

1 The control of alternative splicing by SRSF1 in myelinated afferents contributes to the  
2 development of neuropathic pain

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4 <sup>1,2</sup>Richard P. Hulse, <sup>2</sup>Robert A.R. Drake, <sup>1,2</sup>David O Bates, <sup>2,3</sup>Lucy F. Donaldson

5 **Author affiliations:**

6 <sup>1</sup>Cancer Biology, School of Medicine, University of Nottingham, Nottingham, NG7  
7 7UH, <sup>2</sup>School of Physiology and Pharmacology, University of Bristol, University  
8 Walk, Bristol BS8 1TD, United Kingdom; <sup>3</sup>School of Life Sciences and Arthritis  
9 Research UK Pain Centre, University of Nottingham, Nottingham, NG7 7UH.

10 **\*Corresponding authors:**

11 Dr. R.P. Hulse

12 Email: Richard.Hulse@nottingham.ac.uk

13 Tel: + 44 115 823 1307

14 Dr. L.F. Donaldson

15 Email: Lucy.Donaldson@nottingham.ac.uk

16 Tel: +44 115 823 0158

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1 **Highlights**

- 2       • Regulation of SRPK1 (Serine Arginine-rich Protein Kinase 1)-SRSF1 (Serine  
3       Arginine-rich Splicing Factor 1) mediated alternative RNA splicing in the spinal  
4       cord modulates neuropathic pain.  
5       • SRSF1 is located in myelinated sensory afferent terminals.  
6       • Vascular Endothelial Growth Factor-A (VEGF-A) expression regulates chronic  
7       pain at the level of the spinal cord.

1 Abstract

2 Neuropathic pain results from neuroplasticity in nociceptive neuronal networks. Here  
3 we demonstrate that control of alternative pre-mRNA splicing, through the splice factor  
4 serine-arginine splice factor 1 (SRSF1), is integral to the processing of nociceptive  
5 information in the spinal cord.

6 Neuropathic pain develops following a partial saphenous nerve ligation injury, at which  
7 time SRSF1 is activated in damaged myelinated primary afferent neurons, with  
8 minimal found in small diameter (IB<sub>4</sub> positive) dorsal root ganglia neurons. Serine  
9 arginine protein kinase 1 (SRPK1) is the principal route of SRSF1 activation. Spinal  
10 SRPK1 inhibition attenuated SRSF1 activity, abolished neuropathic pain behaviors  
11 and suppressed central sensitization. SRSF1 was principally expressed in large  
12 diameter myelinated (NF200-rich) dorsal root ganglia sensory neurons and their  
13 excitatory central terminals (vGLUT1+ve) within the dorsal horn of the lumbar spinal  
14 cord.

15 Expression of pro-nociceptive VEGF-A<sub>xxx</sub>a within the spinal cord was increased after  
16 nerve injury, and this was prevented by SRPK1 inhibition. Additionally, expression of  
17 anti-nociceptive VEGF-A<sub>xxx</sub>b isoforms was elevated, and this was associated with  
18 reduced neuropathic pain behaviors. Inhibition of VEGF receptor-2 signaling in the  
19 spinal cord attenuated behavioral nociceptive responses to mechanical, heat and  
20 formalin stimuli, indicating that spinal VEGF receptor-2 activation has potent pro-  
21 nociceptive actions. Furthermore, intrathecal VEGF-A<sub>165</sub>a resulted in mechanical and  
22 heat hyperalgesia, whereas the sister inhibitory isoform VEGF-A<sub>165</sub>b resulted in anti-  
23 nociception. These results support a role for myelinated fiber pathways, and  
24 alternative pre-mRNA splicing of factors such as VEGF-A in the spinal processing of

1 neuropathic pain. They also indicate that targeting pre-mRNA splicing at the spinal  
2 level could lead to a novel target for analgesic development.

3

4 **Keywords**

5 VEGF-A, SRPK1, SRSF1, myelinated, spinal cord, neuropathic pain

6

1 **Abbreviations**

2 VEGF-A = Vascular Endothelial Growth Factor – A

3 SRSF1 = Serine Arginine-rich Splicing Factor 1

4 SRPK1 = Serine Arginine-rich Protein Kinase 1

5 VEGFR2 = Vascular Endothelial Growth Factor Receptor 2

6 PSNI = Partial Saphenous Nerve Ligation Injury

## 1 Introduction

2 Insults to the peripheral nervous system usually result in pain and hypersensitivity to  
3 noxious (hyperalgesia) and innocuous (allodynia) stimuli. These abnormal sensations  
4 arise due to neuronal plasticity leading to alterations in sensory neuronal excitability.  
5 These alterations include peripheral sensitization [20], with enhanced evoked and on-  
6 going activity in primary afferents, and central sensitization, responsible for the  
7 generation and maintenance of chronic pain. The most widely accepted model for  
8 establishment of central sensitization is that ectopic firing/increased activity in C-  
9 nociceptive afferents drives altered spinal sensory processing, particularly the  
10 processing of A-fiber inputs, resulting in secondary hyperalgesia and allodynia (pain  
11 remote from an area of damage) [46,89,90] [42,74,97]. C-nociceptor changes are  
12 reported in the majority of studies of animal or human neuropathies [1,14,20,38,40,68-  
13 70,96] (although not all e.g. [14,38]). Central sensitization can also occur through  
14 neuro-immune interactions, following injury-induced local immune cell infiltration and  
15 cytokine production/release [80]. After nerve injury there is activation of spinal glia,  
16 disruption of the blood-spinal cord barrier, and consequent infiltration of immune cells  
17 [16]. These events can alter the central processing of peripheral inputs, implicated in  
18 the development of chronic pain [27,39,76]. There is, however still debate on how the  
19 processing of A or C fiber inputs is differentially regulated to form the neuronal basis  
20 of chronic pain.

21 During chronic pain, changes in the complement of proteins result in alterations in  
22 sensory neuron excitability, as recently demonstrated whereby expression of voltage  
23 gated potassium channels in the DRG is altered in ATF3 positive sensory neurons  
24 following nerve injury [79]. Furthermore, alternative mRNA splicing allows for  
25 functionally distinct proteins to arise from a single gene. This provides a vast repertoire

1 of actions from a limited source of transcripts, allowing for cell-specific and stimulus-  
2 induced alteration in cellular function. Targeting regulation and expression of  
3 alternative RNA transcripts, and hence proteins, has been proposed as a potential  
4 route for novel drug discovery [73], but this has not been widely investigated with  
5 respect to nociception/analgesia.

6 We recently demonstrated the analgesic effect of targeting alternative mRNA splicing,  
7 by inhibition of peripheral serine-arginine rich protein kinase 1, SRPK1 [35]. SRPK1  
8 controls phosphorylation of serine-arginine rich splice factor 1 (SRSF1), which is  
9 fundamental to the control of the vascular endothelial growth factor A (VEGF-A) family  
10 alternative splicing [2,8,59,60]. Inactive SRSF1 is located in the cytoplasm, but when  
11 phosphorylated by SRPK1 it translocates to the nucleus. There are two VEGF-A  
12 isoform families, VEGF-A<sub>xxx</sub>a and VEGF-A<sub>xxx</sub>b [31] where xxx refers to the number of  
13 amino acids encoded, and a and b denote the terminal amino acid sequence. SRSF1  
14 phosphorylation results in preferential production of the proximal splice site isoforms,  
15 VEGF-A<sub>xxx</sub>a [59]. Little is understood about the contribution of VEGF-A proteins to  
16 nociceptive processing. VEGF receptor-2 (VEGFR2), the principal receptor activated  
17 by both isoform families, has been implicated in nociceptive processing in animal  
18 [29,35,50], and clinical studies [43]. VEGF-A isoforms and VEGFR2 are present in the  
19 spinal cord [6], and contribute to neuroregeneration and neuroprotection [83].

20 We therefore tested the hypothesis that the SRPK1/SRSF1 system contributes to  
21 spinal nociceptive processing in rodent models of neuropathic pain, concentrating on  
22 the effects of SRPK1 inhibition, and VEGF-A<sub>xxx</sub>a/VEGFR2 signaling in central  
23 terminals of myelinated afferents.

1 Materials and Methods

2 Animals

3 Adult male Wistar rats (total 72; 250-350g, Harlan UK) and adult male 129Ola mice  
4 (total 20; 25-30g inbred strain) were used. Animals were provided food and water ad  
5 libitum. All animal procedures were carried out in laboratories at the University of  
6 Bristol in accordance with the U.K. Animals (Scientific Procedures) Act 1986 plus  
7 associated U.K. Home Office guidance, EU Directive 2010/63/EU, with the approval  
8 of the University of Bristol Ethical Review Group.

9

10 Nociceptive Behavior

11 Nociceptive behavioral testing was carried out as previously described [35]. All  
12 animals were habituated to both handling by the tester and the testing environment on  
13 the day prior to testing. Two days of baseline testing were carried out prior to any  
14 intervention (either drug or surgical) followed by testing post-intervention at discrete  
15 time-points as detailed in each experiment. Stimuli were applied to the partially  
16 innervated medial aspect of the plantar surface of the hindpaw, an area innervated by  
17 the saphenous nerve. Mechanical withdrawal thresholds were calculated from von  
18 Frey hair force response curves. Animals were housed in Perspex holding chambers  
19 with metal mesh floors (Ugo Basile) and allowed to habituate for 10 minutes. A range  
20 of calibrated von Frey hairs were applied to the plantar surface of the hindpaw (for a  
21 maximum of five seconds or until paw withdrawal), with a total of five applications per  
22 weighted hair. From these data, force response curves were generated and withdrawal  
23 values were calculated as the weight at which withdrawal frequency = 50%. Tactile  
24 allodynia was assessed in the metal mesh floored enclosures using a brush moved



1 across the plantar surface of the hindpaw where a withdrawal scored one, with no  
2 response zero. This was repeated a total of five times giving a maximum score of five  
3 per session. Cold allodynia: a single drop of acetone was applied to the plantar surface  
4 of the hindpaw using a 1ml syringe a maximum of five times giving a maximum score  
5 of five if the animal exhibited licking/shaking behavior in response to each application.  
6 Thermal hyperalgesia (Hargreaves test[30]): animals were held in Perspex enclosures  
7 with a glass floor. A radiant heat source was positioned under the hindpaw, and the  
8 latency was recorded for the time taken for the animal to move the hindpaw away from  
9 the stimulus. This was repeated three times and a mean value calculated for each test.

