

Supplemental Material for

GPR160 de-orphanization reveals critical roles in neuropathic pain in rodents

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Supplemental Methods

Experimental Animals. Male or female Sprague Dawley rats (200-220g) or male ICR mice (20-30 g) from Envigo (Indianapolis, IN USA) were housed 2-4 per cage (rats) and 5 per cage (mice). Male Sprague Dawley rats (250-300 g) used for electrophysiological experiments (Biological Services, University College London, UK) were housed (maximum of 4). All animals were housed in conventional 12 h: 12h light-dark cycle with food and water available *ad libitum* and regulated temperature (20-22°C) and humidity (55-65%). Animals were randomly assigned to surgical and treatment groups. All behavior testing and biochemical studies were done with the investigator blinded to the surgical and treatment groups.

Defining estrous cycle stage. Given the multi-day nature of the design in freely cycling females, a vaginal smear was taken after the last behavioral time point and stage of estrous defined by cytology as described (1). All animals displayed normal 4-5 day estrous cycle.

Test compounds. All solutions of agents were made fresh each day immediately prior to administration. siRNA constructs: For both in vivo and in vitro assays, we used a cocktail of three (1:1:1 mix) GPR160-targeting siRNA constructs (Sequence 1: UUA AGA UAU CAA CCU AAA AAA AAU GUU UAG GUU GAU; Sequence 2: ACU ACU UCA GGU AAU CAU UAA AAC AAU GAU UAC CUG and Sequence 3: GAA UUU CUC UAA AAC AAC AAG CUU GGU UGU UUU AGA; Integrated DNA Technologies, Coralville, IA USA). All siRNA constructs were rigorously validated prior to experimentation. For both in vivo and in vitro assays, we assessed non-specific targeting of the siRNA constructs first by determining the three most likely non-specific targets using NCBI BLAST, then performing qRT-PCR analysis using primers designed from NCBI PrimerBLAST and purchased from Integrated DNA Technologies in cells or animal tissues using standard protocols in our lab (2, 3) (see Supplemental Figure 5 for example). For controls, we used a non-coding control sequence (siCONTROL; NC-1; Integrated DNA Technologies, Coralville, IA USA) or eGFP-targeted siRNA (sieGfp; 2 µg; #202472256, Integrated DNA Technologies).

Sequences used for in vivo studies were reconstituted and diluted in saline vehicle; whereas sequences used in cell culture were reconstituted in sterile RNase/DNase-free water and diluted in cell culture media vehicle. Immunopharmacological agents: GPR160 neutralizing antibody (#PA5-33650; ThermoFisher Scientific, Waltham, MA USA) targets a 19 amino acid peptide sequence from extracellular domain two of the human GPR160 (Uniprot: Q9UJ42; Entrez Gene ID: 26996). Rabbit IgG Isotype Control (#10500C, ThermoFisher Scientific, Waltham, MA USA) was employed as a control for the GPR160 neutralizing antibody. The CARTp neutralizing antibody (#H-003-62; Phoenix Pharmaceuticals, Burlingame CA, USA) has been reported by the manufacturer to be specific to CARTp evidenced by the loss of the immunohistochemical staining when the antibody was pre-incubated with a neutralizing peptide. All antibodies and control were diluted in saline vehicle. Pharmacological agents: CART peptides (55-102; #003-62) and (62-102; #003-68) (purity $\geq 95\%$), somatostatin (#060-03), and nesfatin-1 (#003-22) were purchased from Phoenix Pharmaceuticals and reconstituted in saline. U0126 monoethanolate (#U120) and 666-15 (#5383410001) were purchased from Millipore-Sigma (St. Louis, MO USA) and reconstituted in 100% DMSO before diluting in saline for injection (vehicle: 10% DMSO/saline).

Chronic Constriction Injury Model. Chronic constriction injury (CCI) to the sciatic nerve of the left hind leg in mice and rats was performed under general anesthesia using the well-characterized Bennett model (4). Briefly, animals were anesthetized with 3% isoflurane/O₂ and maintained on 2% isoflurane/O₂ during surgery. A small incision (1-1.5 cm in length) was made in the middle of the lateral aspect of the left thigh to expose the sciatic nerve, which was loosely ligated around the entire diameter of the nerve (at two distinct sites for mice and three distinct sites for rats, spaced 1 mm apart) using silk sutures (6.0, mice; 4.0, rats). Sham animals underwent the same procedure without nerve ligation.

For siRNA testing, rats were chronically implanted with cannula 7 days prior to surgery using the L5/L6 lumbar approach as previously described (5). The rats were then singly housed throughout the experiment. Daily intrathecal injections of siRNA (2 μ g in 10

μL) followed by a 10 μL sterile saline flush was administered on d0-d8 for prevention paradigms or d7 and d8 for reversal paradigm.

For antibody testing, rats and mice were given a single intrathecal injection of GPR160 ab or its IgG (1 μg in 10 μL) or CARTp ab or its IgG (0.2 μg in 10 μL) using the Wilcox method (6) on d7 or d8 post CCI. Briefly, animals were lightly anesthetized with isoflurane and a 50 μL Hamilton syringe (Hamilton, Reno, NV USA) with a 30-ga (mice) or 25-ga (rat) needle was inserted between the L5/L6 vertebrae of lightly-restrained animal to puncture the dura (confirmed by presence of reflexive tail flick and hind limbs) to deliver vehicle or test substance(s).

Spared Nerve Injury Model. Rats were chronically implanted with cannula to surgery using the L5/L6 lumbar approach as previously described (5) and singly housed throughout the experiment. Seven days after cannula placement, rats underwent spared nerve injury (SNI) or sham surgery according to the method of Decosterd and Woolf (7). Briefly, rats were anesthetized with 3% isoflurane/O₂ and maintained on 2% isoflurane/O₂ during surgery. The sciatic nerve was exposed at the level of its trifurcation into the sural, tibial, and common peroneal nerves. The tibial and common peroneal nerves were tightly ligated with 4.0 silk thread and transected just distal to the ligation, leaving the sural nerve intact. Daily intrathecal injections of siRNA (2 μg in 10 μL) followed by a 10 μL sterile saline flush was administered on d0-d8.

