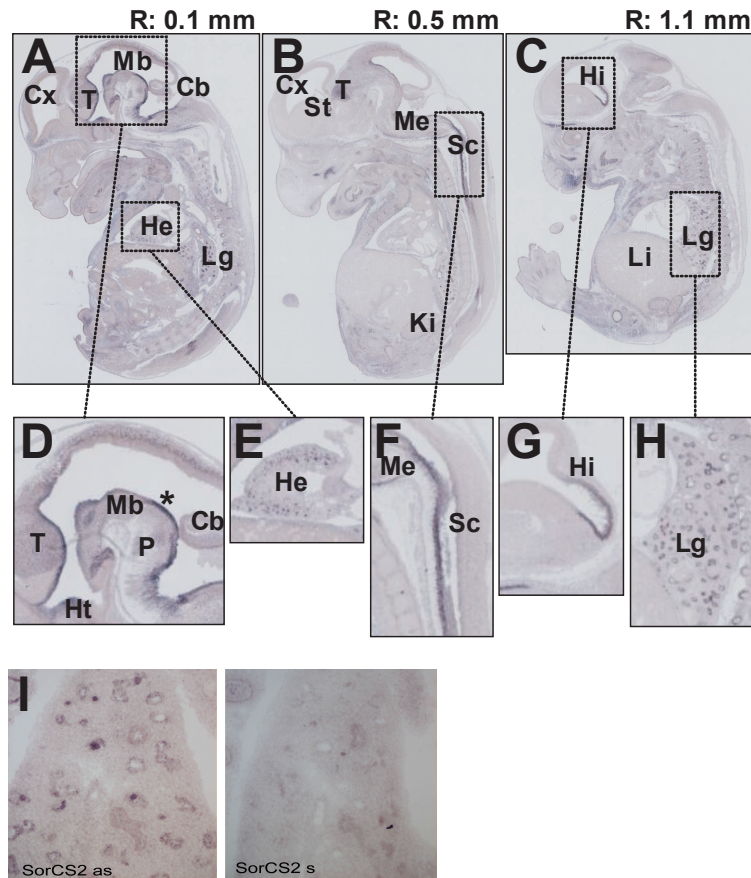


C

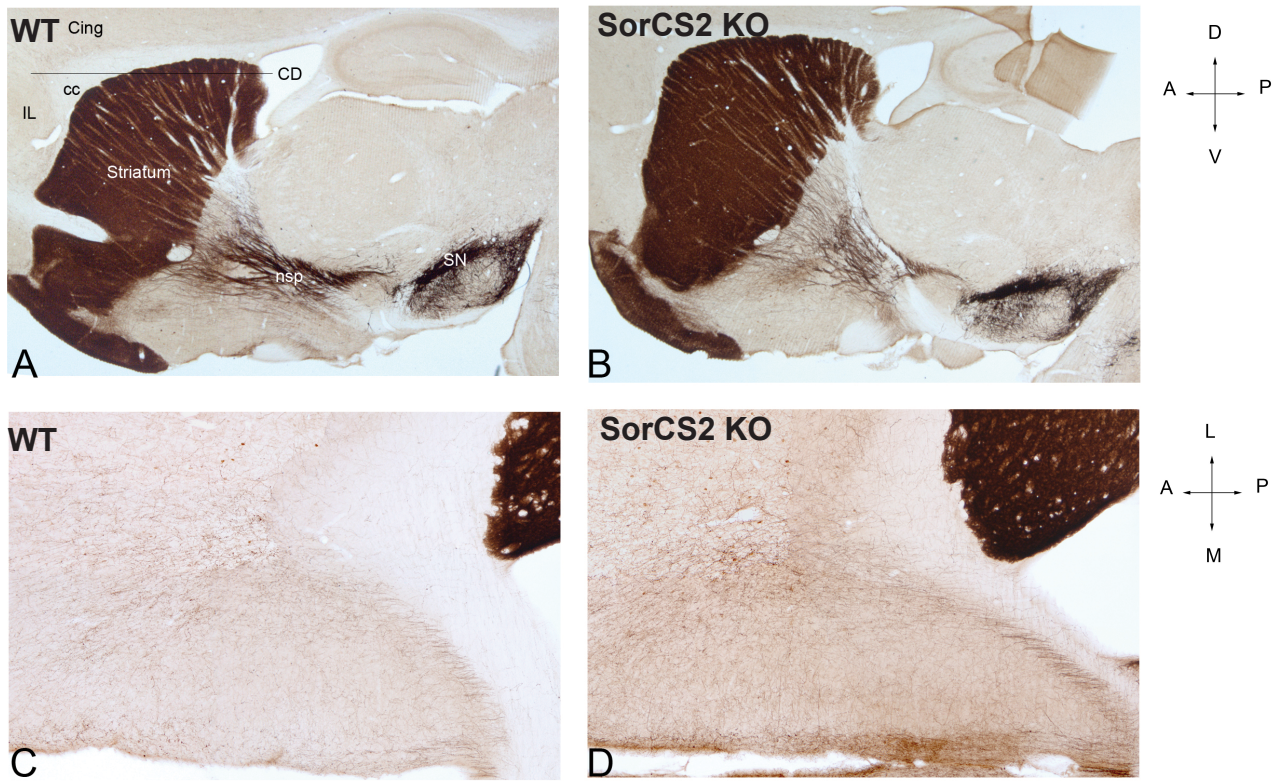
Ngfr^{-/-}



Supplementary figure 4

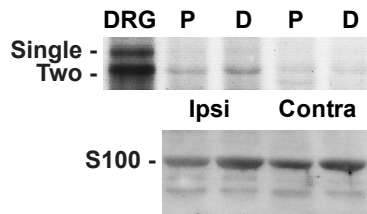


Supplementary figure 5

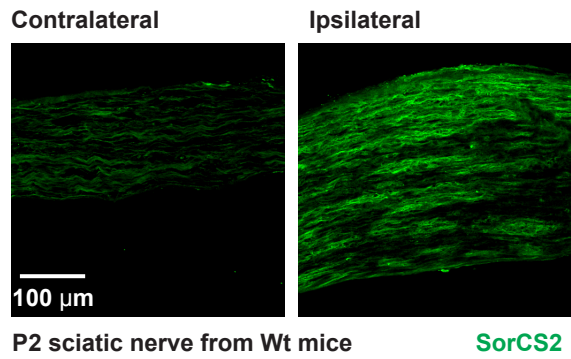


Supplementary figure 6

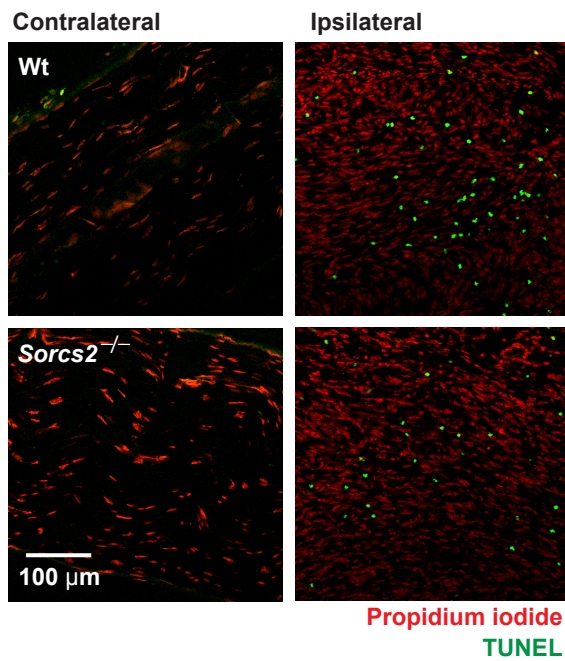
