Title: Visualizing Nerve Injury in a Neuropathic Pain Model with ^{18}F-FTC-146 PET/MR.

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Supplementary Materials:

Table S1 Experimental methods used to assess each group of rats.

Group #	*von Frey*	*S1R Western blotting*	*PET/MR*		*Ex Vivo Autoradiography*		*Immunohistochemistry and Immunofluorescence*	*Pain Treatment*
			baseline	**blocking**	**whole nerve**	**sectioned nerve**		
1[a]	√		√		√			
2[a]	√		√		√			
3[a]	√		√			√	√	
4[a]	√		√			√	√	
5[a]	√			√	√			
6[a]	√			√	√			
7[b]	√						√	
8[b]	√						√	
9-15[b]	√	√						
16[c]	√							√

[a] Each group contains 3 animals: SNI; Sham and Control

[b] Each group contains 2 animals: SNI and Sham

[c] This group contains 6 SNI animals

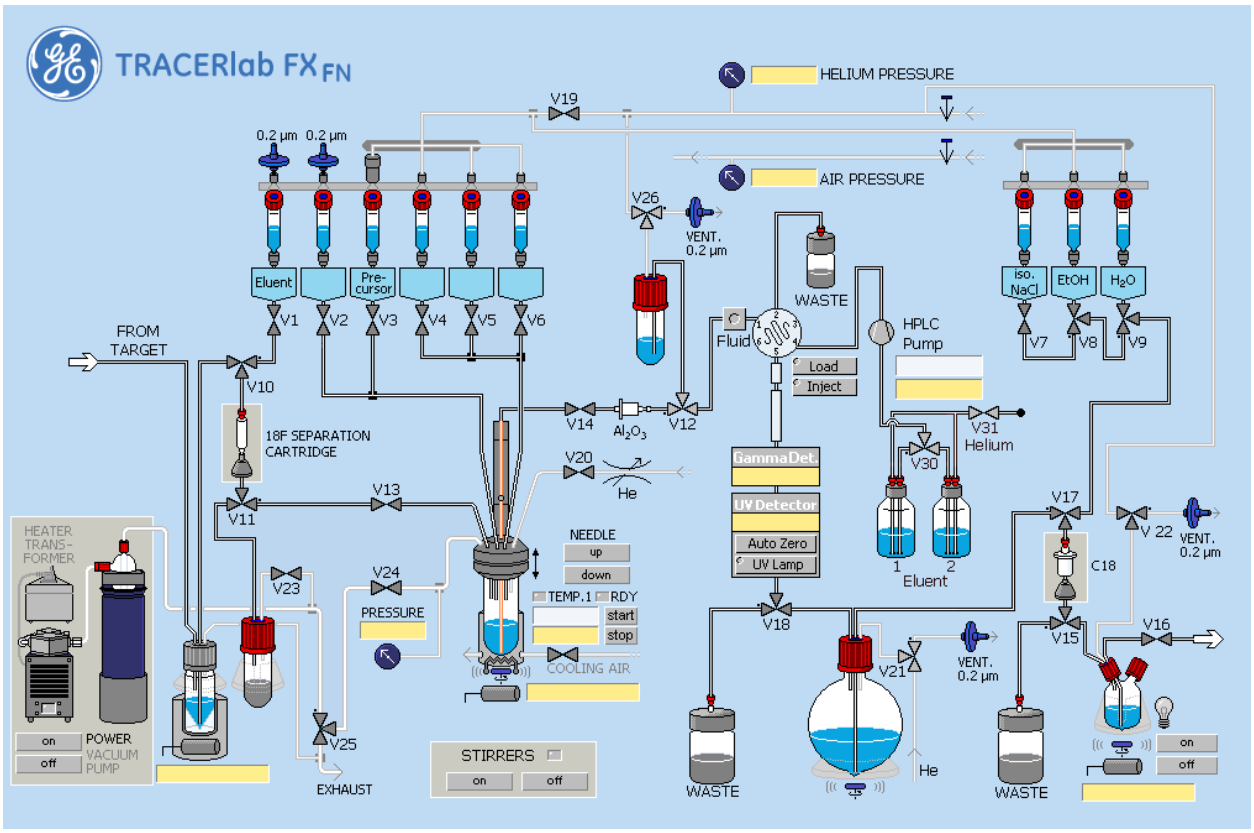


Figure S1 Diagram of interface used with GE TRACERlab FX_{FN} module for the radiosynthesis of [^{18}F]FTC-146.