Spinal nerve ligation (SNL) surgery. SNL surgery was performed as previously described (8). Rats (130-140 g) were maintained under 2% v/v isoflurane anesthesia delivered in a 3:2 ratio of nitrous oxide and oxygen. Under aseptic conditions a paraspinal incision was made and the tail muscle retracted from the spinal column. Part of the L5 transverse process was removed to expose the left L5 and L6 spinal nerves, which were then isolated with a glass nerve hook (Ski-Ry, London, UK) and ligated with a non-absorbable 6-0 braided silk thread proximal to the formation of the sciatic nerve. The surrounding skin and muscle was closed with absorbable 4-0 sutures. Sham surgery was performed in an identical manner omitting the nerve isolation and ligation step. All rats groomed normally and gained weight in the following days post-surgery.

CARTp-induced hypersensitivity. The behaviors mice were measured at baseline (0 h), then an intrathecal injection (10 μ L) of a cocktail of CARTp (30 ng), GPR160 ab (1 μ g), CARTp ab (0.2 μ g), U0126 (5 μ g), 666-16 (2 ng) or their vehicle was administered using the Wilcox method (6). Mechano-allodynia behaviors were measured over 1-6 h, as noted.

Behavioral Testing. All behavior testing was done with the investigator blinded to the surgical and treatment groups. Mechano-allodynia: Mechanical allodynia as a readout for neuropathic pain was assessed by the hind paw withdrawal response to von Frey hair stimulation using the up-and-down method (9). Briefly, the animals were first acclimatized (30 min) in individual clear Plexiglas boxes on an elevated wire mesh platform to facilitate access to the plantar surface of the hind paws. Subsequently, a series of von Frey hairs (0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2 g for mice and 1.4, 2, 4, 6, 8, 10, 15, 26 g for rats; Stoelting, Wood Dale, IL USA) were applied perpendicular to the plantar surface of the hind paw, until the filament buckles, for 2–5 s. A test began with the application of the 0.6 g hair for mice and 4 g for rats. A positive response was defined as clear paw withdrawal or shaking. In the event of a positive response, the next lighter hair was applied; whereas the next higher hair was applied in the event of a negative response. At least four readings are obtained after the first positive response and the pattern of response was converted to a 50% paw withdrawal threshold (PWT), using the method described by Chaplan (10). Mechano-allodynia was defined as a significant (2 SD) decrease in the measured behavior compared to the individual animal's baseline behavior prior to mechanical or pharmacological pain induction.

Acute thermal anti-nociception was measured using the tail flick latency test (11), which measures the withdrawal latency of the tail from a noxious heat source. Briefly, rats were acclimated to the tail-flick instrument (Ugo Basile #37360) and animal restraint. The tail was placed over the infrared thermocouple. When started, the heat source applied sufficient heat to elicit baseline latencies (prior to drug) of 2–3s and a cut-off time of 10s to prevent tissue injury. Time until tail-flick was recorded by the instrument.

Cold allodynia: Animals were placed on an elevated wire mesh floor and the time course of cold-allodynia was assessed with the acetone drop method (12, 13). A drop of acetone (50 μ l) was placed against the center of the plantar hind paw and the following 60s after acetone application the rat's response was monitored. Responses were graded with the following 4-point scale: 0 no response; 1 quick withdrawal, flick or stamp of the paw; 2 prolonged withdrawal or repeated flicking, and 3 repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone was applied alternately three times to each paw and cumulative scores were then generated by adding the three scores for each paw and converting to a percentage of the maximal score, the minimum score being 0 (0%) and the maximum possible score being 9 (100%).

In vivo electrophysiology. In vivo electrophysiology was performed as previously described (14). Rats were initially anaesthetized with 3.5% v/v isoflurane delivered in 3:2 ratio of nitrous oxide and oxygen. Once areflexic, a tracheotomy was performed and rats were subsequently maintained on 1.5% v/v isoflurane for the remainder of the experiment (approximately 4-5 h; core body temperature was maintained with the use of a homeothermic blanket and respiratory rate was visually monitored throughout). Rats were secured in a stereotaxic frame, a midline incision was made across the scalp, and after the skull was exposed co-ordinates for the right ventral posterolateral nucleus (VPL) of the thalamus (contralateral to injury) were calculated in relation to bregma (2.28 mm caudal, 3.2 mm lateral) (15). A small craniotomy was performed with a high-speed surgical micro-drill to expose the cerebrum, and a partial laminectomy was performed with rongeurs to expose the L4-L6 lumbar region with the overlying dura removed. The electrode was manually lowered into the VPL and extracellular recordings were made from thalamic neurons with receptive fields on the glabrous skin of the left paw hind toes (Supplemental Figure 4A) for stereotaxically determined recording sites) using 127 μ m diameter 2 M Ω parylene-coated tungsten electrodes (A-M Systems, Sequim, WA). Searching involved light tapping of the hind paw and neurons were identified as wide dynamic range (WDR) on the basis of neuronal sensitivity to dynamic brushing (i.e., gentle stroking with a squirrel-hair brush), and noxious punctate mechanical (60 g) and heat (48°C) stimulation of the receptive field. The receptive field was then stimulated

using a range of natural stimuli (brush, von Frey filaments – 2, 8, 15, 26 and 60 g and heat – 35, 42, 45 and 48°C) applied over a period of 10s per stimulus. The heat stimulus was applied with a constant water jet onto the center of the receptive field. Acetone and ethyl chloride (100 μ l) were applied as an evaporative innocuous cooling and noxious cooling stimulus respectively (16), and responses quantified over 10s post application. Evoked responses to room temperature water (25°C) were minimal or frequently completely absent, and subtracted from acetone and ethyl chloride evoked responses to control for any concomitant mechanical stimulation during application. Stimuli were applied starting with the lowest intensity stimulus with approximately 40s between stimuli in the following order: brush, von Frey, cold, heat. Stimulus-evoked neuronal responses were determined by subtracting total spontaneous neuronal activity in the 10-second period immediately preceding stimulation; ongoing neuronal activity is plotted as the mean of these 10-second periods. After obtaining baseline evoked responses (mean of three trials), either 1 μ g/10 μ l IgG or anti-GPR160 antibody (in 0.9% saline) were applied directly to the dorsal aspect of the spinal cord. Neuronal responses to natural stimuli were tested at 40, 80 and 120min post-dosing; the 40min time point is plotted for both ongoing and evoked measures (see Supplemental Figure 4B for example histogram traces of single unit responses).