10 Formalin Testing: animals were habituated to glass floored testing enclosures as  
11 above. A single 50µl injection of 5% formalin was administered to the plantar surface  
12 of the right hindpaw by intradermal injection. Immediately following formalin injection,  
13 animals were placed into the testing enclosures. Time (seconds) spent exhibiting pain-  
14 like behaviors and the total number of pain-like behaviors was recorded in five minute  
15 bins for sixty minutes. Data are shown as the classical biphasic response with  
16 behavioral responses pooled for the first phase 0-15 minutes and second phase 20-  
17 60 minutes. Blinding of nociceptive behavioural studies are routine in the laboratory  
18 however where animal welfare/experimental design prohibits this, it cannot be  
19 implemented. For instance, in nerve-injured animals blinding is not possible as  
20 controls are naïve. The lack of blinding may have introduced some subjective bias into  
21 these experiments, which is in part mitigated by behavioural data is supported by the  
22 inclusion of experiments in which measurements are not subjective (e.g. *in vivo*  
23 noxious e.m.g. recording, expression analysis, and neuronal activation using c-fos).

24 Electromyographic Experiments

1 A well-defined method for minimally invasive preferential selection of either C- or A-  
2 fibre mediated nociceptive pathways was used [93,94]. Noxious withdrawal responses  
3 to A- and C-nociceptor selective stimulation were carried out as previously described  
4 [44,45,53], by measurement of electromyographic activity in biceps femoris. Animals  
5 were anaesthetized using isoflurane induction (4% in oxygen), and the external jugular  
6 vein and trachea were cannulated to allow maintenance of airway and anesthesia.  
7 Following surgery, anesthesia was switched to alfaxalone (~30mg/kg/hr i.v.), and  
8 animals were maintained at a steady level of anesthesia by continuous pump perfusion  
9 via the jugular vein for the remainder of the experiment. Bipolar electrodes were made  
10 with Teflon coated stainless steel wire (Advent Research Materials, Oxford UK)  
11 implanted into the bicep femoris. EMG recordings were amplified and filtered by a  
12 combination of in-house built and Neurolog preamplifier and band pass filters  
13 (Digitimer Neurolog System). Animals were maintained at a depth of anesthesia where  
14 a weak withdrawal to noxious pinch could be elicited for the duration of the experiment.  
15 A- and C-cutaneous nociceptors were preferentially activated to elicit withdrawal reflex  
16 EMGs using a well-characterized contact heating protocol [44,45,53]. Two different  
17 rates of heating ( $2.5^{\circ}\text{C}/\text{s}$  and  $7.5^{\circ}\text{C}/\text{s}$ ) were applied to the dorsal surface of the left  
18 hindpaw as these are known to preferentially activate slow/C-nociceptors ( $2.5^{\circ}\text{C}\cdot\text{s}^{-1}$ )  
19 and fast/A nociceptors ( $7.5^{\circ}\text{C}\cdot\text{s}^{-1}$ ) respectively. Contact skin temperature at the time  
20 of onset of the EMG response was taken as the threshold. A cutoff of  $58^{\circ}\text{C}$  for A-  
21 nociceptors,  $55^{\circ}\text{C}$  for C-nociceptors was put in place to prevent sensitization if no  
22 response was elicited. If a withdrawal response was not elicited, threshold was taken  
23 as cut-off  $+2^{\circ}\text{C}$  [22]. Three baseline recordings were performed before i.t. drug  
24 injection with a minimum 8 minutes inter-stimulus interval, and alternating heating  
25 rates, to prevent sensitization or damage to the paw. Digitized data acquisition, digital

1 to analogue conversion, and offline analyses were performed using a CED Micro1401  
2 Mark III and Spike2 version 7 software (Cambridge Electronic Design, UK).

### 3 Nerve injury model

4 The partial saphenous nerve ligation injury (PSNI) model was used to induce  
5 mechanical and cold allodynia, as described previously [34,84]. Under isoflurane  
6 anesthesia (3% in O<sub>2</sub>), the saphenous nerve was exposed via an incision made along  
7 the inguinal fossa region of the right hind leg. Approximately 50% of the nerve was  
8 isolated and tightly ligated using 4.0 silk suture, and the incision was closed using size  
9 4.0 sterile silk suture.

### 10 Drugs and drug delivery

11 I.t. injections were carried out under isoflurane (4% in oxygen) anesthesia, using 0.5ml  
12 insulin syringes (29 gauge, Terumo) in rats and mice. For i.t. administration, 10 µl  
13 injections were made in the midline of the vertebral column through the intervertebral  
14 space between lumbar vertebrae five and six. The injection was deemed to be in the  
15 correct place when it evoked a tail flick response. Rats were used for i.t. anti-VEGF-  
16 A<sub>xxx</sub>b experiments, as the 56/1 mouse monoclonal antibody had not been validated in  
17 mice at that time. All nociceptive behavioural testing was carried out one hour after  
18 intrathecal injection as initial experiments indicated that responses to i.t. PTK peaked  
19 at 1 hour, and returned to normal by 2 hours after injection.

20 All drugs were made up as stock concentrations and then diluted to working  
21 concentration in phosphate buffered saline (PBS) as described in each experiment.  
22 Vehicle controls were used for each drug. PTK787 (LC laboratories, USA) was  
23 dissolved in polyethylene glycol (PEG) 300/PBS, with the final PEG 300 concentration

1 at 0.002%. ZM323881 (Tocris, UK) was made up in DMSO/PBS and given  
2 intrathecally at a final concentration of 100nM ZM323881/0.001% DMSO. Mouse  
3 monoclonal VEGF-A<sub>165b</sub> antibody 56/1 (AbCam ab14994; MRVL56/1), recombinant  
4 human (rh)VEGF-A<sub>165A</sub> (R&D systems, UK) and rhVEGF-A<sub>165b</sub> (R&D Systems UK)  
5 were all dissolved in PBS. SRPIN340 (N-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]  
6 iso nicotinamide; SRPK inhibitor [25] purchased from Ascent Scientific, Bristol, UK)  
7 was dissolved in DMSO and diluted to final concentrations in PBS (to a final DMSO  
8 concentration of 0.03%). All peptides and concentrations used have been previously  
9 shown to exert functional effects in neurons and/or other biological systems [9,35,63].  
10 SRPIN340 has been used in several other studies, different pathological states, and  
11 was used at a known functional concentration (10µM), as previously described  
12 [2,35,51].

### 13 Immunohistochemistry

14 Rats were terminally anesthetized with sodium pentobarbital overdose (i.p. 60mg/kg)  
15 and were perfused transcardially with saline followed by 4% paraformaldehyde. The  
16 L3-4 segments of the lumbar enlargement, containing the central terminals of  
17 saphenous nerve neurons [64], and L3-L4 dorsal root ganglia were removed, post  
18 fixed in 4% paraformaldehyde for 2 hours and cryoprotected in 30% sucrose for 12 h.  
19 Tissue was stored in OCT embedding medium at -80°C until processing. A cryostat  
20 was used to cut spinal cord (20µm) and dorsal root ganglia (8µm) sections that were  
21 thaw mounted onto electrostatic glass slides. Slides were washed in phosphate  
22 buffered saline (PBS) solution 3 times for 5 minutes per incubation, and incubated in  
23 PBS 0.2% Triton X-100 for 5 minutes. Sections were blocked (5% bovine serum  
24 albumin, 10% fetal bovine serum, 0.2% Triton X-100 in PBS) for 2 hours at room  
25 temperature, and then incubated in primary antibodies diluted in blocking solution

1 overnight at 4°C. Sections were washed three times in PBS washes and incubated for  
2 2 h in secondary antibody (e.g. biotinylated or alexafluor-conjugated; 0.2% Triton X-  
3 100 in PBS). For the third stage (i.e. streptavidin-alexafluor conjugate), incubations and  
4 washes were as described for the secondary antibody. Slides were washed in PBS 3  
5 times prior to coverslipping in Vectorshield (H1000 or H1200 containing DAPI for  
6 nuclear staining, Vector Laboratories). Images were acquired on either Nikon Eclipse  
7 E400 and a DN100 camera or Leica TCS SPE confocal microscope using Leica  
8 application suite (Tumor and Vascular Biology Laboratories' imaging suite UoN).

9 Primary antibodies used were as previously reported [2,59]: anti-ATF3 (rabbit  
10 polyclonal; 2µg/ml: Santa Cruz), anti-c-fos (rabbit polyclonal; 2µg/ml: Santa Cruz),  
11 anti-SRSF1 (goat polyclonal; 2µg/ml; sc-10255 Santa Cruz), anti-vGLUT1 (rabbit  
12 polyclonal, 60pg/ml, Synaptic Systems), anti-NF200 (mouse monoclonal; 1.4µg/ml;  
13 N0142 Sigma-Aldrich), anti-NeuN (mouse monoclonal, 1 in 100, Millipore). Use of anti-  
14 VEGF-A and SRSF1 antibodies for both immunolocalisation and immunoblotting has  
15 been previously reported [2,8]. Secondary antibodies (1 in 1000 dilution and from  
16 Invitrogen unless stated): Alexafluor 488 goat anti-mouse, Alexafluor 488 chicken anti-  
17 goat, Alexafluor 555 donkey anti-goat, Alexafluor 555 donkey anti-rabbit; biotinylated  
18 anti-rabbit (Strattech Scientific), Extravidin CY3 (Sigma-Aldrich). Dorsal root ganglia  
19 neuronal cell counts were performed using ImageJ analysis to measure neuronal area  
20 (µm<sup>2</sup>) [67]. The saphenous nerve is approximately equally derived from lumbar DRGs  
21 3 and 4 in rat and human [5,64,95]; the mean number of neurons per section was  
22 quantified from 10 non-sequential random L4 DRG sections per animal. Data are  
23 presented as the mean number of neurons per section and the experimental unit is  
24 the animal. The number of activated SRSF1-positive neurons (defined as those  
25 showing nuclear localization of SRSF1) was calculated as a percentage of total

1 neurons as designated by size (small<600 $\mu\text{m}^2$ , medium 600 $\mu\text{m}^2$ -1200 $\mu\text{m}^2$ ,  
2 large>1200 $\mu\text{m}^2$ ) [79]. The total number of DRG neurons quantified was ~5000 (100  
3 neurons per section, 10 per animal, 3 per group). Determination of SRSF1 spinal cord  
4 expression/localization was determined from 5 non-sequential random spinal cord  
5 sections per animal using Image J analysis. Images were converted to an 8-  
6 bit/grayscale image then thresholding was applied across all acquired images to  
7 determine the area of positive staining. Areas of positive staining were then quantified  
8 across all sections and groups. Colocalisation was determined via coloc2 plugin in  
9 ImageJ. Controls for VEGF-A and SRSF1 immunofluorescence consisted of  
10 incubation with only secondary antibody ('no primary' control) or substitution of the  
11 primary antibody with a species matched IgG.

