



# SURI, newly expressed in astrocytes, mediates neuropathic pain in a mouse model of peripheral nerve injury

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## Abstract

**Background:** Neuropathic pain following peripheral nerve injury (PNI) is linked to neuroinflammation in the spinal cord marked by astrocyte activation and upregulation of interleukin 6 (IL-6), chemokine (C-C motif) ligand 2 (CCL2) and chemokine (C-X-C motif) ligand 1 (CXCL1), with inhibition of each individually being beneficial in pain models.

**Methods:** Wild type (WT) mice and mice with global or *pGfap-cre*- or *pGFAP-cre/ERT2*-driven *Abcc8/SURI* deletion or global *Trpm4* deletion underwent unilateral sciatic nerve cuffing. WT mice received prophylactic (starting on post-operative day [pod]-0) or therapeutic (starting on pod-21) administration of the SURI antagonist, glibenclamide (10 µg IP) daily. We measured mechanical and thermal sensitivity using von Frey filaments and an automated Hargreaves method. Spinal cord tissues were evaluated for SURI-TRPM4, IL-6, CCL2 and CXCL1.

**Results:** Sciatic nerve cuffing in WT mice resulted in pain behaviors (mechanical allodynia, thermal hyperalgesia) and newly upregulated SURI-TRPM4 in dorsal horn astrocytes. Global and *pGfap-cre*-driven *Abcc8* deletion and global *Trpm4* deletion prevented development of pain behaviors. In mice with *Abcc8* deletion regulated by *pGFAP-cre/ERT2*, after pain behaviors were established, delayed silencing of *Abcc8* by tamoxifen resulted in gradual improvement over the next 14 days. After PNI, leakage of the blood-spinal barrier allowed entry of glibenclamide into the affected dorsal horn. Daily repeated administration of glibenclamide, both prophylactically and after allodynia was established, prevented or reduced allodynia. The salutary effects of glibenclamide on pain behaviors correlated with reduced expression of IL-6, CCL2 and CXCL1 by dorsal horn astrocytes.

**Conclusion:** SURI-TRPM4 may represent a novel non-addicting target for neuropathic pain.

## Keywords

Neuropathic pain, astrocyte, sulfonylurea receptor 1 (SURI), SURI-TRPM4, glibenclamide, IL-6, CCL2 or CXCL1

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## Introduction

Peripheral nerve injuries (PNI) are a major source of disability worldwide, causing significant sensorimotor impairment and often leading to neuropathic pain (also called neurogenic pain).<sup>1,2</sup> Non-traumatic causes of PNI are numerous, including metabolic disorders such as diabetes, infections such as herpes zoster and HIV (human immunodeficiency virus), nutritional deficiencies, malignancies and others. Traumatic PNIs may result from motor vehicle accidents, penetrating trauma,

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falls, work-related and iatrogenic injuries. In warfighting, a majority of battlefield wounds involve injuries to exposed limbs, often resulting in injuries to the ulnar, common peroneal or tibial nerve (n.) that results in the development of neuropathic pain.<sup>3,4</sup>

Neuropathic pain is caused by lesions to the somatosensory nervous system that alter its structure and function so that pain occurs spontaneously, and responses to both noxious and innocuous stimuli are pathologically amplified.<sup>5</sup> Both peripheral and central inflammation play critical roles in neuropathic pain after PNI. Neuroinflammation within the spinal cord is characterized by glial activation, blood-spinal barrier (BSB) leakage and infiltration of leukocytes.<sup>6-9</sup> Neuroinflammation within the spinal cord is required for the initiation and maintenance of pain hypersensitivity following PNI.<sup>9-11</sup> Microglia, astrocytes and oligodendrocytes modulate CNS inflammation triggered by PNI. Microglia are the initial responders, whereas astrocytes become involved later, during the chronic phase.<sup>12,13</sup>