Data were captured and analyzed by a CED1401 interface coupled to a computer with Spike2 v4 software (Cambridge Electronic Design, Cambridge, United Kingdom). The signal was amplified (x6000), bandpass filtered (low/high frequency cut-off 150/2000 Hz) and digitized at rate of 20 kHz. Spike sorting was performed *post hoc* with Spike2 using fast Fourier transform followed by 3-dimensional principal component analysis of waveform features for multi-unit discrimination. Neurons were recorded from one site per rat; one to three neurons were characterized at each site. A total of 10 neurons were characterized from 5 SNL rats, 5 neurons from 5 sham rats, and 4 neurons from 4 naïve rats. When multiple WDR neurons were distinguished at a single recording site, the mean neuronal response was calculated per rat to avoid sampling bias.

Cell cultures. KATOIII were maintained in Iscove's Modified Dulbecco's medium (IMDM; ATCC, Manassas, VA USA) with 20% fetal bovine serum (#30-2020, ATCC, Manassas,

VA USA) and 1% penicillin/streptomycin (Sigma Aldrich). Cells were passaged every 2-3 days or until ~80-90% confluency was reached. PC-12 cells were maintained in RPMI-1640 (Sigma Aldrich) with 10% heat inactivated horse serum, 5% fetal bovine serum (#30-2020, ATCC), 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma Aldrich, Saint Louis, MO USA). To differentiate the PC-12 cells, 60mm plates were coated with a solution of 1 mg/ml collagen IV (Millipore-Sigma) in 1X PBS for 3h at 37°C before use. Cells were counted on a LUNA II cell counter (Logos Biosystems, Anyang-si, South Korea) and seeded at 3 million cells per 60 mm plate in RPMI 1640 supplemented with 1% horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA USA), and 25 ng/ml rat NGF-β (Millipore-Sigma). Cells were differentiated for 96h with the media changed after 48 h.

Receptomic approach. Receptomic approach analysis of oGPCRs in neuropathic pain states was based on previously described methods (2). Briefly, GEO DataSets (Accession GDS4625) were queried for non-orphan GPCRs whose expression was significantly altered in neuropathic pain states (17, 18). This approach identified 61 receptors that were then aligned by sequence homology of the protein sequences utilizing the NCBI Constraint-based Multiple Alignment Tool (COBALT) to generate a phylogenetic tree using the Grishin model and Fast Minimum Evolution tree method. This generated four branch clusters containing at least five GPCR members: 1) Opioid, 2) Purinergic, 3) Serotonin/Cannabinoid and 4) Arachidonic Acid clusters. Homologous regions of the branch members were then subjected to BLAST analysis to identify oGPCRs with significant sequence homology. The presence of identified oGPCRs were confirmed by PCR.

PCR analysis. The dorsal horn of the spinal cord (lumbar L4-L6) was harvested at time of peak pain (d7). Total RNA for GPR160 transcript measurement was isolated from tissue using the PureLink Total RNA Isolation Kit according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA USA). Quantitative real-time PCR (qRT-PCR) was performed using iQ SYBRGreen Master Mix and a Bio-Rad CFX96 real-time System (Bio-Rad)

following the manufacturer's instructions. The following primers were designed using PrimerBlast (NCBI, Bethesda, MD USA) and ordered from Integrated DNA Technologies (Integrated DNA Technologies): HPRT1 forward 5'-AGT CCC AGC GTC GTG ATT AGT GAT-3' and reverse 5'-CTC GAG CAA GTC TTT CAG TCC TGT-3'; GPR160 forward 5'-ACT GGT GTA ATC TGT CCA GAG CCA -3' and reverse 5'-AGG ATG AGG ACC TGA AGT GCT ACA-3'; CART forward 5'-CCT ACT GCT GCT GCT ACC TT-3' and reverse 5'-CGC CTT GGC AGC TCC T-3'.

RNA-Seq analysis. The dorsal horn of the spinal cord (lumbar L4-L6) was harvested on d9 post CCI or SHAM surgery. Tissues were immediately preserved in RNALater® overnight at 4°C. Total RNA was isolated the next day using RNeasy® Plus Universal Mini Kit (Qiagen, Germantown, MD USA) and quantitated by NanoDrop™ (ThermoFisher Scientific). Total RNA samples were assessed for RNA quality and sequenced by the Genome Technology Access Center (GTAC) at Washington University in Saint Louis using Illumina HiSeq. Gene expression was normalized using Kallisto (19) followed by Trimmed Mean of M-values methods (20) and differential gene expression between CCI and SHAM groups were tested using negative binomial and generalized linear regression models by edgeR (20). Differentially expressed genes were defined as those with a fold change ≥ 1.5 -fold and a false discovery rate (FDR) ≤ 0.05 .

cFos PCR in KATOIII cells. KATOIII cells were plated onto 12-well plates. After 48 h, the cells were transfected with either Vehicle (Lipofectamine 2000, ThermoFisher Scientific) or Lipofectamine-coated GPR160 siRNA or eGFP siRNA according to the manufacturer's instructions and our previous report (2). Five hours later, media was changed to serum-free IMDM. Twenty-four hours following transfection, cells were treated with vehicle (serum-free IMDM), 10 nM CART peptide or IMDM + 20% FBS (positive control) and incubated for 1h at 37°C. Cells then were lysed and total RNA isolated (PureLink RNA Isolation Kit, Invitrogen). RNA was used as a template for cDNA production (iScript cDNA Synthesis Kit, BioRad) and qPCR was used to detect changes in cFos mRNA expression, as we have previously described (2), using a BioRad CFX96 Real Time PCR system.

Changes in cFos mRNA expression between siRNA treatments were calculated using the $\Delta\Delta\text{Ct}$ method (21) using GAPDH as a housekeeping control.

Immunoprecipitation. KATOIII cells were treated for 10min with 10 nM CARTp (55-102) in serum free media. Media was removed and unbound CARTp (55-102) was washed quickly with 1X PBS. Cells were fixed with 4% paraformaldehyde (PFA) for 10min at room temperature. After fixation, the PFA was removed and the cells were washed with 1X PBS before centrifugation for 5min at 292 x g and 4°C. Next, cells were lysed for 15min on ice using lysis buffer containing (1% NP40, 1 mM EDTA, 5% glycerol, 10 mM Tris) pH 7.4 supplemented with protease inhibitor cocktail (#P8340, Sigma-Aldrich, St. Louis, MO). Lysed cell pellets were centrifuged at 9500 x g at 4°C on a tabletop centrifuge. Lysate supernatant protein concentration was quantified by BCA assay (#23225, ThermoFisher Scientific, Waltham, MA USA) and 100 µg was immunoprecipitated with antibodies to GPR182 (5 µg; #PA5-32811, Invitrogen) or GPR160 (5 µg; #ab117074, Abcam, Cambridge UK) for 6h at room temperature. Next, Protein A dynabeads (#10002D, Life Technologies, Carlsbad, CA USA) were incubated with the antibody complex rotating overnight at 4°C. The dynabead complex was washed 3 times with lysis buffer, a 1:1 ratio of 2X Laemmli loading buffer was added, and the mixture was boiled for 10min at 95°C. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel (10%), transferred to PVDF membrane at 30V for 16h. Bands were labeled for CARTp by incubating with CARTp antibody (1:1000; #ab192364, Abcam) overnight at 4°C and then with donkey anti-rabbit HRP (#711-035-152, Jackson ImmunoResearch, West Grove, PA USA). Bands were visualized using chemiluminescence (Clarity Max Western ECL; Bio-Rad) and Chemidoc (Bio-Rad). We confirmed its specificity of the CARTp antibody by Western blot (Supplemental Figure 5D).