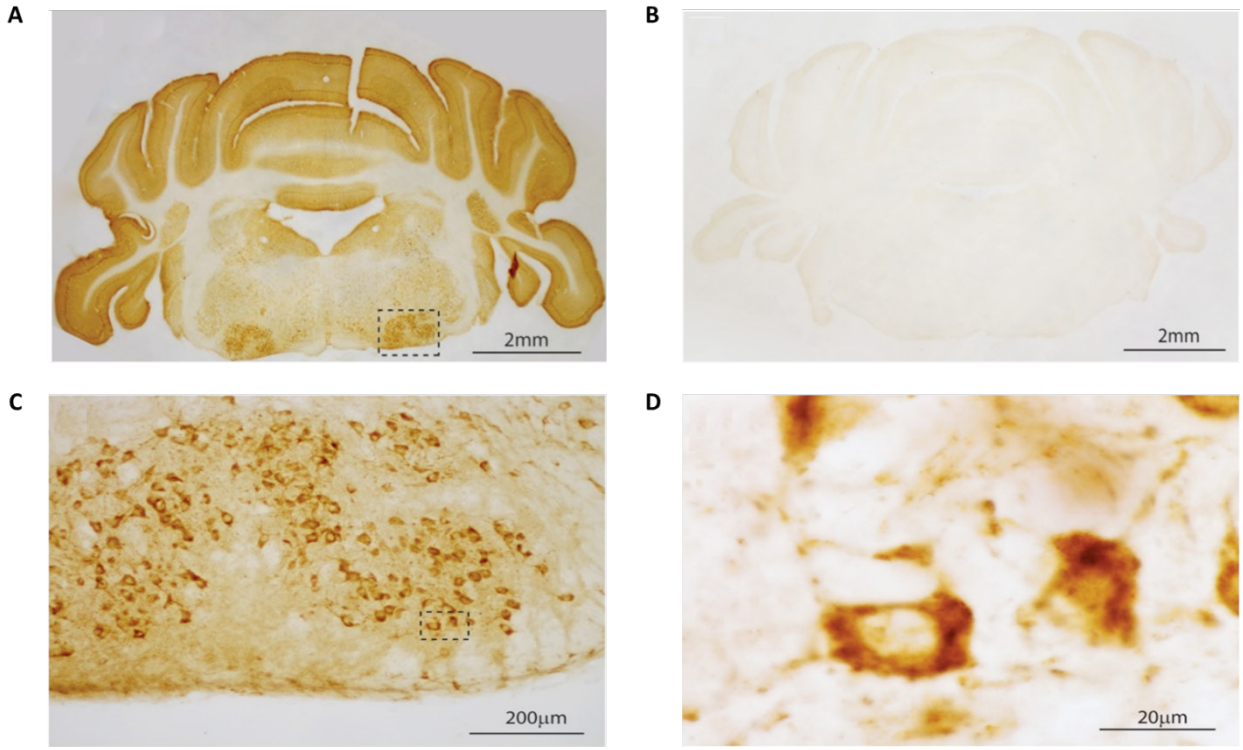


Figure S2 Validation of anti-sigma-1 receptor (S1R) rabbit polyclonal antibody specificity through immunohistochemical staining of (**A)** wild-type and (**B**) S1R knockout mouse brain tissue. Panel (**C**) and (**D**) show 10× and 100× images of S1R staining in facial nucleus of wild type mouse from panel (**A**) – highlighted by dashed box.