Sulfonylurea receptor 1 (SUR1), which is encoded by the *Abcc8* gene, is a regulatory subunit that co-assembles with the inward rectifier potassium-selective channel, Kir, to form  $K_{ATP}$  channels.<sup>14</sup> Various channel subunits (SUR1, SUR2, Kir6.1 and Kir6.2) are expressed in primary afferent neurons of dorsal root ganglia (DRG),<sup>15,16</sup> in paranodal sites of nodes of Ranvier and in the Schmidt-Lanterman incisures of Schwann cells.<sup>16</sup> In DRG neurons, opening  $K_{ATP}$  channels reduces excitability whereas inhibiting  $K_{ATP}$  increases excitability and may amplify sensory signaling.<sup>15</sup> Consistent with a role for  $K_{ATP}$  in DRG neurons, intrathecal injection of *Abcc8* shRNA (AAV9-GFP-U6-m-ABCC8-shRNA) in uninjured mice results in the appearance of GFP (green fluorescent protein) and in reduced expression of *Abcc8* mRNA predominantly within the DRG, accompanied by a decrease in mechanical paw-withdrawal latencies.<sup>17</sup> In studies on neuropathic pain induced by nerve injury, the SUR1 antagonist, glibenclamide, has been shown to blunt the effect of numerous analgesics that signal via the L-arginine/nitric oxide/cyclic GMP/ $K_{ATP}$  channel pathway.<sup>18-28</sup> In the same studies, however, in the absence of analgesic, SUR1 inhibition by itself with a one-time dose of glibenclamide had no effect on pain thresholds.

Apart from  $K_{ATP}$ , SUR1 also co-assembles with the non-selective cation channel, transient receptor potential melastatin 4 (TRPM4) to form SUR1-TRPM4 channels, which are also blocked by the SUR1 inhibitor, glibenclamide.<sup>29</sup> Recent work has implicated SUR1-TRPM4 in neuroinflammatory responses in the central nervous system, including brain<sup>30-34</sup> and spinal cord.<sup>35,36</sup> In these reports, neuroinflammation and its neurofunctional phenotypes were ameliorated by global deletion of

*Abcc8*/SUR1 (a.k.a., *Abcc8*<sup>-/-</sup>) or by repeated daily administration of glibenclamide over the course of many days.

Based on the foregoing, it is possible that following PNI, inhibiting *Abcc8*/SUR1 could inhibit spinal cord neuroinflammation by acting via SUR1-TRPM4, thereby ameliorating neuropathic pain. However, it is also conceivable that inhibiting *Abcc8*/SUR1 could amplify sensory signaling by acting via  $K_{ATP}$  in the DRG, thereby exacerbating neuropathic pain. Here, we used a murine model of sustained neuropathic pain induced by sciatic n. cuffing<sup>37,38</sup> to examine the effects of global, *pGfap*-cre- and *pGFAP*-cre/ERT2-driven *Abcc8*/SUR1 deletion, global *Trpm4* deletion, as well as pharmacological inhibition of SUR1 by repeated daily administration of glibenclamide, on pain behaviors and neuroinflammation in the spinal cord.

## Methods

### Ethics statement

The article was written in accordance with ARRIVE guidelines.<sup>39</sup> We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore, and in accordance with the relevant guidelines and regulations as stipulated in the National Research Council Publication, "Guide for the Care and Use of Laboratory Animals", NIH Publication 86-23. All efforts were made to minimize the number of animals used and their suffering.

### Subjects

Adult male C57BL/6 mice, 22–25 gm, were obtained from Envigo (Indianapolis, IN). Global *Abcc8* knock-out (a.k.a., *Abcc8*<sup>-/-</sup>) mice and *Abcc8*-floxed mice were generated, validated and gifted by Dr. Joseph Bryan and colleagues.<sup>40,41</sup> Global *Trpm4* knock-out (a.k.a., *Trpm4*<sup>-/-</sup>) mice were generated, validated and gifted by Prof. Dr. Marc Freichel and Prof. Dr. Veit Flockerzi.<sup>42</sup> *Gfap*-cre mice (B6.Cg-Tg(*Gfap*-cre)73.12Mvs/J; stock #012886) and *GFAP*-cre/ERT2 mice (B6.Cg-Tg(*GFAP*-cre/ERT2)505Fmv/J; stock #012849), in which Cre recombinase is under the control of the murine *Gfap* or human *GFAP* promoter, either constitutive or conditional, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice with constitutive deletion of *Abcc8* driven by the *Gfap* promoter, i.e., *Abcc8*<sup>fl/fl</sup>;+*Gfap*-cre (henceforth, *pGfap*-cre-driven *Abcc8* deletion) and littermate controls