Western Blot to Confirm CARTp Antibody Specificity. Lyophilized CARTp 55-102, CARTp 62-102, somatostatin, nesfatin-1 (10 µg) were suspended at 0.1µg/µl in 1X PBS. Each peptide (1 µg) was mixed with an equal volume of 2X Laemmli buffer. The mixture was boiled for 5min at 95°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel (10%), then transferred to a PVDF membrane at 30 V for 16h. Bands

were labeled for CARTp by incubating with CARTp antibody (1:1000; #ab192364, Abcam) for 1h at room temperature, and then labeled with donkey anti-rabbit HRP (#711-035-152, Jackson ImmunoResearch, West Grove, PA USA). Bands were visualized using chemiluminescence (Clarity Max Western ECL; Bio-Rad) and Chemidoc (Bio-Rad).

Immunofluorescence and RNAScope®. Tissue preparation. Animals were perfused 1X PBS then fixed overnight in 4% PFA (22). Spinal cords were removed as and post fixed overnight in 4% PFA (22). The tissues were then sequentially treated for cryopreservation in 20% and 30% sucrose for 24h each. The fixed and cryopreserved lumbar segment was isolated and bisected to produce a coronal section between approximately L3 and L4. The left side of the spinal cord was marked with a nick and the spinal cord sections were snap frozen in liquid nitrogen cooled 2-methyl butane.

Immunocytochemistry. KATOIII cells were plated onto chamber slides (ibidi GmbH, Martinsried, Germany) and incubated under normal culturing conditions for 24h to allow for cell attachment. Cells then were serum-starved in filtered IMDM (ATCC) for 24 h. On the day of the experiment, cells were treated with vehicle (serum-free IMDM) or vehicle containing 10 nM CARTp (55-102) labeled with FAM (FG-003-60A, Phoenix Pharmaceuticals, Inc., Burlingame, CA) for 15min at 37°C. Cells were fixed with 4% paraformaldehyde for 10 min, then washed 3x in phosphate buffered saline. Cells were blocked with 5% donkey serum and stained for GPR160 (5 µg; #ab117074, Abcam, Cambridge UK) for 1h at room temperature followed by incubation with anti-rabbit Alexa 594 (Jackson ImmunoResearch, West Grove PA) at 1:300 for 1 h. Cells were imaged on an Olympus FV1000 Confocal Microscope.

Immunofluorescence. Spinal cord sections (30 µm) were free-floated in 1X PBS before antigen retrieval in 1 mM EDTA, pH 8.0 for 30min at 90°C in a water bath. Sections were blocked for 1h at room temperature in 5% donkey serum in 1X PBS with 0.25% Tween-20. Primary antibodies to NeuN (1:500; #MAB377, Millipore-Sigma), GFAP (1:1000; #PA5-18598; ThermoFisher Scientific), and GPR160 (1:250; #PA5-33650; ThermoFisher) were incubated with sections for 72h at room temperature in a sealed

humidified chamber. Sections were washed extensively and stained for 2h at room temperature with a cocktail (1:300 dilution each) of anti-goat Alexa 488 (#705-545-147; Jackson ImmunoResearch, West Grove, PA), anti-mouse Alexa 594 (#715-585-151; Jackson ImmunoResearch), anti-rabbit Alexa 647 (#711-605-152; Jackson ImmunoResearch) and DAPI (ThermoFisher). Slides were mounted in Prolong® Gold (ThermoFisher) and images were captured on an Olympus VS120 slide scanning microscope (Olympus, Waltham, MA) to image the entire section. The medial portion of the dorsal horn was determined to be free of artifact across all sections and was selected for quantification. The mean gray value of the GPR160 channel was measured using FIJI (23) from images of the medial portion of lamina 1 and 2 from the dorsal horn ipsilateral and contralateral to injury.

Proximity Ligation Assay (PLA). When two protein targets in close proximity *in situ* are immunolabeled and detected by secondary oligo-labeled antibodies, PLA assays produce a fluorescently-labeled PCR product by rolling DNA synthesis. To this end, ARPE-19 cells were plated onto chamber slides (ibidi, Fitchburg, WI) and incubated under normal culturing conditions for 24h to allow for cell attachment. Cells were serum-starved in filtered DMEM/F12 (Sigma Aldrich, St. Louis, MO) for 24 h. On the day of the experiment, cells were treated with vehicle (serum-free IMDM) or vehicle containing unlabeled 10 nM CARTp (55-102) for 15min at 37°C. Cells were fixed with 4% paraformaldehyde for 10 min, then washed 3x in phosphate buffered saline. An antibody to the GPR160 second extracellular domain (PA5-33650, ThermoFisher, Waltham, MA) and an antibody to CART (Abcam, ab192364, Cambridge, MA) were dialyzed against PBS to remove sodium azide and subsequently concentrated before using the Duolink probemaker PLUS and MINUS kits (DUO9209 and DUO9210, Sigma Aldrich) according to manufacturer's directions to conjugate PLA oligonucleotides directly. We then performed a commercially available PLA assay (DUO92008, Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions.