## 12 Western blotting

13 Naïve and PSNI rats (treated with i.t. vehicle or SRPIN340) were terminally  
14 anesthetized (i.p. 60mg/kg sodium pentobarbital) and perfused with saline solution.  
15 The lumbar region of the spinal cord was extracted and frozen immediately on dry ice,  
16 then stored at -80°C. Protein lysates (80 $\mu\text{g}$ /well) were prepared using lysis buffer  
17 (RIPA buffer, Sigma-Aldrich) with protease inhibitors (Sigma-Aldrich) and samples  
18 were homogenized. Protein extracts were stored at -80°C until required. Samples were  
19 run on a 4% stacking gel/12 % running SDS-PAGE gel (90V, 1hr 30min) and  
20 transferred (wet transfer) to nitrocellulose membrane for 1hr @ 100V. Membranes  
21 were then incubated with either  $\alpha$ -SRPK1 (mouse; 1 $\mu\text{g}$ /ml; Sigma-Aldrich),  $\alpha$ -SRSF1  
22 (ASF/SF2; rabbit; 0.5 $\mu\text{g}$ /ml; Abcam),  $\alpha$ -SRSF1 (ASF/SF2; mouse; 0.5 $\mu\text{g}$ /ml;  
23 SantaCruz),  $\alpha$ -Actin (SantaCruz; 2 $\mu\text{g}$ /ml)  $\alpha$ -VEGF-A<sub>165b</sub> (mouse; 4 $\mu\text{g}$ /ml; Abcam;),  $\alpha$ -  
24 pan-VEGF-A (rabbit; Santa Cruz A20; 2 $\mu\text{g}$ /ml) or  $\alpha$ -tubulin (mouse; 1 in 4000; Sigma-  
25 Aldrich) antibodies and visualized with Femto chemoilluminescence kit (exposure

1 between 1 second and 1 minute, Thermo Scientific) or Licor IRdye secondary  
2 antibodies (as previously reported [2,25,59])

### 3 Statistical Analysis

4 All data are represented as means  $\pm$  SEM. Data were extracted and analyzed using  
5 Microsoft Excel 2010, Graphpad Prism v6 and ImageJ [67]. Nociceptive behavioral  
6 analyses were between-subjects designs comparing effects of drugs by two way  
7 ANOVA with post-hoc Bonferroni tests. In those experiments involving intrathecal and  
8 intraperitoneal administration of drugs in naïve animals, both hind paws were included  
9 in the analysis as replicates. EMG experiments used a within-subjects design and  
10 immunofluorescence experiments a between-subjects design with the effects of drug  
11 treatment compared to baseline values using one-way ANOVA with post-hoc  
12 Bonferroni tests. Immunofluorescence analysis of spinal cord (c-fos quantification)  
13 was taken from entirety of dorsal horn. DRG (SRSF1+ve) and spinal cord (c-fos)  
14 neuron counts were ascertained from multiple representative images, at least 10 per  
15 animal and the mean value of those 10 calculated. Coloc2 analysis (Image J plugin)  
16 was used to ascertain the pixel intensity spatial correlation (co-localization) of SRSF1  
17 and vGLUT1 staining in the spinal cord. This provides an automated measure of the  
18 correlation of pixel intensity for the two independent immunofluorescence channels for  
19 each sample, given as the Pearson's correlation co-efficient [17,47]. Western blot  
20 analyses of SRSF1 and VEGF-A family expression were determined from ImageJ  
21 densitometry analysis (gel analysis plug in) and compared using Mann Whitney U  
22 tests. All F test statistics are described as a column factor with reference to  
23 drug/experimental grouping. NS designates not significant.

1 Results

2 **SRSF1 is predominantly expressed in myelinated neurons in rats**

3 SRPK1 and SRSF1 are key factors in the control of VEGF-A<sub>xxx</sub>a preferential splicing  
4 particularly in disease [2,59]. SRSF1 is expressed in the cytoplasm of dorsal root  
5 ganglia (DRG) neurons in naïve animals [35](Fig. 1A-C). Upon activation  
6 (phosphorylation), SRSF1 is known to translocate from the cytoplasm to the nucleus  
7 [2,59], where it is involved in pre-mRNA processing. Following PSNI, SRSF1  
8 immunoreactivity in sensory DRG neurons was found to be nuclear (Fig. 1E-G) in  
9 some but not all neurons. Matched IgG (Fig. 1D) and omission of primary antibody  
10 (Fig.1H) controls showed no signal. PSNI injury induces activating transcription factor  
11 3 (ATF3) expression in injured DRG sensory neurons [9]. There was an increase in  
12 ATF3-positive DRG neurons after PSNI (Fig. 1I-K), with 43% of DRG neurons  
13 expressing ATF3 post-PSNI compared to only 1% in naïve animals (Fig. 1K). After  
14 PSNI, all nuclear localized SRSF1-positive (Fig 1L) DRG neurons (Fig 1M) were also  
15 ATF3 positive (Fig 1N), indicating nuclear SRSF1 was exclusively found in damaged  
16 neurons (Fig. 1O). This represents that 45% of ATF3 -positive neurons were also  
17 SRSF1 positive, with the remaining 55% of ATF3 positive neurons negative for  
18 SRSF1.

19 SRSF1 was expressed predominantly in the cytoplasm of 96% of larger (cross  
20 sectional area  $>1200\mu\text{m}^2$ ) neurofilament-200 (NF200) positive DRG neurons in naïve  
21 animals (Fig. 2A-C, L), and 71% of medium (area  $601-1200\mu\text{m}^2$ ) neurons, but was in  
22 only a small proportion (14%) of neurons of area  $<600\mu\text{m}^2$  (small,  $<30\mu\text{m}$  diameter).  
23 NF200 is a marker for myelinated neurons indicating that SRSF1 expression is  
24 principally found in the somata of A-fiber DRG neuronal population, but it was also



1 found in peripheral sensory nerve fibers in PSNI animals (Fig. 2I-K). Following PSNI,  
2 activated (nuclear) SRSF1 co-localized with ATF3 and NF200 in DRG sensory  
3 neurons (Fig. 2D-F), The size distribution of activated (nuclear) SRSF1 in injured  
4 neurons was similar to that in naives, - 69% of large cells, 21.5% of medium cells but  
5 a small proportion (1.7%) of small neurons. In contrast, only a minority of the IB4-  
6 binding, largely unmyelinated DRG neurons from nerve-injured animals were positive  
7 for SRSF1 (Fig. 2G-H). The size distribution profile of DRG sensory neurons indicated  
8 that SRSF1-positive neurons are medium/large in size (Fig. 2L).

9

10 SRSF1 immunofluorescence was also identified in the lumbar region of the spinal cord  
11 of PSNI rats, where it was co-localized with the marker of myelinated primary afferent  
12 central terminals, the vesicular glutamate transporter 1 (vGLUT1, Fig. 3A-C)  
13 [11,58,92]. There was an increase in SRSF1 expression in the central sensory  
14 terminals 2 days after PSNI, as assessed by immunofluorescence (Fig. 3D-I) and  
15 quantified by Western blot (Fig. 3J-K;  $p=0.055$ ). Co-localization analysis of vGLUT1  
16 and SRSF1 staining showed a stronger colocalization in the PSNI animals (indicative  
17 of increased SRSF1 expression) in PSNI (Fig. 3L). vGLUT1 is found in large diameter  
18 myelinated neurons, and is not found in either the peptidergic or IB<sub>4</sub>-binding C-  
19 nociceptor populations [11,62]. Furthermore, SRSF1 (Fig. 3M) was co-localized with  
20 vGLUT1 (Fig. 3M-O) in DRG sensory neurons. There was no SRSF1 expression in  
21 the contralateral dorsal horn of either naïve or PSNI rats, although vGLUT1 expression  
22 was evident, indicating that the increased spinal SRSF1 expression was associated  
23 with injury to peripheral neurons and not a systemic response (Fig. 3P-S).

24

1 **Attenuation of SRSF1 mediated alternative splicing prevents A-nociceptor**  
2 **mediated neuropathic pain in rats**

3 The increased SRSF1 immunoreactivity in vGLUT1-positive central terminals after  
4 PSNI (Fig. 3) was accompanied by an increase in total VEGF-A expression in spinal  
5 cord (Fig. 4A-F) assessed with the pan-VEGF-A antibody A20 [2]). VEGF-A was also  
6 co-localized with SRSF1 in some, but not all central terminals (Fig. 4G-I). VEGF-A<sub>xxx</sub>b  
7 remained unchanged in spinal cord after PSNI whereas total (pan)-VEGF-A  
8 significantly increased (Fig. 4J&K). This indicates an increase in the expression of  
9 VEGF-A<sub>xxx</sub>a isoforms, resulting in a decrease in VEGF-A<sub>xxx</sub>b as a proportion of total-  
10 VEGF-A (Fig. 4L).