(*Abcc8*<sup>fl/fl</sup>; -*Gfap*-cre) were obtained by crossing homozygous *Abcc8*-floxed mice with transgenic *Gfap*-cre mice. Mice with conditional deletion of *Abcc8* driven by the *GFAP* promoter, i.e., *Abcc8*<sup>fl/fl</sup>; +*GFAP*-cre/ERT2 (henceforth, *pGFAP*-cre/ERT2-driven *Abcc8* deletion) and littermate controls (*Abcc8*<sup>fl/fl</sup>; -*GFAP*-cre/ERT2) were obtained by crossing homozygous *Abcc8*-floxed mice with transgenic *GFAP*-cre/ERT2 mice.

Genotyping of mice used for breeding and experiments was performed by Transnetyx (Cordova, TN). Mice were given free access to food and water, except during neurofunctional testing. They were housed in plastic cages in specially constructed rooms with controlled humidity, exchange of air and controlled lighting (12/12 hour light/dark cycle). Male mice were used for all experiments.

### Surgery

Mice were anesthetized (100 mg/kg ketamine plus 10 mg/kg xylazine, intraperitoneal [IP]) and breathed room air spontaneously. Core temperature was maintained at 37°C using a heating pad (Deltaphase® Isothermal Pad, Braintree Scientific, Braintree, MA, USA). Hair was clipped from the right proximal lateral thigh, and the surgical site was prepared using iodine and alcohol. A sterile environment was maintained throughout the procedure. Lidocaine solution (2%) was injected subcutaneously prior to making an incision.

The procedure for sciatic n. cuffing was as previously described,<sup>37,38</sup> with only minor modification, as we described.<sup>43</sup> In this model, mechanical allodynia is sustained unabated for at least 9 weeks.<sup>43</sup> Using a surgical microscope, the common branch of the right sciatic n. was exposed by separating the muscles and the nerve by blunt dissection. After isolation, the nerve was gently stretched for 15 minutes by placing a 5-mm diameter plastic rod beneath it, which caused the nerve to blanch. A 2-mm long section of PE-20 tubing, pre-split and sterilized, was placed around the nerve. Animals in the sham group received surgery identical to that described but without nerve manipulation or cuffing. After the surgical procedure, mice were nursed on a heating pad to maintain body temperature ~37°C until they emerged from anesthesia. The day of surgery is called post-op day 0 (pod-0).

No mice became infected, required early euthanasia or died. No mice in the control groups failed to develop stable mechanical allodynia. There were no exclusions.

### Glibenclamide treatment

A stock solution of glibenclamide (#G2539; meets USP testing; Sigma-Aldrich) of 2.5 mg/mL in dimethyl sulfoxide was prepared. We diluted 40 µL of the stock solution

into 960 µL phosphate buffered saline (PBS); mice received 100 µL of the final solution (10 µg glibenclamide) or an equal volume of vehicle IP daily until the end of the experiment. IP injections were performed using a 27-gauge needle with the depth of the injection limited to 3 mm by a sleeve of PE20 tubing placed over the shaft of the needle.

### Sample size calculation

For mechanical allodynia, we based our sample size calculation on a previous study that used the same model of neuropathic pain but tested a different drug. Values derived from Figure 2 of Yalcin et al.<sup>38</sup> suggested an effect size (Cohen's *d*) of  $\approx 2$ , where  $d = (M_1 - M_2) / SD_{\text{pooled}}$ ,  $M_1$  and  $M_2$  are the means, and  $SD_{\text{pooled}} = [(SD_1 + SD_2) / 2]^{1/2}$ . Using the following assumptions: two-tailed hypothesis,  $\alpha$ , 0.05; desired power, 80%;  $d$ , 2.1, sample size calculation indicated a minimum sample size of 5 mice per group. This group size is similar to other reports using this model.<sup>37,43</sup>