RNAScope®. Stained sections were prepared according to the manufacturer's instructions (Advanced Cell Diagnostics, Newark, CA). Briefly, 5 µm sections on glass

slides were dried for 2h first in the cryostat and then at 60°C before treating with hydrogen peroxide, antigen retrieval and protease. A cocktail of probes for *Rbfox3* (Rn-Rbfox3), *Aif1* (Rn-Aif1) or *Gfap* (Rn-Gfap-C2) with *Gpr160* (Rn-Gpr160-C3) was incubated for 2h at 40°C in a humidified chamber before amplification and detection with either fluorescein, Cy3.5 or Cy5 TSA® reagents (Perkin Elmer, Waltham, MA). Slides were counterstained with DAPI and mounted in ProLong® Gold (ThermoFisher Scientific) and sequential channels imaged of the Laminae 1 and 2 of the dorsal horn using a Leica SP8 TCS confocal microscope (Leica Microsystems, Buffalo Grove, NY). For quantification, 40x image stacks (5 µm total stack height sampled at 350 nm intervals) from Laminae 1 and 2 of the dorsal horn were scanned at 512 x 512 pixels. FIJI (23) was used to measure the count of *Gpr160* signal within dilated (x0.5) counting masks based on either *Aif1*, *Gfap*, or *Rbfox3* signals. These comparisons were done using a custom macro written for ImageJ that allows input of empirically determined intensity thresholds for counting. The macro is provided below. For the total *Gpr160* RNAscope quantification, individual image planes were first merged into a maximum intensity projection before counting particles based on a threshold highlighting the *Gpr160* signal. Particle counts performed in ImageJ. The mean number of *Gpr160* RNA *in situ* hybridization signals within proximity to *Aif1*, *Gfap*, and *Rbfox3* ipsilateral to CCI were related to the mean number on the contralateral side. FIJI (23) was also used on 2048x2048 representative images from the same regions counted for small global adjustments to brightness and contrast for each channel of the display for presentation.

ERK and CREB Western blot. PC-12 cells were plated on collagen 4- (#C7521, Millipore-Sigma) coated plates and differentiated for 96h by incubation with Rat Nerve Growth Factor (25 nM, #N2513, Millipore-Sigma). Differentiated cells were serum starved overnight and treated for 5min with 300 nM CARTp (55-102) at 37°C. Reaction was quenched with addition of ice cold 1X PBS and cells were scraped on ice before centrifugation for 5 min, 292 x g, 4°C. Cell pellet was lysed using PhosphoSafe™ Extraction Reagent (#71296, Millipore-Sigma) supplemented with Phosphatase Inhibitor cocktail 2 (#P5726) and 3 (#P0044; Millipore-Sigma) for 10min on ice. Lysates (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel (10%), transferred to PVDF

membrane at 30V for 16 h. Bands were labeled for phosphorylated ERK (1:2500; #9101, Cell Signaling Technologies, Danvers MA) and total ERK (1:2500; #9102, Cell Signaling Technologies) overnight at 4°C and then with donkey anti-rabbit horseradish peroxidase (#711-035-152, Jackson ImmunoResearch). Bands were visualized using chemiluminescence (Clarity Max Western ECL, Bio-Rad) and Chemidoc (Bio-Rad).

For in vivo tissues, saline-perfused lumbar segments (L4-L6) were harvested from mice 1h following intrathecal treatments and behavioral measurements. Tissues were homogenized in 100 µl of ice-cold homogenization buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 5% glycerol, 1 mM PMSF, 1X protease inhibitor cocktail (#P8643, Millipore-Sigma), 1 mM Na₃VO₄, 1 mM Na₃MbO₄, 50 mM NaF and 1X phosphatase inhibitor cocktail (#P0444, Millipore-Sigma)]. The homogenates were incubated for 10min on ice before being pulse sonicated (Sonic Dismembrator 60; ThermoFisher Scientific). The samples were clarified by centrifugation at 15,000 x g, 15min and 4°C. The total protein concentration in the clarified lysates was measured using bicinchoninic acid (Thermo Fisher Scientific, Carlsbad CA, USA). Lysate proteins (10 µg) were resolved by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membrane. Membranes were blocked for 1h at room temperature in 1X TBS, 3% BSA (#A7030, Millipore-Sigma) with 0.01% thimerasol and subsequently probed with specific antibodies to phosphorylated (Thr202/Tyr204; #4370) and total (#4696) ERK1/2 (1:1000, Cell Signaling Technologies), phosphorylated (Ser133; #9198) CREB (1:1000, Cell Signaling Technologies) or β-actin (1:5000; #A5441, Millipore-Sigma) in 1X TBS, 1% BSA and 0.01% thimerasol at 4°C overnight. The phosphorylated CREB antibody detects phosphorylated forms of CREB (~43-48 kDa) as well as phosphorylated form of the CREB-related protein ATF-1, as described by the manufacturer. The membranes were washed in 1X TBS-T (15 mM Tris pH 7.6, 150 mM NaCl, 1% Tween-20). The bound antibodies were then visualized following incubation with horseradish peroxidase-conjugated bovine anti-mouse IgG secondary antibody (ERK: 1:3000, CREB: 1:1000 or β-actin: 1:5000; #04-18-15, Jackson ImmunoResearch) or peroxidase-conjugated goat anti-rabbit IgG (ERK: 1:5000 or CREB: 1:1000; #7074, Cell Signaling) for 1h at room temperature. Horseradish peroxidase (HRP)-conjugated antibodies were visualized by enhanced chemiluminescence (Clarity, Bio-Rad, Hercules

CA) and documented using Chemidoc XRS+ documentation system and ImageLab™ software (BioRad). Blot images were captured using image accumulation mode (100 images; 30-300 s). Blots were treated twice for 15min with 30% hydrogen peroxide to deactivate the HRP (24) between phosphorylated ERK and total ERK and between phosphorylated CREB and β -actin. Relative protein expression was quantified by measuring band densitometry. Images for the blots of phosphorylated and total ERK were selected for analyses and presentation based on densitometric distribution curve with a predetermined range of lower value (0 units) and upper grayscale value (27000-37000 units) in order to assure linear densitometric values. Images were analyzed using the lane and band functions of the ImageLab™ software. For presentation purposes, the grayscale range was set between 0 units and the predetermined upper grayscale value (27000-37000 units) prior to exporting as an image file. Post-export modifications of the images were limited to cropping to the regions of interest.

Statistics. Data are expressed as mean $\pm$ SD or SEM for N animals as noted. Data were analyzed by two-tailed paired or unpaired t-test, one-way ANOVA with Dunnett's comparisons or two-way with Bonferroni comparisons as noted. All data were analyzed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego CA USA, www.graphpad.com). Data for the electrophysiological studies were analyzed by two-tailed, two-way repeated measures (RM) ANOVA with Bonferroni comparisons and the Greenhouse-Geisser correction applied based on Mauchly's test of sphericity using SPSS v25 (IBM, Armonk, NY). Ongoing firing frequencies, and cold and brush evoked firing were compared with a two-tailed paired Student's t-test. Significant differences in receptomic and RNA-Seq studies were defined at $P < 0.05$ and a FDR of $q < 0.05$. Significant differences in biochemical, behavioral and electrophysiological studies were defined at $P < 0.05$.