11 These results suggest that SRSF1 phosphorylation and activation at the level of the  
12 spinal cord is induced by PSNI, and is accompanied by a change of the balance of  
13 VEGF isoforms toward VEGF-A<sub>xxx</sub>a. As VEGF-A<sub>165a</sub> has been shown to be pro-  
14 nociceptive, and VEGF-A<sub>165b</sub> anti-nociceptive [35], it is therefore possible that  
15 changes in SRSF1 and VEGF-A expression at the level of the spinal cord are  
16 associated with the development of neuropathic pain behaviors. SRSF1 activity is  
17 activated through phosphorylation by serine-arginine-rich protein kinase SRPK1 [2].  
18 To test the hypothesis that PSNI neuropathic pain is dependent upon SRSF1  
19 activation, we inhibited SRPK1 in the spinal cord of rats, with intrathecal (i.t) injection  
20 of the SRPK1 antagonist, SRPIN340 (N-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]  
21 isonicotinamide, Ascent Scientific, Bristol UK) [24] (10µM i.t. injection) at the time of  
22 nerve injury surgery (time point day 0). SRPIN340 has been used extensively to inhibit  
23 SRPK1 activity and a multitude of studies have demonstrated its involvement with  
24 controlling alternative splicing for VEGF-A isoforms [2,51,59], through suppression of  
25 SR protein phosphorylation and stabilization [24]. SRPIN340 inhibits both SRPK1 and

1 SRPK2 at concentrations equal or less than 10 $\mu$ M [24], and this has been shown  
2 previously to inhibit VEGF-A<sub>xxx</sub>a production *in vitro* [59] and *in vivo* [2]. PSNI induced  
3 a reduction in mechanical withdrawal thresholds in the ipsilateral hindpaw as  
4 expected, and this was blocked by i.t. SRPIN340 (Fig. 5A; PSNI+vehicle n=9,  
5 PSNI+SRPIN n=6). Tactile and cooling allodynia which also developed in the  
6 ipsilateral hindpaw (Figs. 5B & C) were also inhibited by SRPIN340. Contralateral  
7 hindpaws from vehicle and SRPIN340 treated groups did not differ from each other,  
8 indicating no effect of central SRPK1 inhibition on noxious processing from uninjured  
9 tissue. The PSNI model does not in itself lead to the development of heat hyperalgesia  
10 [34], but Hargreaves latencies did increase as a result of SRPIN340 treatment  
11 compared to vehicle treated PSNI animals, both ipsilateral (Fig. 5D) and contralateral  
12 (Fig. 5E) to the nerve injury, indicating a possible contribution of SRPK1/SRSF1 in  
13 normal nociceptive processing. SRPIN340 treatment also resulted in a significant  
14 inhibition of the increase in SRSF1 immunoreactivity in the central terminals of the  
15 dorsal horn of the spinal cord induced by PSNI (Fig. 6A-H). Furthermore, the  
16 administration of SRPIN340 resulted in increased distal splice site, anti-nociceptive  
17 isoform VEGF-A<sub>xxx</sub>b with no overall change in total VEGF-A expression (Fig. 7A),  
18 indicating a switch from proximal to distal splice site transcripts following SRPIN  
19 treatment in peripheral nerve injury (Fig. 7B-C). Intrathecal SRPIN340 not only blocked  
20 the development of nociceptive behaviors and altered alternative splicing in the dorsal  
21 horn, it also blocked indicators of central sensitization. The number of c-fos positive  
22 neurons in the spinal cord, a marker of central sensitization [36] as assessed by  
23 immunofluorescent staining (Fig. 7D), was increased after PSNI and was significantly  
24 reduced by i.t. SRPIN340 (Fig. 7E-F). SRPK1 protein expression within the spinal cord  
25 was not significantly altered following nerve injury alone (Fig. 6G)

1

## 2 **VEGF-R2 activation at the level of spinal cord contributes to nociceptive** 3 **processing**

4 VEGF-A<sub>xxx</sub>a and VEGF-A<sub>xxx</sub>b differ only in their terminal 6 amino acids. The C-terminal  
5 sequence determines the efficacy of VEGFR2 signaling of the isoforms and their  
6 functional properties [13]. On binding to VEGFR2, VEGF-A<sub>xxx</sub>a leads to full  
7 phosphorylation and activation of VEGFR2, whereas VEGF-A<sub>xxx</sub>b activates only partial  
8 VEGFR2 phosphorylation, leading to receptor degradation [4]. VEGF-A<sub>165</sub>b also  
9 antagonizes VEGF-A<sub>xxx</sub>a binding [88]. The different C-terminal sequences also  
10 determine the anti- or pro-nociceptive effects of the VEGF-A<sub>165</sub>b and VEGF-A<sub>165</sub>a  
11 isoforms respectively [35] but both isoforms promote neuroprotection [9,71]. Our  
12 findings above show that VEGF-A alternative splicing is altered in neuropathic states  
13 (Fig. 3-5), and this is associated with pain behaviors. These results suggest that spinal  
14 cord VEGFR2 activation by different VEGF isoforms could contribute to nociceptive  
15 processing. Despite evidence from clinical studies that demonstrate an involvement of  
16 VEGF receptors in pain [43,52], and experimental evidence showing that spinal VEGF  
17 levels are associated with pain [57], there are few published findings on the effects of  
18 VEGF-A in spinal nociceptive processing. As spinal VEGF-A splicing and isoform  
19 expression, and therefore by inference VEGFR2 activation, were altered in PSNI we  
20 determined the effect of VEGFR antagonism on central nociceptive processing.

21 PTK787 (or vatalanib) is a tyrosine kinase inhibitor that has non-selective inhibitory  
22 actions on VEGFR1 and 2. It is 18-fold more selective for VEGFR1 and 2 over  
23 VEGFR3, and has slight selectivity for VEGFR2 (IC<sub>50</sub> <50nM) over VEGFR1 (IC<sub>50</sub>  
24 ~100nM) [86]. In naïve rats, systemic VEGFR antagonism with PTK787 (30mg/kg, i.p.)

1 increased thermal withdrawal latencies to heat (Fig. 8A n=5/group) indicating an  
2 analgesic effect. To determine the effect of PTK787 on one aspect of central  
3 nociceptive processing, we used the formalin test. Injection of formalin into the hind  
4 paw allows for the investigation of two distinct phases of acute nociceptive behavior.  
5 The initial phase (0-15min) is largely mediated by peripheral nerve activation, whereas  
6 the second has both a peripheral and central component. One hour prior to formalin  
7 injection, rats were treated with either (i.p.) vehicle or PTK787. The acute phase was  
8 unaffected (0-15min) by PTK787 treatment (Fig. 8B-E; n=7/group). In contrast the  
9 second phase (20-60 min) was significantly reduced by systemic PTK787 treatment  
10 for both the time of flinching (Fig. 8B& D) and the number of flinches (Fig. 8C & E).  
11 These results suggest a central component of VEGFR inhibition. To determine the  
12 targets of VEGF-A/VEGFR signaling in naïve rats, given the effects of the VEGFR  
13 antagonist on the second phase of the formalin test, we recorded electromyographic  
14 nociceptive withdrawals to selective nociceptor activation. Fast heating (fast heating  
15 rates  $\sim 7.5^{\circ}\text{C/s}$ ) preferentially activates myelinated A-nociceptors and slow heating  
16 activates unmyelinated C-nociceptors, both inducing a withdrawal from the stimulus.  
17 To determine VEGFR2 specific actions, ZM323881 (5-[[7-(benzyloxy) quinazolin-4-  
18 yl]amino]-4-fluoro-2-methylphenol) was used locally. ZM323881 which has sub-  
19 nanomolar potency and specificity for VEGFR2 ( $\text{IC}_{50} < 2\text{nM}$ ) [85], with an  $\text{IC}_{50}$  greater  
20 than  $50\mu\text{M}$  for VEGFR1 and PDGFR [85]. I.t. ZM323881 (100nM, specific VEGFR2  
21 inhibitor, [85]) led to a prolonged (up to 60 min) increase in the temperature at which  
22 the rats withdrew during A-nociceptor stimulation (Fig. 8F, n=3-5 per group).  
23 ZM323881 did not have a significant effect on C-nociceptor withdrawals (Fig. 8F).  
24 These results show that VEGFR2 signaling is mediated, at least in part, by A-nociceptor  
25 activation in the spinal cord.

1 Taken together, these results are consistent with the hypothesis that the VEGF-A  
2 isoforms may have different functions in the spinal cord, as in the periphery [35]. We  
3 tested this by giving VEGF agonists and antagonists intrathecally (i.t.), and measuring  
4 pain behaviors in mice and rats. PTK787 increased both mechanical withdrawal  
5 thresholds (Fig. 9A; n=3 mice/group, 6 hind-paws treated as replicates) and heat  
6 nociceptive withdrawal time (Fig. 9B) compared with vehicle treated mice. In contrast  
7 injection of 2.5nM VEGF-A<sub>165a</sub> reduced mechanical withdrawal thresholds (Fig. 9C;  
8 n=4 mice/group, 8 hind-paws treated as replicates) and heat withdrawal latencies (Fig.  
9 9D), indicating a central pro-nociceptive action of VEGF-A<sub>165a</sub> in naïve mice.  
10 Conversely, 2.5nM VEGF-A<sub>165b</sub> increased mechanical thresholds (Fig. 9E n=4 mice  
11 group, 8 hind-paws treated as replicates) and heat withdrawal latencies (Fig. 9F)  
12 indicating a central anti-nociceptive effect. In rats, administration of a neutralizing  
13 antibody against VEGF-A<sub>xxx</sub>b had a similar effect to that of VEGF-A<sub>165a</sub>, decreasing  
14 withdrawal thresholds to mechanical stimulation (Fig. 9G; n=3 rats group, 6 hind-paws  
15 treated as replicates) and the time taken for withdrawal from heat (Fig. 9H), indicating  
16 that loss of endogenous VEGF-A<sub>xxx</sub>b from the spinal cord is painful in naïve animals.

17

### 18 **Attenuation of central VEGFR2 signaling leads to alleviation of neuropathic pain**

19 We mimicked the effect of spinal SRPK1 inhibition by increasing the proportion of  
20 spinal VEGF-A<sub>165b</sub> with exogenous protein, 2 days after the onset of neuropathic pain  
21 behavior in rats. Intrathecal VEGF-A<sub>165b</sub> reversed both mechanical (Fig. 10A) and cold  
22 allodynia (Fig. 10B) and increased thermal withdrawal latencies both ipsilaterally (Fig.  
23 10C) and contralaterally (Fig. 10D). IP (30mg/kg) PTK787 led to the increase in

- 1 withdrawal latencies to heat both ipsilateral (Fig. 10E) and contralateral (Fig. 10F) in
- 2 PSNI injured rats.

## 1 **Discussion**

2 We show that the splicing factor kinase SRPK1 is a key regulator of spinal nociceptive  
3 processing in naïve and nerve injured animals. We present evidence for a novel  
4 mechanism in which altered SRSF1 localization/function in neuropathic pain results in  
5 sensitization of spinal cord neurons. Inhibiting the splicing factor kinase SRPK1 can  
6 control alternative splicing of VEGF-A isoforms in spinal cord, and can prevent the  
7 development of neuropathic pain.

## 8 **Alternative splicing and pain**

9 The development of neuropathic pain and associated neuronal excitation, results from  
10 alterations in neuromodulatory protein function, leading to sensitization of peripheral  
11 and central nociceptive systems. Both short and long term changes occur in the  
12 expression and function of ion channels, receptors, excitatory and inhibitory  
13 neurotransmitters/modulators and second/third messenger systems [15,78,79]  
14 leading to the regulation of neuronal excitability through modulation of excitatory  
15 and/or inhibitory networks. Many of these alterations can be attributable to altered  
16 protein expression (e.g. [61,66]). Alternative pre-mRNA splicing is a rapid, dynamic  
17 process, recognised to be important in many physiological processes, including in  
18 nociception [37]. Such splicing of many channels and receptors particularly calcium  
19 channels, is altered in pain states [3,56], but prior to our studies the control of  
20 mechanisms of alternative pre-mRNA splicing had not been considered as a  
21 contributory factor in nociceptive processing [35].