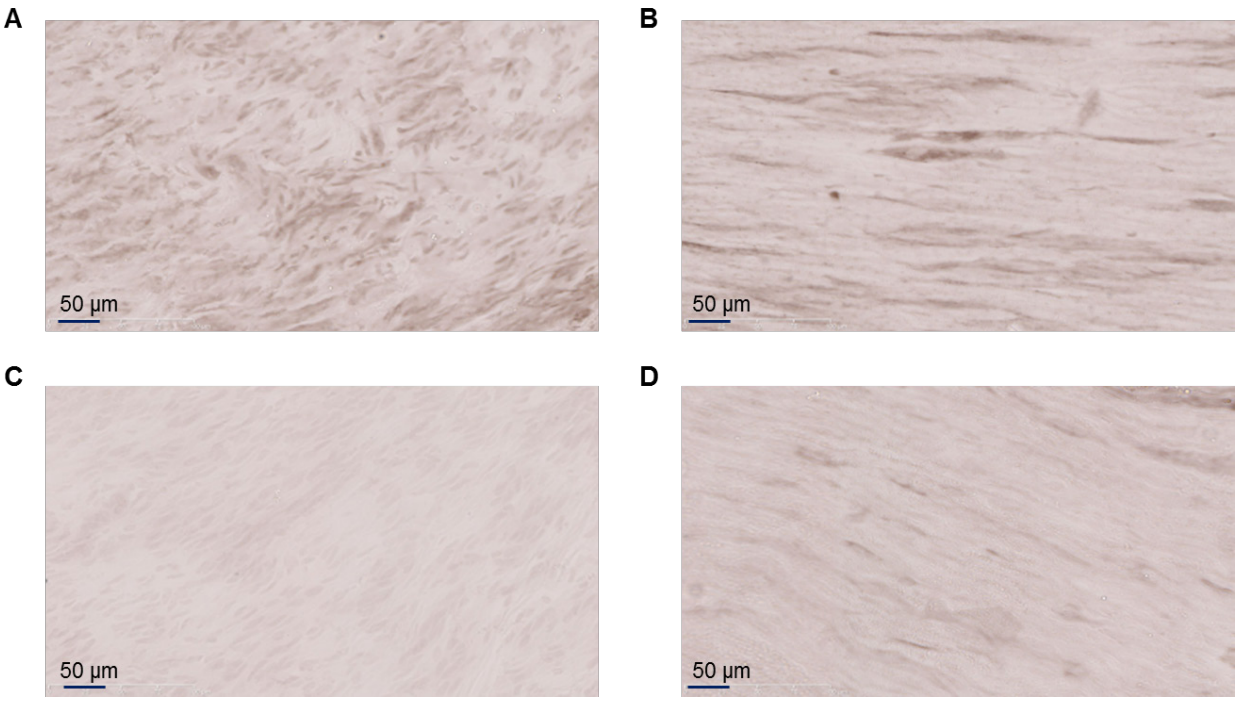


Figure S3 Sigma-1 receptor (S1R) immunohistochemical staining of dissected sciatic nerve. (**A**) S1R staining at the site of nerve injury (i.e., neuroma). (**B**) S1R staining at a site distal to the neuroma in injured nerve. (**C**) No primary control of neuroma in injured nerve. (**D**) Uninjured nerve in Sham group. All pictures were taken at 40× magnification.