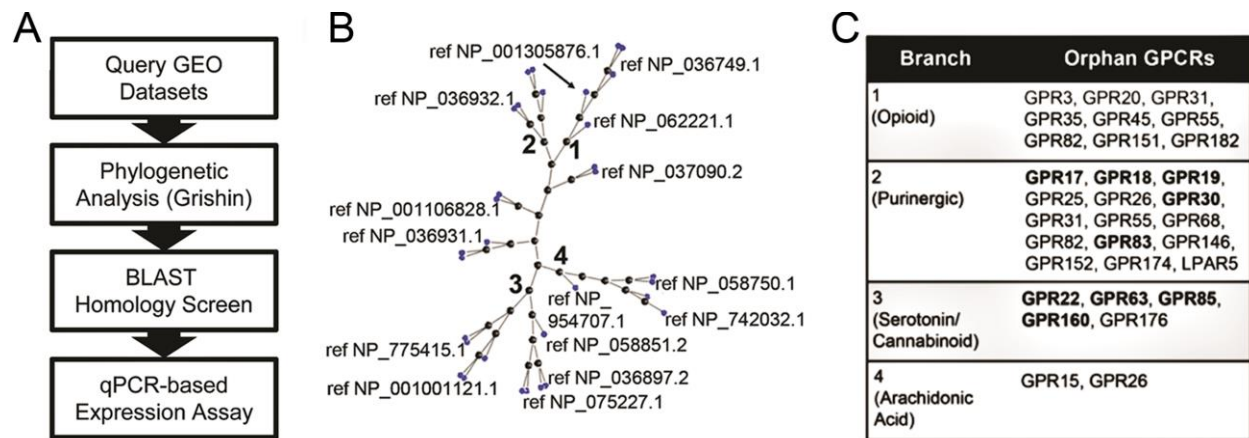
Imagej Macro for automated RNAscope counts. #The XX values must be manually empirically defined per experiment:

```
Resultname = getTitle()
setOption("Stack position", true);
```

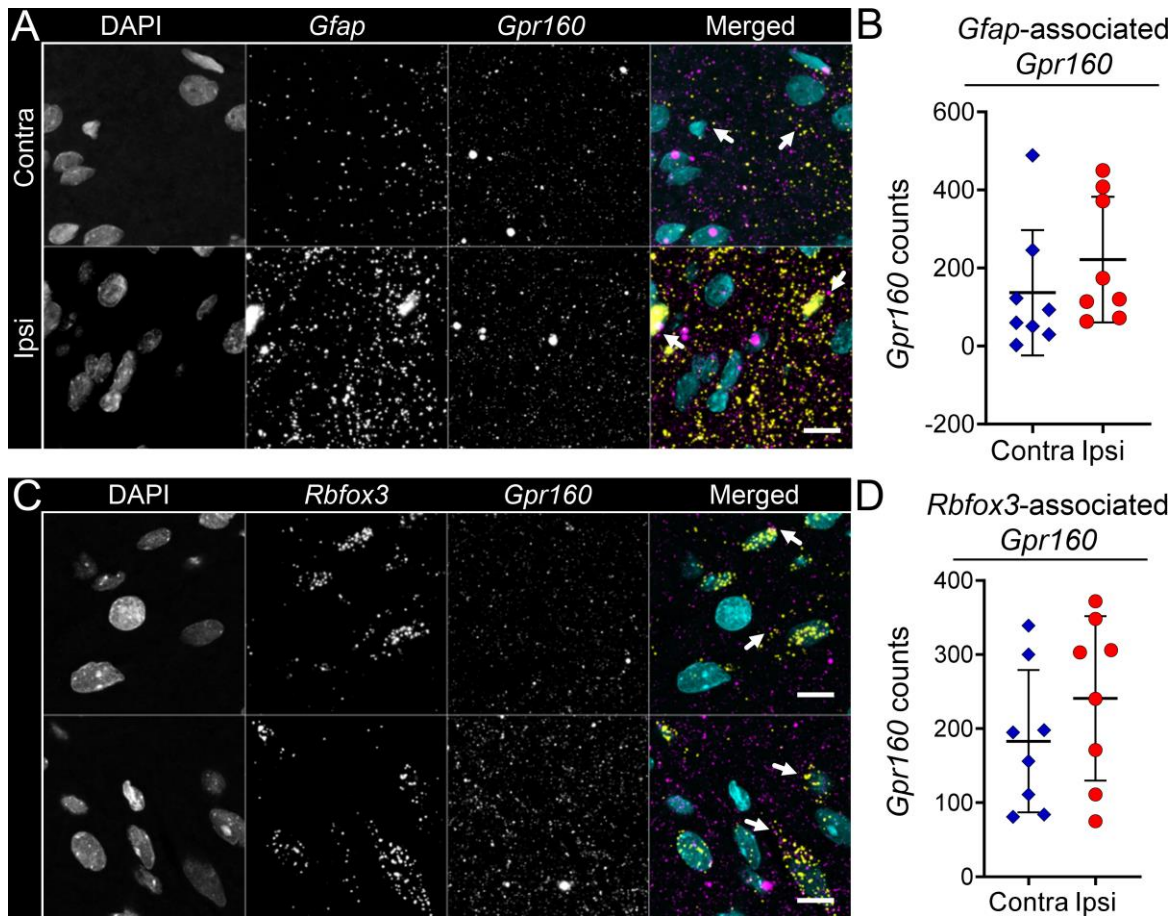
```

for (n=1; n<=nSlices; n++) {
    setSlice(n);
    Stack.setChannel(2)
    setThreshold(XX, 255);
    run("Create Selection");
    run("Enlarge...", "enlarge=0.5 pixel");
    Stack.setChannel(3);
    setBackgroundColor(0, 0, 0);
    run("Clear Outside", "slice");
    run("Select None");
    setThreshold(XX, 255);
    run("Analyze Particles...", "summarize slice");
}
Resultname = getTitle()
saveAs("Results", "/Users/gkolar/Desktop/results/Resultname.csv");

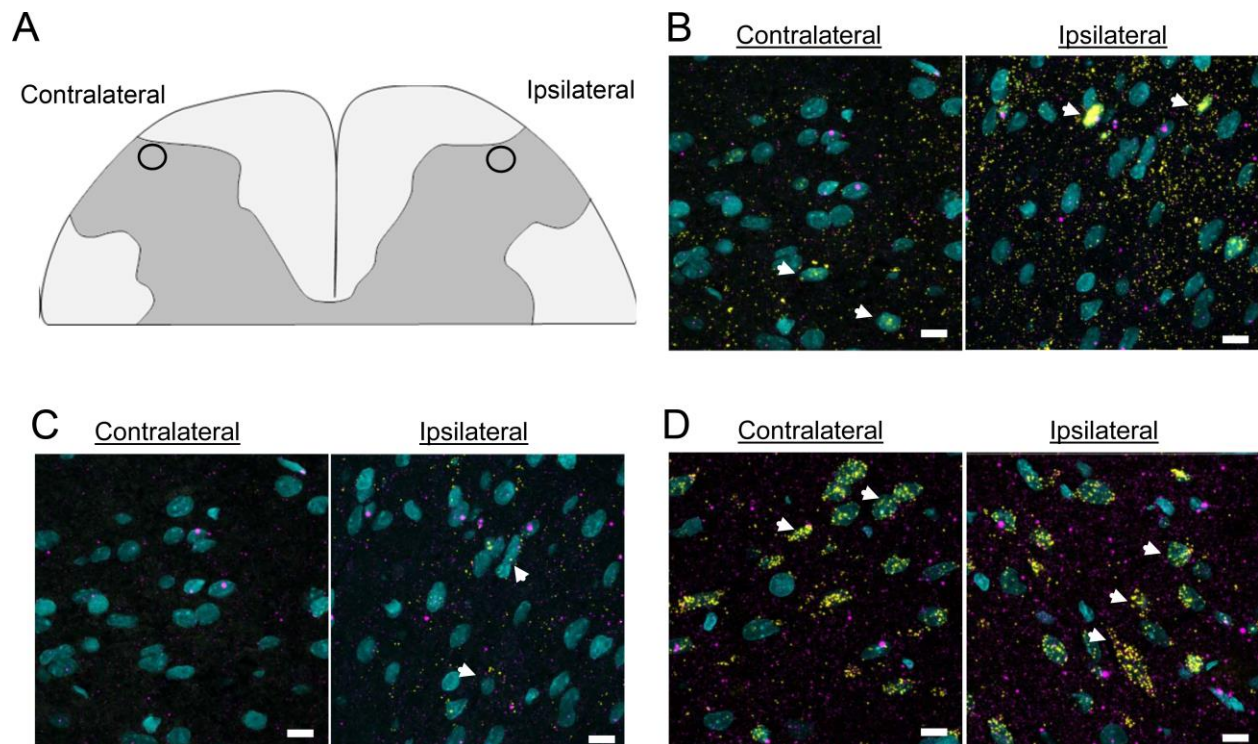
```