22 **Inhibition of SRPK1 alleviates neuropathic pain and reduces SRSF1 activation.**



1 The splicing kinase SRPK1, a member of the serine-arginine-rich kinases, controls  
2 alternative pre-mRNA splicing of a relatively small number of identified RNAs [35]. To  
3 date, there is strong evidence for the involvement of only one of these, VEGF-A, in  
4 nociception [35,49,50,65,83]. SRPK1 controls the activity of splice factor SRSF1 that  
5 is fundamental to the processing of pre-mRNA transcripts [28], their cellular  
6 localization/transport [10], and it may also be involved in translational repression [19].  
7 Phosphorylation and activation of SRSF1 results in nuclear translocation in a number  
8 of cell types [2,59]. After nerve injury activated SRSF1 was only found in the nuclei of  
9 injured (ATF-3 positive) large excitatory (vGLUT1 positive) neurofilament-rich DRG  
10 neurons whereas it was found in the cytoplasm of uninjured DRG neurons.  
11 Interestingly, SRSF1 was also seen in the central terminals of myelinated neurons  
12 after injury, but was not in central terminals in naïve animals. The nuclear localization  
13 suggests that neuronal SRSF1 is activated in mRNA processing in injured myelinated  
14 neurons [2]. The redistribution of cytoplasmic SRSF1 to central terminals may reflect  
15 a change in neuronal function or mRNA transport [77]. Little is understood of this  
16 function of SRSF1 in sensory neurons, although mRNA transport is closely linked to  
17 splicing, and specific mRNA splice variants can be targeted to axons [54].

18 After traumatic nerve injury, injured DRG neurons (e.g. ATF3 positive) demonstrate  
19 ectopic and/or increased evoked activity. These neuronal phenomena arise due to  
20 expression changes in key mediators of sensory neuronal excitability, ultimately  
21 underlying chronic pain phenotypes [20,79]. Local neuro-immune interactions  
22 resulting from damage to neurons alter the properties of adjacent 'uninjured' afferents  
23 [20,79], including sensitization of A-fiber afferents [96], and together these drive  
24 excitability changes in the spinal cord [18]. Mechanisms such as SRPK1/SRSF1-  
25 mediated alternative pre-mRNA splicing could underpin this 'phenotypic switch'

1 change in properties, for example by controlling relative expression of ion channel  
2 splice variants in damaged neurons [3,79]. Increased release of neurotransmitters and  
3 modulators from primary afferent central terminals is seen in the spinal cord following  
4 nerve injury [26]. The cellular SRSF1 redistribution also suggests that phosphorylated  
5 SRSF1 could act to transport RNAs to the central terminals in nerve injury, and hence  
6 enable translation of specific isoforms (e.g. VEGF-A<sub>165a</sub>) in the nerve terminals [26].  
7 This reduction in the amount of SRSF1 present in afferent central terminals following  
8 intrathecal SRPK1 inhibition could be due to increased degradation of the SRPK1-  
9 SRSF1 complex and/or reductions in transport of mRNA to the central terminals of  
10 primary afferents.

11 In addition to peripheral sensitization, PSNI results in mechanical and cold  
12 hypersensitivity [34] and central sensitization [84]. Intrathecal administration of the  
13 SRPK1 inhibitor SRPIN340 abolished pain behaviors including mechanical allodynia  
14 and hyperalgesia, and cold allodynia, and the central sensitization indicated by spinal  
15 c-fos expression. Central hyperalgesic priming of primary afferent nociceptors is  
16 dependent on local protein translation in central terminals [23], so we speculate that  
17 SRPK1/SRSF1 actions on RNA localization or protein translation [10,19] may also  
18 contribute to this sensitization mechanism. As heat hyperalgesia was also reduced but  
19 PSNI animals did not display sensitization to radiant heat [33,84], this suggests that  
20 central SRPK1 inhibition not only prevents central sensitization, but also reduces  
21 activation of non-sensitized spinal nociceptive networks.

## 22 **VEGF splicing and VEGF-dependent nociceptive processing in spinal cord.**

23 SRPK1/SRSF1 controls the splice site choice in the alternative splicing of the vascular  
24 endothelial growth factor A (VEGF-A) family, leading to increased expression of

1 VEGF-A<sub>xxx</sub>a isoforms [2,25,59]. VEGF-A<sub>xxx</sub>a isoforms are widely known as pro-  
2 angiogenic/cytoprotective factors and this splicing pathway is strongly associated with  
3 solid tumor development [2]. Peripheral administration of VEGF-A<sub>165a</sub> resulted in pain,  
4 as did, somewhat surprisingly, VEGFR2 blockade [35]. These findings are supported  
5 by observations that systemic VEGF-A receptor blockers result in pain in clinical  
6 studies [12,43] and painful experimental neuropathy [83]. In contrast, given  
7 intrathecally, the VEGF-R2 antagonist, PTK787 *decreased* hypersensitivity in naïve  
8 and neuropathic rodents (Fig. 8, and [50]), but VEGF-A<sub>165a</sub> again increased  
9 hypersensitivity in naïve (Fig. 8) and spinal cord injury rats [57]. This latter increase in  
10 pain was associated with aberrant myelinated fiber sprouting in dorsal horn and dorsal  
11 columns that may be VEGF-A dependent [57]. In contrast, van Neervan and  
12 colleagues [82] found only very small anti-nociceptive effects of intrathecal VEGF-  
13 A<sub>165a</sub> on pain, and no effect on neuronal function. Observed differences in VEGF-A  
14 effects could be attributable to different concentrations used, the source of VEGF-  
15 A<sub>165a</sub>, the degree of injury, or different endogenous isoform complement [6]. Clinically,  
16 elevated levels of VEGF-A in the spinal cord of neuropathic pain patients correlate  
17 with reported pain [52]. VEGF-A and VEGF-A receptor 2 are present in both peripheral  
18 and central nervous systems including spinal cord [7,9,72]. rhVEGF-A<sub>165a</sub> has  
19 consistent pro-nociceptive actions peripherally [35] and centrally, and our findings  
20 demonstrate that the different VEGF-A isoform subtypes have opposing actions on  
21 nociception in the spinal cord, as they do in the periphery [35]. We are the first to show  
22 that the alternatively spliced isoform, VEGF-A<sub>165b</sub> has anti-nociceptive actions in the  
23 spinal cord.

24 Taken together our observations of: increased spinal splicing factor expression,  
25 increased spinal pro-nociceptive VEGF-A<sub>165a</sub> but unchanged VEGF-A<sub>165b</sub> expression,

1 and blockade of pain behavior and VEGF-A expression changes by SPRK1 inhibition,  
2 suggest that exogenous and endogenous VEGF-A isoforms modulate spinal  
3 nociceptive processing in naïve animals and after peripheral nerve injury. The sites of  
4 ligand/receptor expression, the differences in peripheral and central administration,  
5 and the current clinical use of many anti-VEGF treatments to treat varied diseases  
6 highlight the importance of recognizing the different functions and sites of action of the  
7 alternative VEGF-A isoforms.

### 8 **Myelinated afferents and neuropathic pain.**

9 We found that VEGFR2 blockade resulted in inhibition of A fiber nociceptor-mediated  
10 nociception, suggesting that endogenous VEGF is involved in spinal processing of A  
11 fiber nociceptor inputs. Irrespective of the animal model or human condition of  
12 neuropathic pain, the prevailing evidence is that afferents are sensitized [20,34] both  
13 C-fiber [1,14,20,38,40,68-70,96] and A-fiber nociceptors [81,96], increasing the  
14 afferent barrage to the spinal cord through enhanced stimulus-evoked responses  
15 and/or increases in spontaneous/ongoing firing. Other mechanisms, such as neuro-  
16 immune interactions, can also contribute to changes in spinal excitability [80]. The  
17 result of increased input to and excitability of spinal neurons is central sensitization  
18 [46] leading to hyperalgesia and allodynia. It has been hypothesized that central  
19 sensitization allows low threshold A-fiber afferents to “access” pain pathways [48,79]  
20 although precise mechanisms are unknown. Early reports of low threshold A $\beta$  fiber  
21 mechanoreceptors (LTMs) sprouting into superficial laminae [91] are still debated  
22 [32,87]. A-fiber nociceptive afferents, as opposed to LTMs, have similar central  
23 terminals in superficial dorsal horn laminae (I and II<sub>o</sub>) in both naïve and nerve injured  
24 animals [87] and may represent the afferents expressing SRSF1. What is clear is that

1 altered central processing of myelinated nociceptor information contributes to  
2 neuropathic pain [55,75,98], such as secondary dynamic allodynia [41]. Both C-fiber  
3 (unmyelinated) and A-fiber (myelinated) pathways can contribute to chronic pain  
4 [48,98], but this is the first time that VEGFR2 has been implicated in the processing of  
5 information in these pathways. If VEGFR2 is involved in A-fiber nociceptive pathways,  
6 then this provides a potential new mechanism for the modulation of nociception.

## 7 Conclusion

8 Here we identify a novel pathway of nociceptive processing through a SRPK1-SRSF1-  
9 VEGF-A<sub>xxx</sub>a axis in myelinated nociceptors that is involved in nociception at the level  
10 of the spinal cord. During neuropathic pain development SRPK1 drives expression of  
11 pro-nociceptive VEGF-A<sub>xxx</sub>a at the level of the spinal cord. Therefore the development  
12 of SRPK1 targeted therapy, or other controls for alternative splicing, would be  
13 interesting targets for novel analgesic agent development [21]. These findings  
14 highlight the importance of understanding control of RNA function, including alternative  
15 splicing in relation to pain, and considering specific interactions of splice factors in  
16 excitatory networks following peripheral nerve trauma.