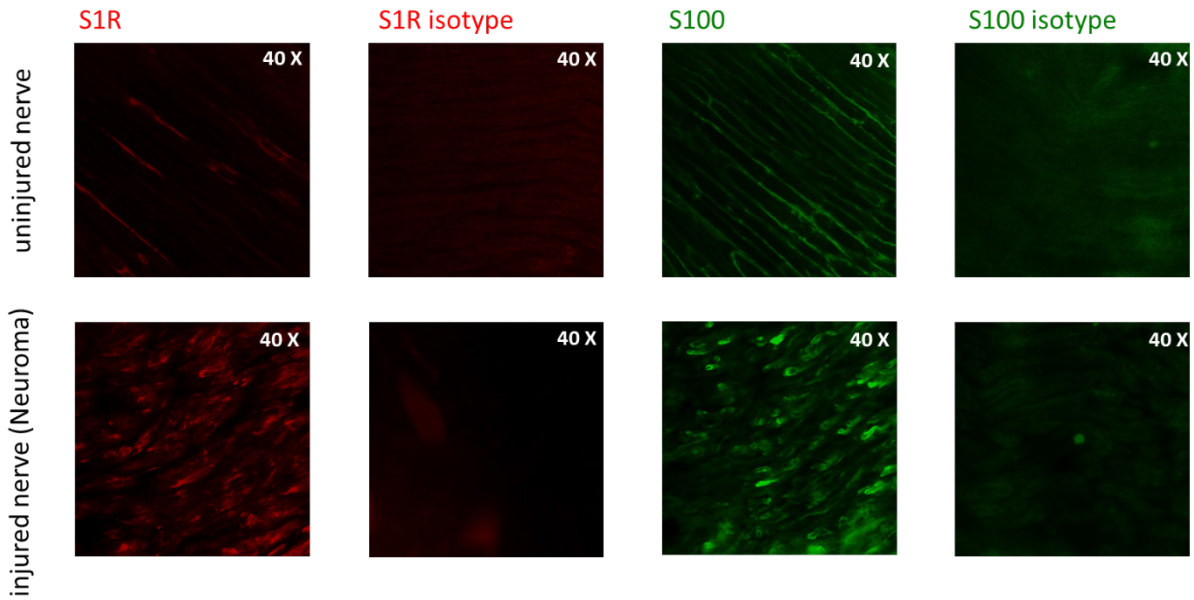
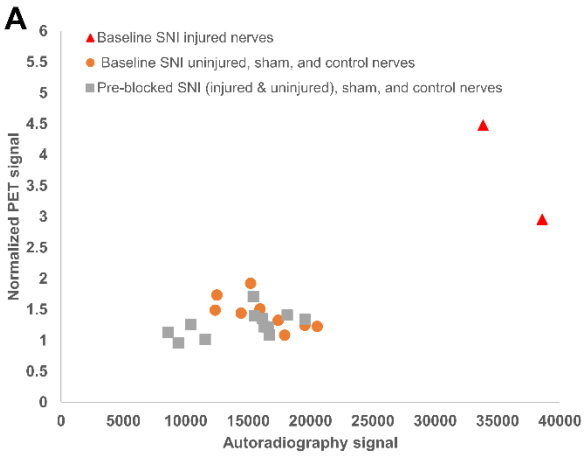


Figure S4 Immunofluorescent staining of S100β/S1R sciatic nerve tissue (i.e. neuroma, and uninjured nerve) using anti-S100β, anti-S1R, and/or isotype control antibody.



B

Group#	Nerve type	Autoradiography signal	Normalized PET signal
▲ = Baseline SNI injured nerves			
1	SNI injured	33844	4.48
2	SNI injured	38589	2.96
● = Baseline SNI uninjured, sham, and control nerves			
1	SNI uninjured	19541	1.25
2	SNI uninjured	15191	1.93
1	Sham uninjured left	15945	1.51
2	Sham uninjured left	17925	1.09
1	Sham uninjured right	17404	1.33
2	Sham uninjured right	20531	1.23
1	Control uninjured left	12357	1.49
2	Control uninjured left	16075	1.34
1	Control uninjured right	12496	1.74
2	Control uninjured right	14428	1.44
■ = Pre-blocked SNI (injured & uninjured), sham, and control nerves			
5	SNI injured	16116	1.35
6	SNI injured	15404	1.71
5	SNI uninjured	16295	1.22
6	SNI uninjured	15511	1.40
5	Sham uninjured left	10392	1.25
6	Sham uninjured left	16589	1.21
5	Sham uninjured right	11556	1.02
6	Sham uninjured right	16720	1.09
5	Control uninjured left	8545	1.13
6	Control uninjured left	19544	1.34
5	Control uninjured right	9384	0.96
6	Control uninjured right	18126	1.41