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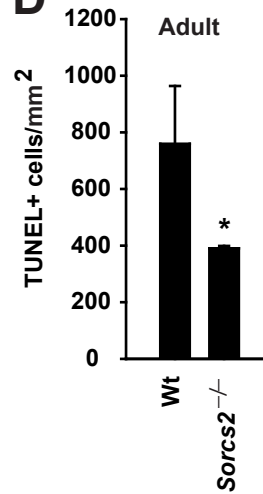
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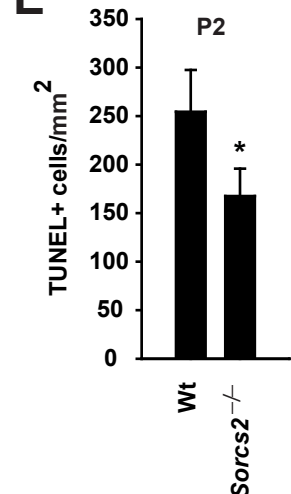
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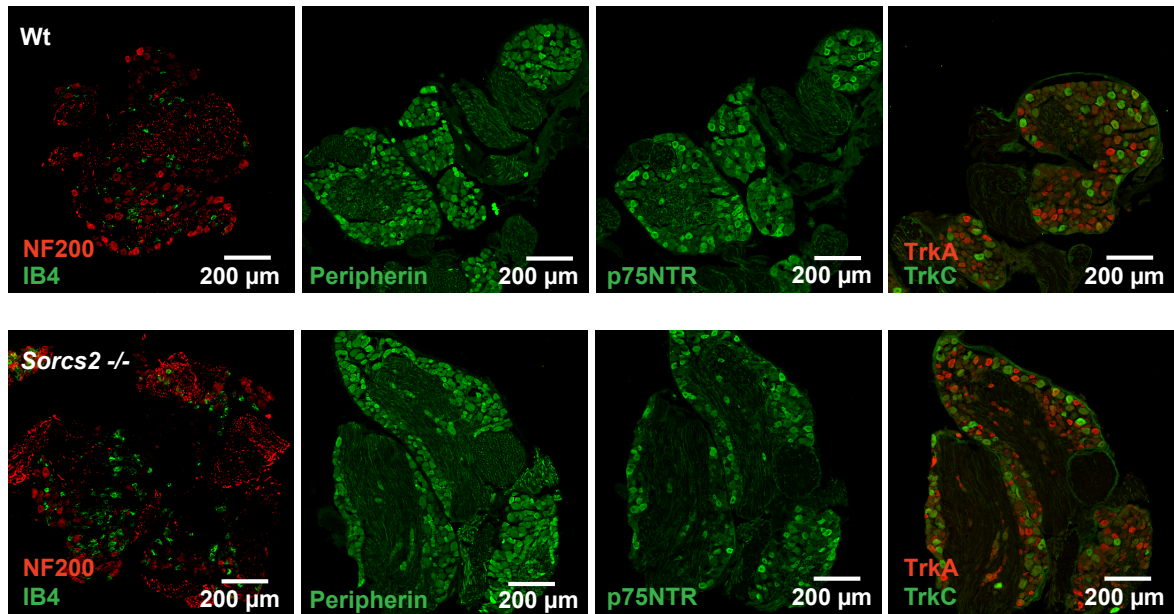
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E