17

18

19

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6

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10 LFD and DOB are co-inventors on patents protecting VEGF-A<sub>165b</sub> and alternative  
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12 LFD and DOB are founder equity holders in, and DOB is director and CSO of  
13 Exonate Ltd, a company with a focus on development of alternative RNA splicing  
14 control for therapeutic application in a number of different conditions, including  
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16

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11

1 Figures and Legends

2 Figure 1. SRSF1 expression and activation in DRG sensory neurons following PSNI  
3 injury

4 [A-C] SRSF1 (Red) was expressed in the cytoplasm (not co-localized with DAPI) of  
5 the DRG sensory neurons in naïve animals. [D] Replacement of the primary antibody  
6 with a species matched IgG control DRG image resulted in no staining. [E-G] SRSF1  
7 was co-localized with nuclear stain DAPI in DRG sensory neurons following PSNI  
8 injury (arrows). In some neurons cytoplasmic SRSF1 is still evident (arrowheads). [H]  
9 Omission of the primary antibody resulted in no staining. [I & J] Representative  
10 examples of ATF3 expression in NeuN-co-labeled DRG sensory neurons in [I] naïve  
11 and [J] PSNI animals. [K] The number of ATF3 positive DRG neurons was significantly  
12 increased in the L4 from PSNI animals (unpaired t test, n=5/group). [L-O] High  
13 magnification representative images of SRSF1/ATF3/NeuN co-labeled DRG neurons.  
14 (white arrows). \*\* p<0.001. Scale bars = 50µm low magnification and 20µm high  
15 magnification.

16

17 Figure 2. SRSF1 expression in NF200 sensory neurons

18 [A-C] SRSF1 expression in the cytoplasm of NF-200-positive L4 dorsal root ganglia  
19 neurons in the naïve animal. [C] Note the clear cytoplasmic localization of the  
20 SRSF1 (arrows). [D-F] Following PSNI, clear SRSF1 nuclear translocation was  
21 evident in the NF200 positive neurons (arrows in F). [G] SRSF1 was not expressed  
22 in IB4 positive dorsal root ganglia neurons, [H] though SRSF1 is co-localized with  
23 nuclear marker DAPI. [I-K] SRSF1 was also localized to NF200-rich sensory nerve

1 fibers of the PSNI saphenous nerve. [L] Quantification of the SRSF1 positive and  
2 total number of sensory neurons in the dorsal root ganglia by cell cross-sectional  
3 area ( $\mu\text{m}^2$ ) in naïve and PSNI injured rats. Scale bars = 50 $\mu\text{m}$ . N=5 per group.

4

5 Figure 3. SRSF1 is expressed in myelinated central terminals in the dorsal horn of  
6 the spinal cord and increased after PSNI

7 [A] SRSF1 was expressed at low levels in the dorsal horn of the spinal cord in naïve  
8 animals. [B] vGLUT1 was used as a marker of myelinated sensory fiber central  
9 terminals. [C] Merged image of SRSF1 and vGLUT1. [D-F] Two days after PSNI  
10 nerve injury there was an increase of SRSF1 expression in the spinal cord, still co-  
11 localized with vGLUT1. [F] Inset images of no primary SRSF1 (i) and vGlut1 (ii)  
12 antibodies. [G-I] High power views of boxes marked in D-F. [J] Increased SRSF1  
13 expression/localization within the lumbar spinal cord following PSNI was  
14 demonstrated by western blot with two different primary antibodies (Santa Cruz  
15 mouse monoclonal and Abcam rabbit polyclonal antibodies). [K] Quantification of  
16 increased expression post-PSNI in spinal cord vs. naïve rats (Abcam antibody, Mann  
17 Whitney U test,  $p=0.055$ ,  $n=3$ ) [L] Using coloc2 analysis through determination of  
18 Pearson correlation coefficient, there was an increase in the degree of co-  
19 localization between vGLUT1 and SRSF1 immunoreactivity in the spinal cord  
20 following PSNI, compared to naïve (\*\* $p<0.01$  Mann Whitney test,  $n=4$  per group). [M]  
21 SRSF1 was expressed in DRG neurons that were [N] positive for vGLUT1, a marker  
22 of excitatory large diameter DRG neurons. [O] Overlay of vGLUT1 and SRSF1  
23 images. [P & Q] Representative images of SRSF1 stained spinal cord sections used  
24 for analysis, showing the contralateral dorsal horn from [P] a naïve and [Q] PSNI

1 animal. [R & S] The same images of contralateral dorsal horns showing VGLUT1  
2 staining in [R] naïve and [S] PSNI animals (Scale bars = 50 $\mu$ m).

3

4 Figure 4. VEGF<sub>xxx</sub>a isoform expression increases in the spinal cord following PSNI.

5 [A-F] Immunofluorescence of VEGF in the naïve ([A] ipsilateral [B] contralateral),

6 PSNI ([C] ipsilateral [D] contralateral) and PSNI+SRPIN ([E] ipsilateral [F]

7 contralateral) spinal cord (superficial dorsal horn located in top right of images) using

8 the pan-VEGF-A antibody A20. [G-I] Co-localization of pan-VEGF-A with SRSF1 in

9 the dorsal horn of the lumbar spinal cord (high magnification images). [J] Western

10 blot of protein extracted from spinal cords of 6 animals, three naïve and three after

11 PSNI. Pan-VEGF-A but not VEGF-A<sub>165b</sub> increased after PSNI. [K]. Densitometric

12 analysis of the Western blot showed a large increase in pan-VEGF-A expression, no

13 increase in VEGF-A<sub>xxx</sub>b expression and [L] a reduction in the proportion of VEGF-

14 A<sub>xxx</sub>b after PSNI versus naïve animals (one way ANOVA, Sidak post hoc test,

15 \* $p < 0.05$ , ( $F(3,6) = 1.347$ ),  $n = 3$  per group). Scale bars = 50 $\mu$ m.

16

17 Figure 5. Inhibition of SRPK1 activity in the spinal cord prevents neuropathic pain

18 Intrathecal (i.t.) SRPIN340 treatment in rats completely prevented [A] mechanical (F

19 test (2,20) = 3.539), [B] dynamic brush allodynia (F (2,20) = 5.526) and [C] cooling

20 allodynia (F (2, 20) = 7.8) after PSNI ( $n = 9$ , PSNI + vehicle,  $n = 6$ , PSNI + SRPIN340)

21 in the ipsilateral hindpaw. Contralateral hind-paws were not different between groups

22 following mechanical, brush and cooling nociceptive testing. Withdrawal latencies

23 were increased both [D] ipsilaterally (F(2,20) = 25.86) and [E] contralaterally (F(2,

1 20) = 12.72) following i.t. SRPIN340 treatment. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  two  
2 way ANOVA with post-hoc Bonferroni tests).

3

4 Figure 6. PSNI increases and intrathecal SRPIN340 reduces SRSF1 expression in  
5 the spinal dorsal horn.

6 [A-C] SRSF1 immunoreactivity in vGLUT1-positive terminals in the spinal cord after  
7 PSNI. (C shows the co-localization of SRSF1 and vGLUT1). [D-F] Intrathecal 10 $\mu$ M

8 SRPIN340 reduced SRSF1 immunoreactivity in vGLUT1-positive terminals. [F]

9 indicates that there is a loss of expression of SRSF1 but not vGLUT-1. [G]

10 Quantification of SRSF1/vGLUT1 fluorescence intensity by area. PSNI increased

11 SRSF1 staining and SRPIN340 treatment led to a reduction in SRSF1

12 immunostaining within the dorsal horn 2 days after PSNI ( $F(2,9) = 11.16$ , \* $p < 0.05$ ,

13 \*\* $p < 0.01$  one way ANOVA with post-hoc Bonferroni test;  $n = 4$  per group). [H]

14 Intrathecal SRPIN 340 treatment in PSNI injured animals demonstrate a reduction in

15 colocalisation between vGLUT1 and SRSF1 compared to PSNI+vehicle group

16 (\*\* $p < 0.01$ , Mann Whitney test,  $n = 4$  per group).

17

18 Figure 7. Inhibition of SRPK1 in the spinal cord following PSNI leads to reduction in  
19 VEGF-A<sub>xxx</sub>a expression.

20 [A] Immunoblotting for pan-VEGF-A, VEGF-A<sub>xxx</sub>b and tubulin expression in spinal

21 cord from 4 PSNI animals treated with vehicle or SRPIN340. [B] Quantification of

22 intensity showed that the amount of VEGF-A<sub>xxx</sub>b increased slightly, and pan-VEGF-A

23 reduced resulting in [C] a restoration of the VEGF-A<sub>165b</sub> ratio in PSNI towards that in



1 naïve control animals (compare with Fig. 3H, one way ANOVA,  $*p < 0.05$  Sidak test  
2 ( $F(3,6) = 3.529$ )  $n = 3$  per group). [D-E] C-fos immunostaining in spinal cord dorsal horn  
3 in PSNI animals treated with either i.t. vehicle or SRPIN340. [F]. Increased spinal  
4 neuronal activation, indicated by increased numbers of c-fos expressing dorsal horn  
5 neurons after PSNI, was blocked by PSNI+SRPIN340 treatment (one way ANOVA  
6 with post Bonferroni test,  $*** p < 0.001$ , ( $F(2, 9) = 36.50$ ),  $n = 4$  per group for c-fos  
7 expression). [G] SRPK1 was expressed in the lumbar spinal cord in the naïve  
8 animal, and was unchanged post-PSNI ( $n = 3$  per group, NS) Scale bar =  $40\mu\text{m}$ .

9

10 Figure 8. VEGF receptor 2 blockade leads to attenuation of nociceptive pain  
11 behavior in rats.

12 [A] Intraperitoneal injection of 30mg/kg PTK787 led to an increased withdrawal  
13 latency to heat (two way ANOVA with post-hoc Bonferroni test  $n = 5$ /group,  $**p < 0.01$ ,  
14 ( $F(1,20) = 5.388$ ). Intraperitoneal 30mg/kg PTK787 attenuated both [B] time ( $F(11,$   
15  $132) = 13.39$ ) and [C] number ( $F(11, 132) = 4.015$ ) of formalin-induced pain  
16 behaviors within the second phase (two way ANOVA with post-hoc Bonferroni test,  
17  $*p < 0.05$ ,  $**p < 0.01$ ,  $n = 7$ /group). Area under the curve analysis of [D] duration ( $F(1,12)$   
18  $= 5.874$ ) and [E] number ( $F(1,12) = 8.739$ ) for the two phases of nociceptive  
19 behaviors shown in B & C ( $**p < 0.01$ ,  $***p < 0.001$  two way ANOVA with post-hoc  
20 Bonferroni test). [F] Intrathecal injection of 200nM of VEGFR2 antagonist ZM323881  
21 led to an increase in EMG response threshold only to A-nociceptor stimulation  
22 versus baseline and vehicle groups ( $**p < 0.01$ ; two way ANOVA with post Bonferroni)  
23 ( $n = 3-5$ /group).