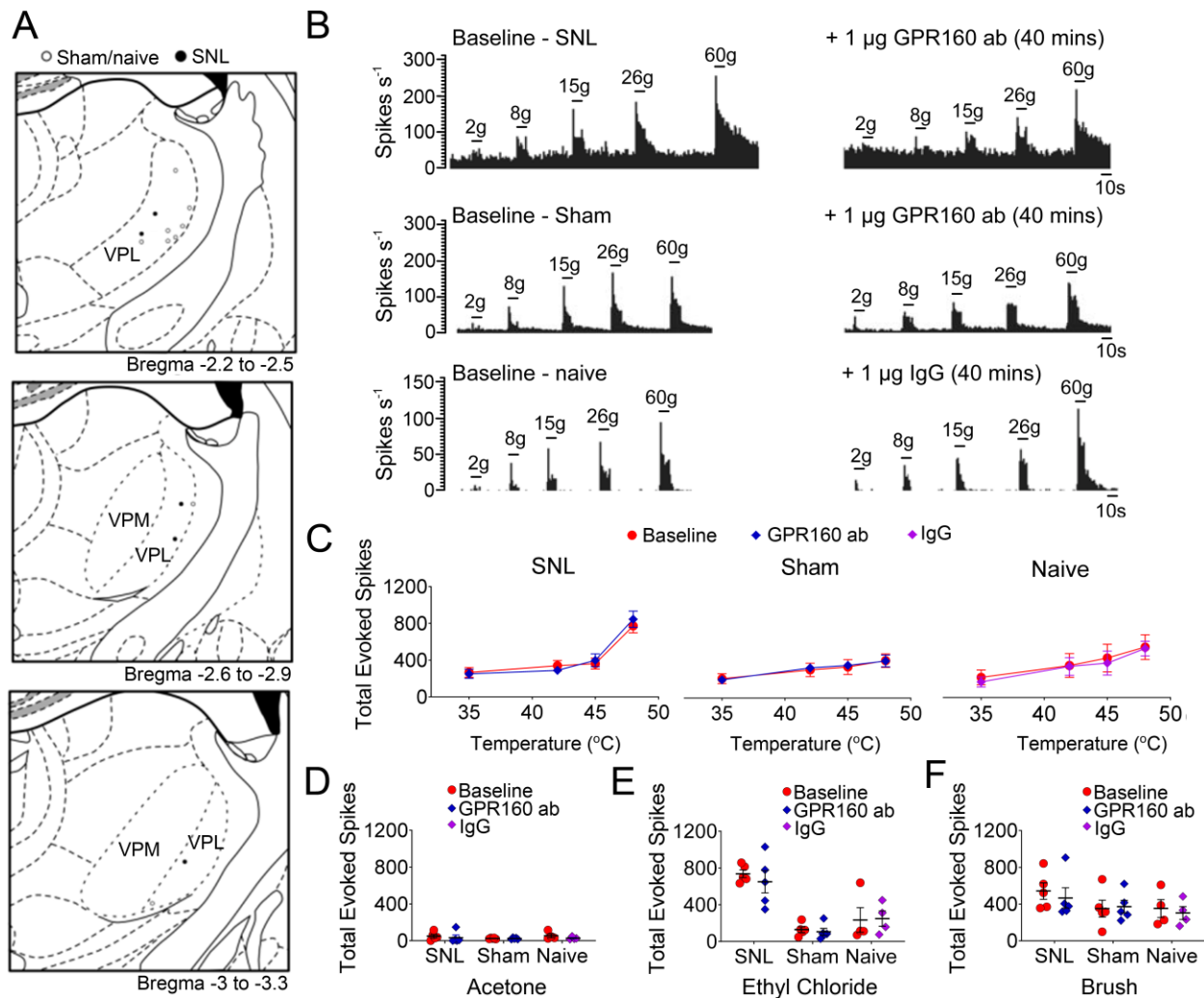
Supplemental Figure 1. Receptomic and unbiased transcriptomic analyses of oGPCR expression in the spinal cord reveals GPR160 expression increases during the development of neuropathic pain. **(A)** Diagram of the receptomic approach. **(B)** The phylogenetic tree of the 61 oGPCRs identified by receptomics approach generated using the Grishin model and Fast Minimum Evolution tree method. **(C)** The oGPCRs encompassed within the specific branch clusters of the phylogenetic tree. Receptors endogenously expressed in normal rat spinal cord are in bold.