Figure S5 The SNI injured nerves (which has increased S1R-expression) showed a strong correlation for higher PET and ARG signals unlike the other baseline and pre-blocked nerves that demonstrated an association for lower PET and ARG signals consistent with either lower S1R-expression or blocked S1Rs.

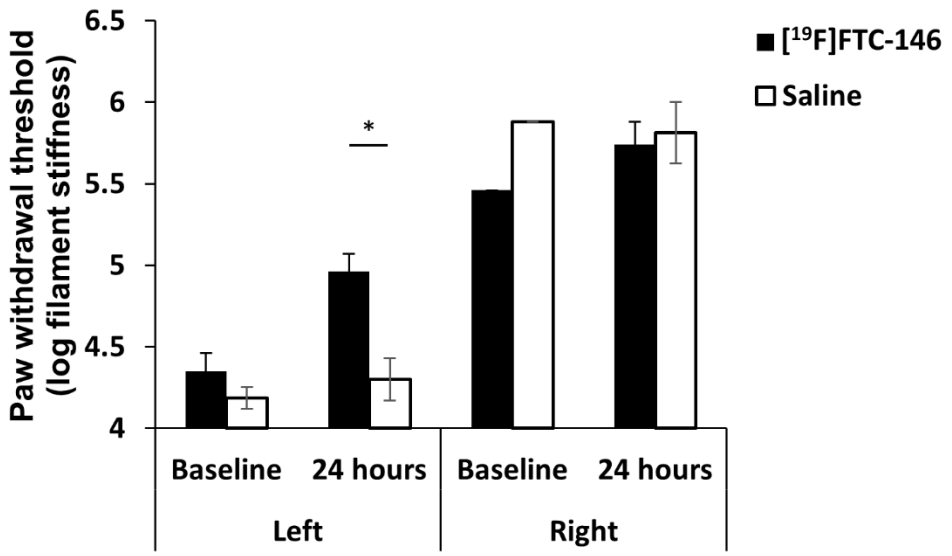


Figure S6 Effect of local treatment of neuroma with [^{19}F]FTC-146 (7.2 mg/mL, 50 μL) under ultrasound guidance. Mechanical allodynia, as evidenced by a reduction in the pressure leading to withdrawal of paw using standard von Frey testing, was used to assess response to treatment with [^{19}F]FTC-146. Average paw withdrawal thresholds are depicted for left and right paws of [^{19}F]FTC-146 and saline (control) treated rats (n = 3 in each group). Error bars represent standard errors, *$p<0.05$.