24

1 Figure 9. Alteration of spinal VEGFR activation attenuates nociceptive behavior in  
2 naïve mice and rats

3 [A] Intrathecal administration of 200nM PTK787 increased mechanical withdrawal  
4 thresholds ( $F(1,10) = 12.47$ ) and [B] increased withdrawal latency to heat in mice  
5 ( $F(1, 12) = 8.165$ ,  $n=4$ /group vehicle, (8 hind paws used as replicates),  $n=3$ /group  
6 PTK787, (6 hind paws used as replicates),  $**p<0.01$  two-way ANOVA with post-hoc  
7 Bonferroni test). [C] Intrathecal VEGF-A<sub>165a</sub> reduced mechanical thresholds ( $F(1,12)$   
8  $= 17.18$ ) and [D] heat ( $F(1,12) = 18.61$ ) withdrawal latencies in mice ( $n=4$ /group (8  
9 hind paws used as replicates). [E] Intrathecal VEGF-A<sub>165b</sub> increased mechanical  
10 thresholds ( $F(1,12) = 25.26$ ) and [F] thermal ( $F(1,16) = 5.631$ ) response latencies in  
11 mice ( $n=4$  vehicle group (8 hind paws used as replicates),  $n=5$  VEGF group, (10 hind  
12 paws used as replicates)). [G] Treatment of rats with a VEGF-A<sub>165b</sub> neutralizing  
13 antibody decreased both mechanical thresholds ( $F(1, 15) = 18.66$ ) and [H] thermal  
14 latencies ( $F(1,15) = 1.400$ ,  $n=3$  group (6 hind paws used as replicates), two way  
15 ANOVA with post-hoc Bonferroni test,  $***p<0.001$ ).

16

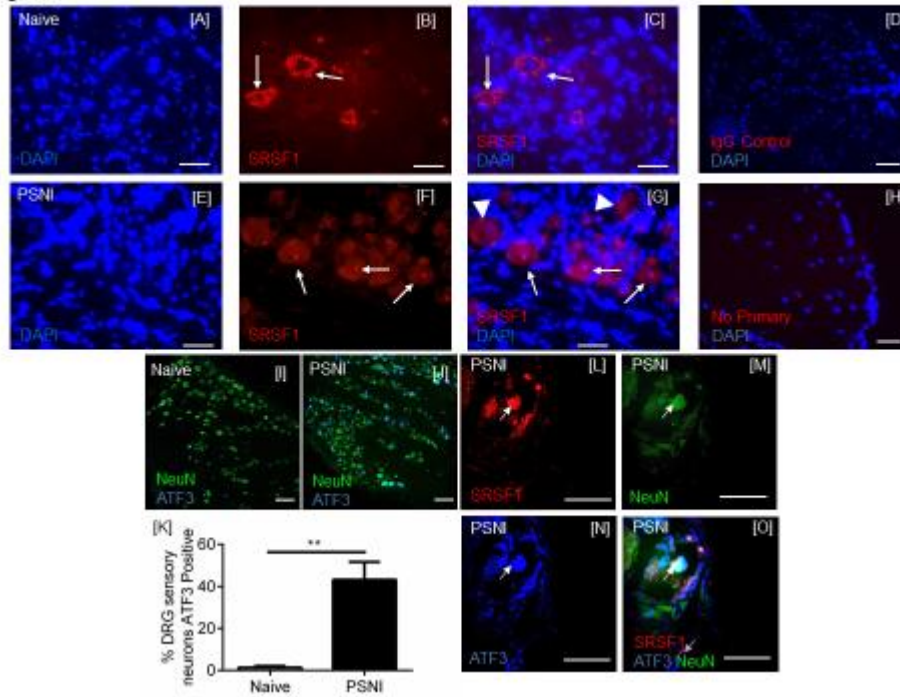
17 Figure 10. Attenuation of VEGFR2 signaling leads to alleviation of neuropathic pain  
18 in rats

19 Intrathecal application of VEGF-A<sub>165b</sub> two days after PSNI surgery abolished [A]  
20 mechanical ( $F(2, 10) = 32.39$ ) and [B] cooling ( $F(2, 20) = 14.03$ ) allodynia ( $n=6$  per  
21 group), and increased withdrawal latencies to heat in both [C] ipsilateral ( $F(2,20) =$   
22  $4.201$ ) and [D] contralateral hind paws ( $F(2,10) = 3.476$ , two way ANOVA with post-  
23 hoc Bonferroni test,  $*p<0.05$ ,  $**p<0.01$ ,  $***p<0.001$ ,  $n=6$  per group). Contralateral  
24 hind-paws from both groups did not differ in nociceptive behavioral response to [A]

1 mechanical and [B] cooling stimulation. IP 30mg/kg PTK787 led to increased  
2 withdrawal latencies to heat in the [E] ipsilateral ( $F(2,12)=2.45$ ) and [F] contralateral  
3 limb ( $F(2,12)=1.38$ ) (two way ANOVA with post-hoc Bonferroni test,  $**p<0.01$ ,  $n=4$   
4 per group).

5

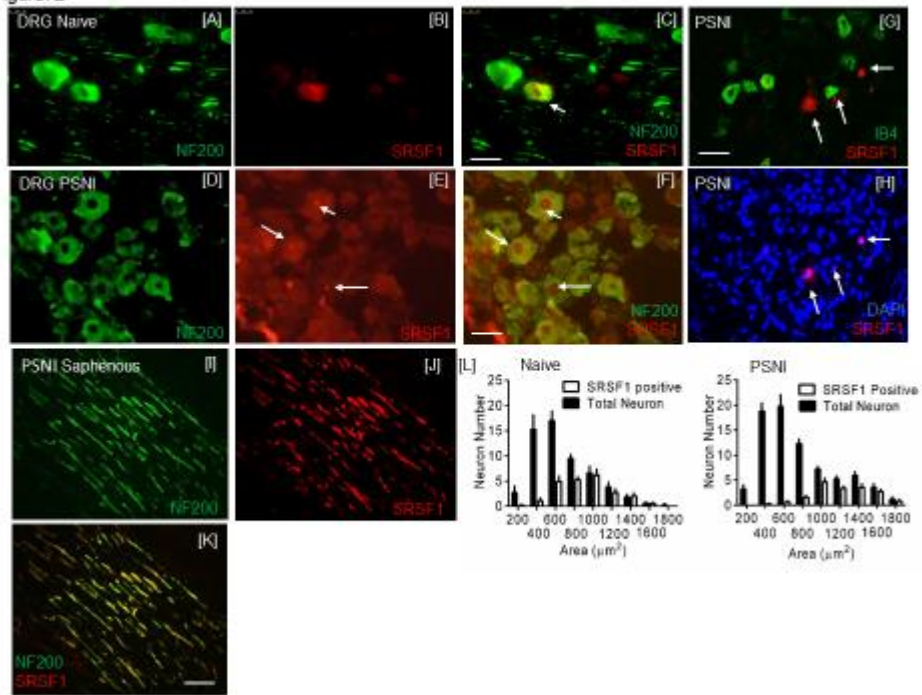
Figure 1



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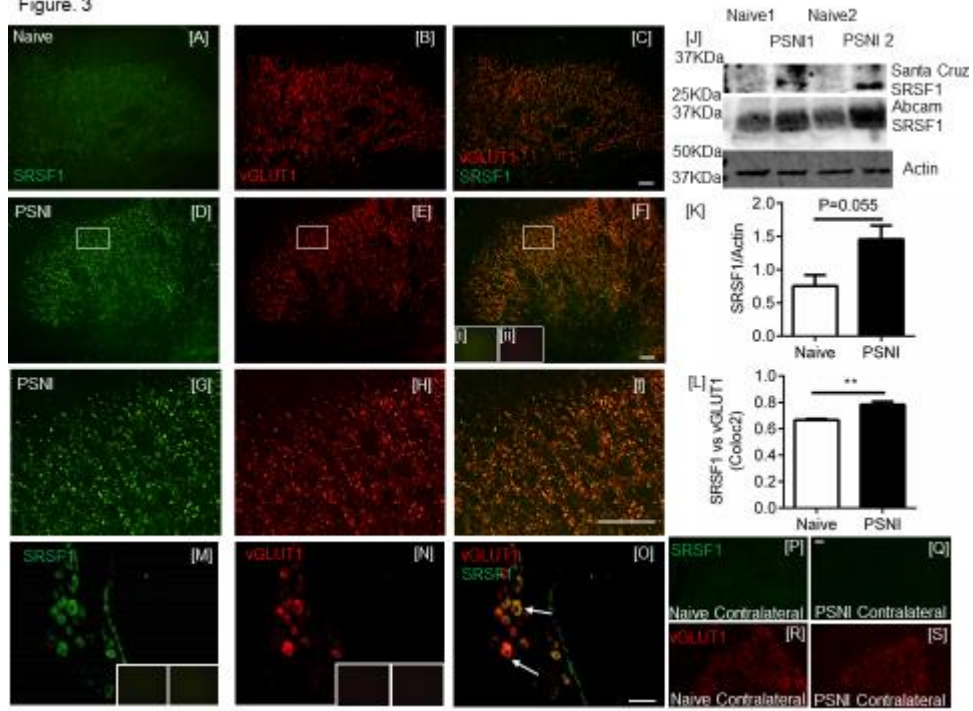
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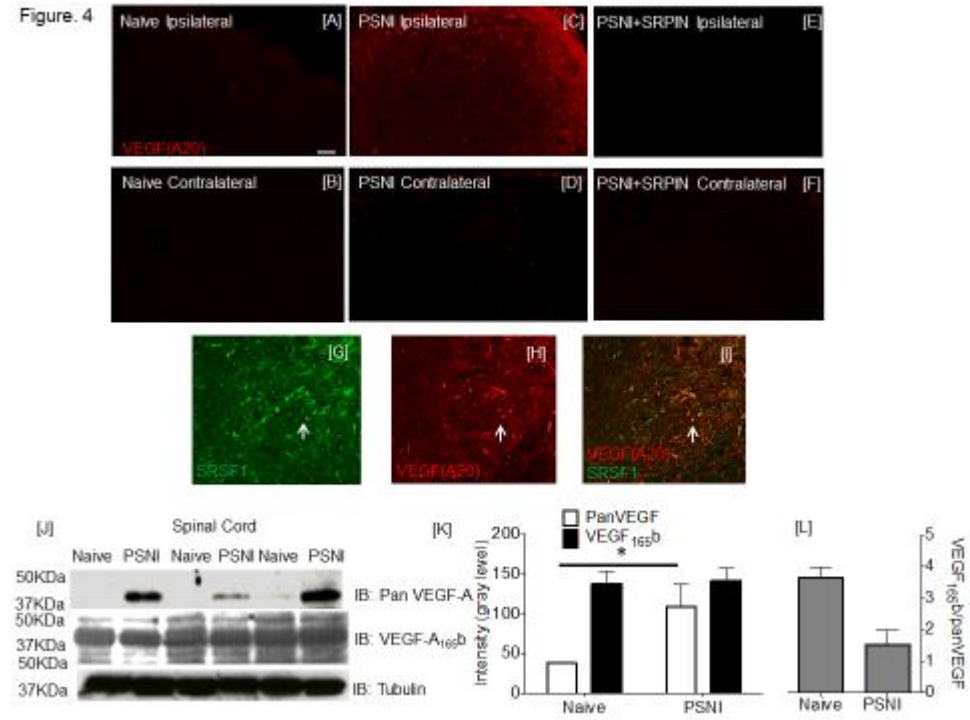
Figure 3