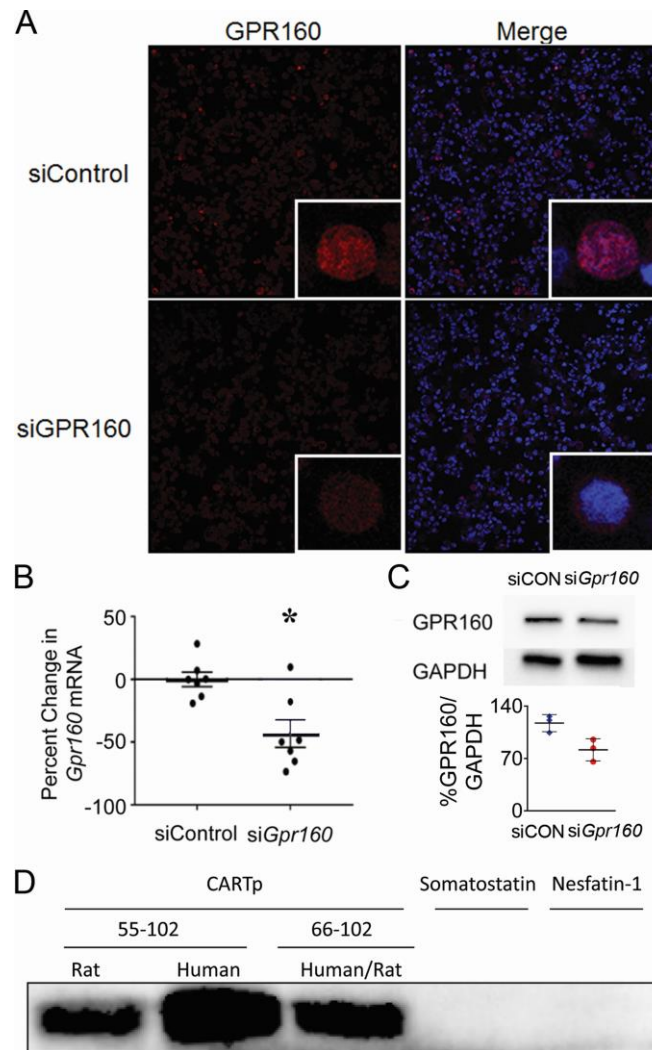
Supplemental Figure 2. RNAscope analysis of nerve injury-induced *Gpr160* expression in the spinal cord following CCI and its association with astrocytes and neurons. RNAscope imaging of *Gpr160* and *Gfap* (astrocytes, **A,B**) or *Rbfox3* (neurons, **C,D**) transcripts in the rat spinal cord on day 10 following CCI revealed that the increase in ipsilateral *Gpr160* (magenta) was not associated (white arrows) with regions of *Gfap* (yellow, **A**) or *Rbfox3* (yellow, **C**). DAPI (cyan). Panels of DAPI and *Gpr160* for ipsilateral and contralateral spinal cord in Figure 1F are reshown in **A** with the corresponding *Gfap* expression. Scale bar = 10 μ m. Data are expressed as mean $\pm$ SD and analyzed by two-tailed Student's *t*-test.



Supplemental Figure 3. Lower magnification RNAscope analysis of nerve injury-induced *Gpr160* expression in the spinal cord following CCI and its association with microglia, astrocytes and neurons. (A) Medial regions of lamina 1 and 2 of the dorsal horn of the spinal cord imaged. (B-D) Lower magnification of images in Figure 1F and Supplementary Figure 2A,C for *Gpr160* (magenta; B-D) expression in cells positive for (B) *Gfap* (astrocytes; yellow), (C) *Aif1* (microglia; yellow) or (D) *Rbfox3* (neurons; yellow). Merged image: DAPI (cyan); *Rbfox3* (yellow) and *Gpr160* (magenta). Scale bar = 10 μ m.



Supplemental Figure 4. Recording thalamic response to low-threshold mechanical stimuli in SNL and sham/naive rats. **(A)** Recording sites within the ventral posterolateral nucleus of the thalamus of rats with spinal nerve ligation (SNL; ●; n=5) or sham/naïve groups (○; n=9). **(B)** Representative histogram traces of single unit responses prior to and post spinal delivery of antibody. There were no changes in the neuronal responses to heat **(C)**, cold **(D,E)** or dynamic brush **(F)** stimuli in rats with SNL (n=5) or sham surgery (n=5) prior to and following spinal administration of GPR160 antibody (1 μ g/10 μ l) or in naïve rats prior to and following spinal administration of IgG (1 μ g/10 μ l; n=4). Data represent mean $\pm$ SEM. and analyzed by two-tailed, two-way repeated measures ANOVA with Bonferroni comparisons.



Supplemental Figure 5. Validation of GPR160 siRNA and antibodies targeting GPR160 and CARTp. **(A)** GPR160 expression (red) in KATO III cell 48h after treatment with control (siControl) or Gpr160-targeting (siGpr160) siRNA (25nM). DAPI = blue. The loss of GPR160 immunoreactivity following siRNA treatment serves as an additional control for the specificity of the GPR160 antibody. **(B,C)** *Gpr160* mRNA (**B**; n=7/group) and protein (n=3/group) expression in PC-12 cells treated with control (siControl; siCON) or Gpr160-targeting siRNA (siGpr160). Blot is representative 3 blots with n=1/group/blot. Results are expressed as mean±SD and analyzed by *t*-test; *P<0.05. **(D)** Western blot analysis of the CARTp antibody for Western blot reaction with CARTp, Somatostatin and Nesfatin-1 peptide (1 µg/lane).

Supplemental References

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