Automated radiolabeling of [^{18}F]FTC-146

No carrier added-aqueous [^{18}F]-fluoride ion was produced on a PETtrace cyclotron (GE Healthcare, Sweden) by irradiation of a 1.6 mL water target using a 16 MeV proton beam on 95% enriched [^{18}O]H_2O by the [^{18}O(p,n)^{18}F] nuclear reaction. [^{18}F]Fluoride in [^{18}O]H_2O was TRACERlab FX_{FN} synthesizer and passed through an anion exchange resin (QMA cartridge in carbonate form, prepared by washing with 1 mL of EtOH and 1 mL of water) under vacuum. Trapped [^{18}F]Fluoride ions were then eluted from the QMA cartridge and transferred to the reactor using an eluent solution containing 3.5 mg of K_2CO_3 and 15 mg of Kryptofix 222 (K222: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosan) in a mixture of acetonitrile (0.9 mL) and water (0.1 mL). The solution was then evaporated at 65 °C under helium flow and vacuum, followed by heating at 88 °C under vacuum. Tosylate precursor 17, 3-(2-oxo-3-(2-(azepan-1-yl)ethyl)-2,3-dihydrobenzo[d]thiazol-6-yl)propyl 4-methylbenzenesulfonate (2 mg) was dissolved in DMSO (1 mL) and added to the dry Kryptofix-222/K^+[^{18}F]F^- complex. The mixture was allowed to react at 150 °C for 15 min. Upon completion, the reaction mixture was diluted with sterile water (8 mL) and passed through a C18 Sep-Pak cartridge. The C18-trapped, radiolabeled product was then eluted from the C18 Sep-Pak with acetonitrile (1.5 mL) and sterile water (1.5 mL). The resulting crude mixture was then injected onto two serial HPLC Phenomenex Gemini C-18 (5 μm, 10 mm × 250 mm) semipreparative reversed-phase columns. A mobile phase of H_2O (0.1% TEA)/ acetonitrile (0.1% TEA), (pH 8): (20/80, v:v) was used with a flow rate of 5.0 mL/min, and the retention time (t_R) of [^{18}F]FTC-146 was 13 min. Tosylate precursor and decomposed hydroxyl derivative eluted at 17.5 and 10 min, respectively. The radioactive fraction corresponding to [^{18}F]FTC-146 was collected in a round-bottom flask containing sterile water (15 mL) and then passed through a C18 Sep-Pak. An additional 10 mL of sterile water was passed through to wash the C18 Sep-Pak again. The trapped, purified radiolabeled product was eluted from the C18 Sep-Pak using ethanol (1 mL) and saline (9 mL). The formulated solution was then filtered through a sterile 13 mm Millipore GV 0.22 μm filter into a sterile pyrogen-free evacuated 30 mL vial. The final formulated product in saline contained no more than 10% ethanol by volume and was subsequently used for the studies described in this article.

Animal model of neuropathic pain

Animal experiments were approved by Stanford IACUC. Rats had access to food and water *ad libitum* and were kept under a 12 h light/dark cycle. Experiments were carried out using adult male Sprague-Dawley rats weighing 200-250 g.

1. Spared Nerve Injury (SNI): Rats underwent a left SNI surgery, which creates a well-characterized nerve injury and rat neuropathic pain model, showing chronic mechanical and thermal hypersensitivity with symptoms arising 24 h post-surgery and lasting several months. Rats were briefly anesthetized with inhalational 2-3% isoflurane and placed on a warming bed. Hair was removed from the posterolateral aspect of the left thigh. Following a longitudinal skin incision, the left sciatic nerve was identified, exposed and followed distally until its trifurcation into the tibial, common peroneal, and sural nerves. An axotomy and ligation of the tibial and common peroneal nerves were performed with cautious sparing of the sural nerve. The muscle layer was closed with absorbable interrupted sutures (4-0, plain gut; Ethicon) and the skin was apposed with staples. After recovery from anesthesia, rats were returned to their cages and allowed free access to food and water. The staples were removed five days after the surgery. The right hind limb was used as control. Rats were permitted to heal for a period of four weeks following surgery.

2. Sham: Rats underwent a surgery similar to that of SNI rats, but without axotomy or ligation of the tibial and common peroneal nerves; the trifurcation of the sciatic nerve was exposed, and the wound was closed. Post-surgical care was similar to that of SNI rats.
3. Control: Rats did not undergo any surgical procedure prior to imaging. Rats were similar in age and weight to the rats in the SNI and Sham-operated groups.