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Figure 4

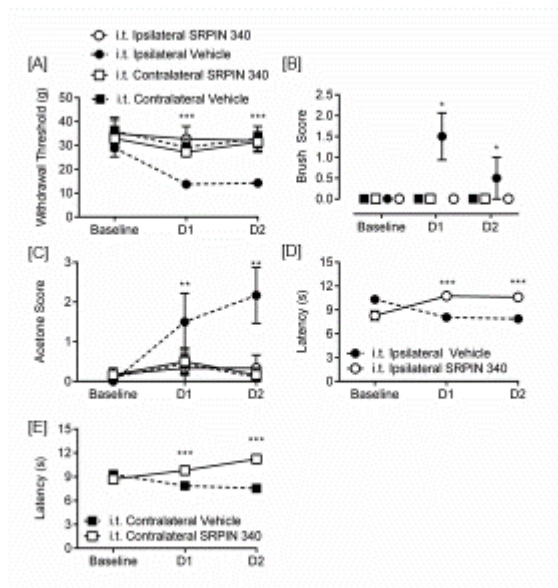


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Figure 5

PSNI

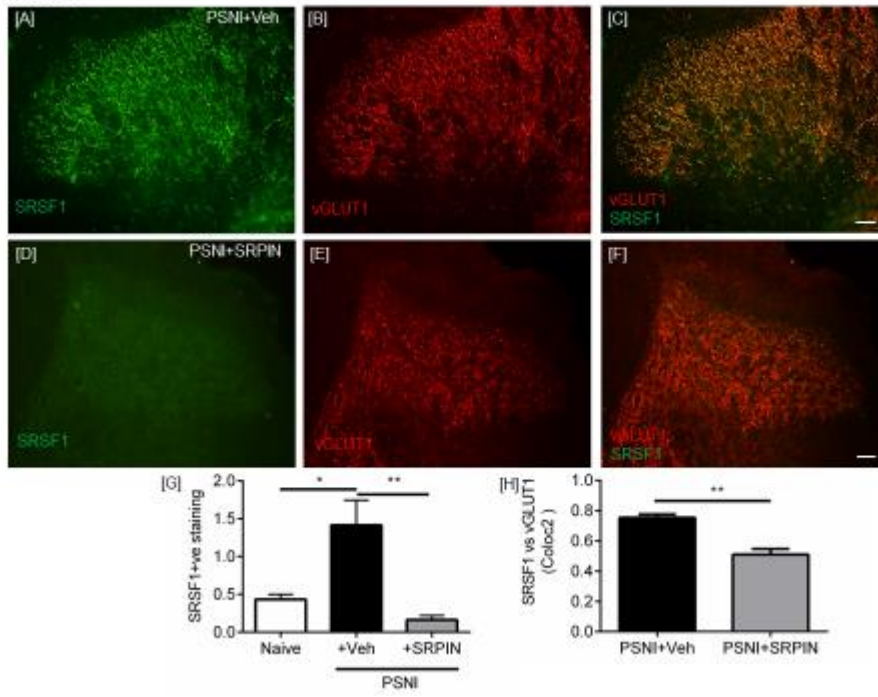


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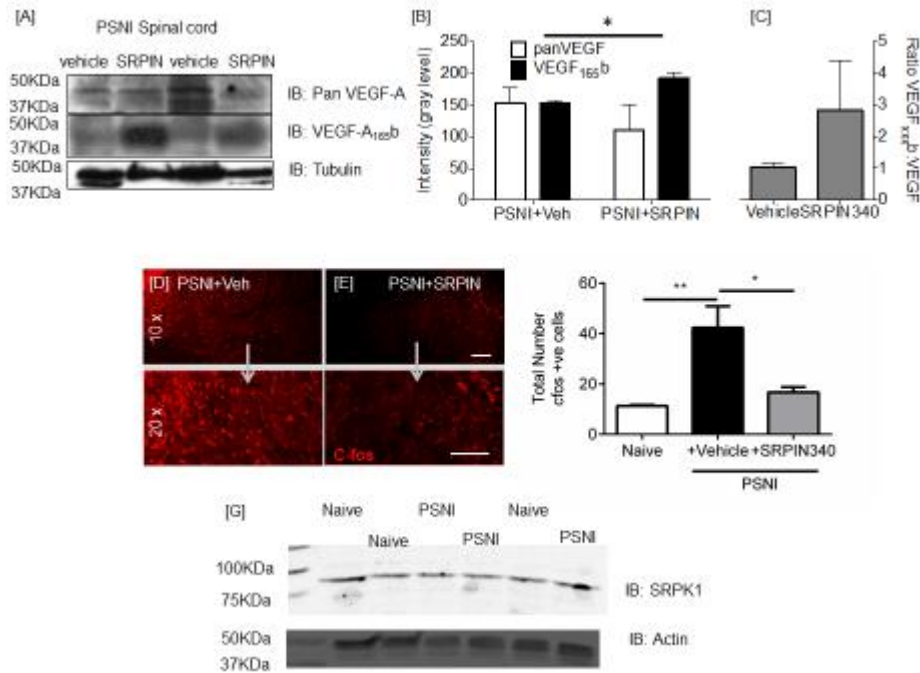
Figure 6



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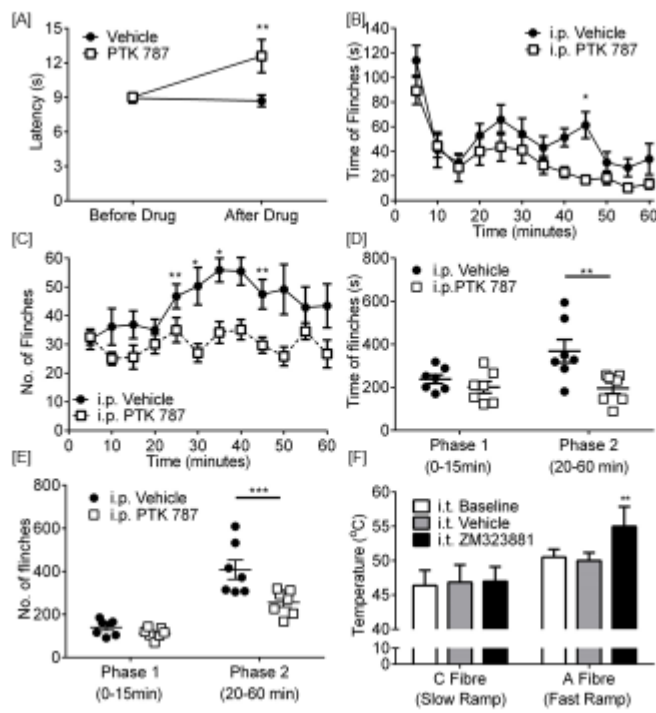
Figure 7



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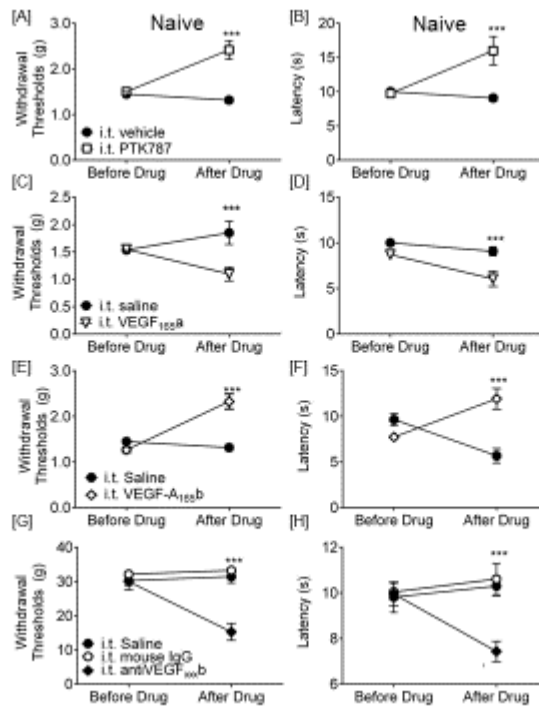
Figure 8



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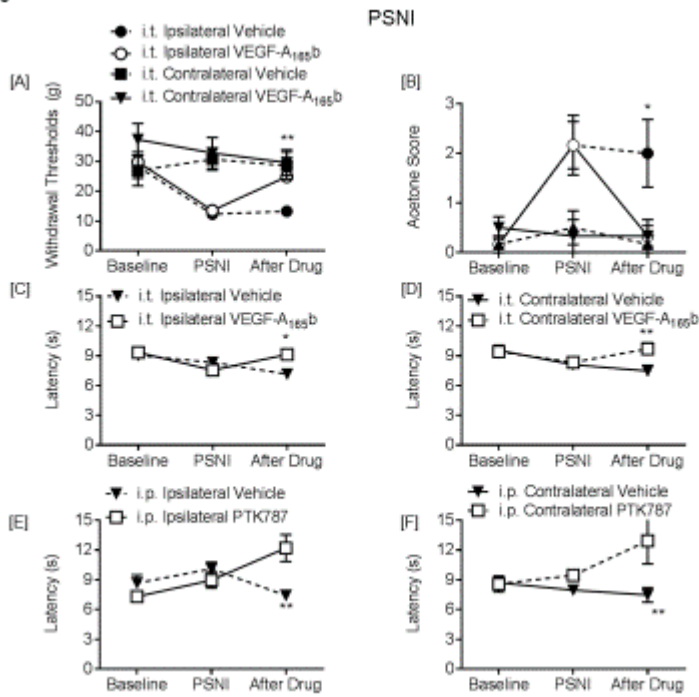
Figure 9



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Figure 10



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