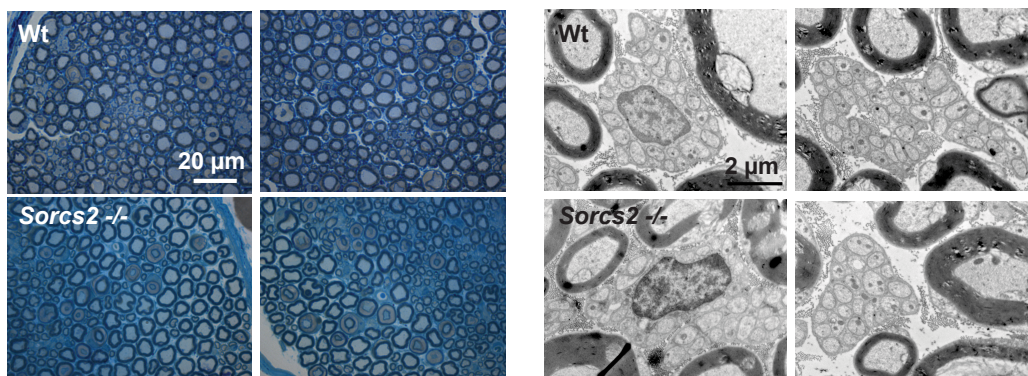


A

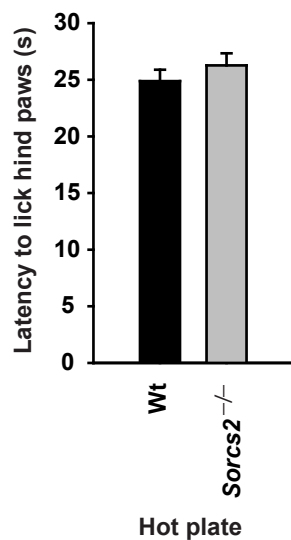


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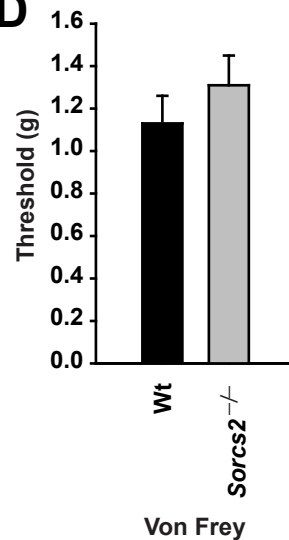
	Fiber number	Axon diameter (μm)	Myelin thickness (μm)
Wt	3992 +/- 106	3.81 +/- 0.12	0.69 +/- 0.02
<i>Sorcs2</i> ^{-/-}	4131 +/- 130	3.98 +/- 0.12	0.70 +/- 0.03
t-test (n=7)	0.42	0.32	0.87



C



D



E