Assessment of pain (allodynia)

Development of allodynia in the rats was evaluated using the von Frey test, which assesses mechanical allodynia using thin nylon filaments. Tests were performed before surgery (baseline) and on the day before imaging. Sensitivity to mechanical stimulation was measured by recording the paw withdrawal response to serially increasing filament stiffness. On each of the 4 days immediately preceding testing, the rats were placed on a raised, wire-mesh platform and acclimatized for 2 h at a time. They were also acclimatized to the platform for 1 h just before testing. The filament was applied to the lateral portion of the plantar aspect of each hind paw through the mesh floor, pressed until it bowed, and kept in place for 8 s. A brisk paw withdrawal upon application of the fiber was recorded as a positive response, which was confirmed by repeating the test with the same filament after a waiting period of at least 1 min. Testing of the paw was terminated if the same filament elicited 3 consecutive positive responses, or if the filament did not elicit a positive response but was stiff enough to raise the paw from the floor. The data thus collected was fitted to a normalized sigmoid curve to calculate the 50% withdrawal threshold value (in log filament stiffness units) by the Psychofit program (http://psych.colorado.edu/~lharvey/html/software.html). The threshold is defined as the stimulus intensity at which the withdrawal is detected 50% of the time.

Western blotting

Brain tissue, uninjured nerves, and neuromas were harvested from n=7 SNI and SHAM rats (4 weeks post-surgery) and immediately snap-frozen. Frozen tissues were homogenized with a 1 mL douncer in 15 µl per 1 mg tissue of RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors (Thermo Scientific, Rockford, IL) followed by 12 pulses with an Omni Homogenizer. Lysates were spun down at 700 g for 10 min at 4°C, then the supernatant was collected in a new centrifuge tube and spun down at 10,000 g for 30 minutes. The supernatant was used for protein estimation using Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein (40 µg) were separated on 4-20% polyacrylamide gel (Bio-Rad) and transferred onto a nitrocellulose membrane (BioRad). After blocking with 5% milk in TBS-T (Tris-buffered saline plus 0.05% Tween 20), blots were washed 4 times 5 minutes in TBS-T and were probed overnight at 4°C with anti-S1R rabbit polyclonal antibody (1:250, Invitrogen, catalog number 42-3300, polyclonal affinity-purified antibody raised against a synthetic peptide derived from the C-terminus region of the rat S1R). The blot was also probed for β-actin (to serve as a loading control) using anti-β-actin rabbit polyclonal antibody (1:2500, Sigma-Aldrich, catalog number A2066). After four times 5 min washes in TBS-T, blots were probed with secondary antibody - horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000, Bio-Rad, catalog number 170-6515) with StrepTactin-HRP conjugate (1:10,000, Bio-Rad, catalog number 161-0381). Enhanced chemiluminescence (Bio-Rad) was used to detect protein bands using a Bio-Rad ChemiDoc MP. The bands obtained were quantified using Bio-Rad Image Lab, and β-actin was used to normalize the protein loading. The normalized values (to β-actin) of uninjured nerves and neuromas were used for the statistical analysis.

Despite inclusion of one outlying data point of the SNI group (not all treated animals produced a successful pain model), there was still an overall significant difference observed between neuroma and injured nerve, and between neuroma and uninjured nerve.

Immunohistochemistry (sciatic rat nerve)

Staining was performed on sections of sciatic rat nerves and adjacent muscle tissue. Serial frozen longitudinal sections (6 μm thick) from sciatic nerve/muscle tissue blocks embedded in OCT were cut in a cryostat (Leica CM1950) and collected onto plus-plus slides (Fisherbrand Superfrost Plus Microscope Slides). The sections were then washed (3 × 5 min) in a solution of tris-buffered saline (TBS). Following washing, the sections were incubated in a 1% H_2O_2, 50% TBS/MeOH solution for 30 min to quench the endogenous peroxidase activity. After subsequent washing (3 × 5 min) in TBS, the sections were placed in a 10% normal goat serum (NGS, Vector Laboratories), TBST (1% Triton X-100) for 1 h in order to block unspecific staining and permeabilize the cells.

Finally, without further washing, the sections were then incubated with the S1R specific primary antibody 1:200 containing 5% NGS and TBST (0.1% Triton X-100) for 24 h at room temperature. The sections were then washed (3 × 5 min) in TBST (0.1% Triton X-100) and incubated with biotinylated anti-rabbit secondary antibody 1:400 (Vector Laboratories) in 5% NGS and TBST (0.1% Triton X-100) for 1 h at room temperature. The sections were then washed again in the Triton-TBS solution (3 × 5 min), and an avidin-biotin complex was applied (diluted 1:1000 in TBS, Vector Laboratories) for 90 min at room temperature. The sections were then washed (3 × 5 min) in TBS again, before being incubated with 3,3'-Diaminobenzidine (DAB) for 15 min. Finally, the sections were washed (3 × 5 min) with ice-cold TBS to stop the reaction. The immunohistochemical stained sections were dehydrated and cover-slipped with Permount (Sigma Aldrich) for microscopic observation.

Omission of the primary antibody abolished the staining. To confirm the specificity of the primary antibody, 50 μm slices from a S1R knockout-mouse were processed immunohistochemically and no staining was seen.

Double immunofluorescence staining (sciatic rat nerve)

Staining was performed on sections of sciatic nerves, brain and adjacent muscle tissue. Serial frozen longitudinal sections (6 μm thick) from sciatic nerve/muscle and brain tissue blocks embedded in OCT were cut in a cryostat (Leica CM1850) and collected onto plus-plus slides (Fisherbrand Superfrost Plus Microscope Slides). The sections were defrosted at room temperature for 45 minutes and washed for 10 minutes in a solution of tris-buffered saline (TBS). Following washing, the sections were then placed in a 10% normal goat serum (NGS, Vector Laboratories), TBST (1% Triton X-100) for 1 h in order to block unspecific staining and permeabilize the cells. Finally, without further washing, the sections were incubated with a mixture of S1R specific primary antibody and S-100 specific primary antibody both 1:100 containing 5% NGS and TBST (0.1% Triton X-100) overnight in a dark humidified chamber at room temperature. The sections were then washed (3 × 10 min) in TBST. The slides were then incubated with secondary antibodies 1:1000 (Alexa 488-conjugated goat anti-mouse IgG1, and Alexa 594-conjugated goat anti-rabbit IgG) in blocking solution for 1 hour. The sections were then washed again in TBS solution (3 × 10 min) and washed in DI water for 5 minutes. The sections were then cover slipped with Vectashield with DAPI (Vector Labs, H-1200) and imaged using an Olympus IX89 microscope using Metamorph software. Additionally, to further confirm specificity of the primary antibody,

isotype control for mouse IgG1 (Sigma Aldrich, F6397), or rabbit IgG (Novus Biologicals, NBP1-43957) 1:1000 were applied on 6 µm slices of rat sciatic nerve, and no specific staining was shown.

Treatment of neuroma under ultrasound guidance

After a baseline von Frey's test, the rats were anesthetized with inhalational isoflurane 2-3% in oxygen. They were immobilized with tape, in a lateral position with the left side up, on a firm raised platform next to the ultrasound machine (Vevo 2100, Fujifilm VisualSonics Inc., Ontario, Canada). Hair was removed from the posterolateral aspect of the left thigh. The left sciatic nerve and neuroma were located under ultrasound, the position of the ultrasound probe and the rat leg were fixed, and the position of the sciatic nerve neuroma in the field of view noted. The rat was moved out of the field of view by lowering the platform by a fixed distance using a built in lever, without moving the rat relative to the platform. Then, a needle guide was used to determine the path of the needle under the probe and adjusted to allow the needle tip to reach the position of the neuroma as previously determined. The rat platform was replaced, and a syringe with 50 µl of the drug ([^{19}F]FTC-146; 7.2 mg/mL or saline) was loaded on to the needle guide. Under real time ultrasound imaging, the needle guide was used to inject the dose at the neuroma. The rats were then allowed 24 hours to recover from the anesthesia and return to their normal activities in the cage. 24 hours after treatment, von Frey tests were performed to assess pain.