### Experimental series

In *series 1*, 8 WT mice underwent sciatic n. cuffing and, on pod-3, pod-7 and pod-14, 2–3 mice per time point were euthanized to examine SUR1 expression in the spinal cord; 3 uninjured sham mice were used as controls. In *series 2*, 5 mice with global deletion of *Abcc8* and 5 WT littermate controls underwent sciatic n. cuffing and were assessed for pain behaviors until pod-14. In *series 3*, 10 mice with *pGfap*-cre-driven *Abcc8* deletion and 10 littermate controls underwent sciatic n. cuffing and were assessed for pain behaviors until pod-14; tissues from these mice were used for immunohistochemistry and PLA for SUR1-TRPM4. In *series 4*, 5 mice with *pGFAP*-cre/ERT2-driven *Abcc8* deletion and 5 littermate controls underwent sciatic n. cuffing, were assessed for pain behaviors until pod-35, and on pod-14–18, were administered tamoxifen in corn oil (75 mg/kg; 100 µL IP). In *series 5*, 7 mice with global deletion of *Trpm4* and 7 WT littermate controls underwent sciatic n. cuffing and were assessed for pain behaviors until pod-14. In *series 6* (prophylactic treatment), 20 WT mice underwent sciatic n. cuffing and were randomly divided into two groups, one receiving vehicle, the other, glibenclamide. Treatments were administered daily, beginning on pod-0, and pain behaviors were assessed up to pod-14. Tissues from these mice were used for immunohistochemical assessment of neuroinflammation in the affected dorsal horn. In *series 7* (therapeutic treatment), 10 WT mice underwent sciatic n. cuffing and were randomly divided into two groups, one receiving vehicle, the other, glibenclamide. Treatments were administered daily, beginning on pod-21, after pain behaviors had

been stably established. Pain behaviors were assessed up to pod-45.

In all cases, mice were handled 2–3× weekly for acclimatization to handlers, and the non-injured contralateral hind paw served as the control.<sup>44,45</sup> In *series 3–7*, outcomes were assessed by investigators blinded to treatment group; in *series 2*, blinding was not possible because *Abcc8*<sup>-/-</sup> mice are white and littermate WT controls are black.

### Mechanical sensitivity

Mechanical sensitivity was assessed using von Frey filaments.<sup>44,45</sup> Mice were placed in elevated Perspex cages with a wire mesh floor (15 × 10 × 10 cm) (ITCC Life Science, Woodland Hill, CA, USA) and were acclimatized for 30 minutes prior to testing. The paw withdrawal threshold to mechanical stimulation of both the ipsilateral and contralateral hind paws was measured using a series of von Frey filaments that exerted forces ranging from 0.16–4 g (North Coast Medical, San Jose, CA, USA). The von Frey filaments were pressed perpendicularly onto the plantar surface of the hind paw for 2 seconds (five times for each filament), and a positive response was noted if there was a sharp flinching or licking of the hind paw. To determine the withdrawal threshold, we used the “ascending” method, to preclude potential aversive behavior associated with the “up-down” method.<sup>45</sup> The Kolmogorov-Smirnow test for normality showed that, at the  $P=0.05$  level, the data on mechanical sensitivity were drawn from a normally distributed population.

### Thermal sensitivity

Thermal sensitivity was assessed using the Hargreaves method<sup>44,45</sup> and a Hargreaves-type apparatus (Ugo Basile Thermal Plantar Test Instrument, Stoelting Co., Wood Dale, IL) that uses a fiber optic sensor to record time latency automatically. Unrestrained mice were placed in a Perspex enclosure atop of a glass pane and were acclimatized for 30 minutes prior to testing. An infrared generator placed below the glass pane was aimed at the plantar surface of the hind paw and the time to withdrawal was recorded automatically by the fiber optic sensor. Paw-withdrawal latency was calculated as the mean of 3–5 different measurements for each of three trials taken at 15-minute intervals.

### Antibody validation

The custom anti-SUR1 and anti-TRPM4 antibodies used for immunolabeling were validated previously using lysates from appropriate SUR1 and TRPM4 expression systems, tissues from wild-type, *Abcc8*<sup>-/-</sup> and *Trpm4*<sup>-/-</sup> mice, and using mass spectrometry of

immunoisolated proteins,<sup>29</sup> in accordance with recommendations for antibody validation by Uhlen et al.<sup>46</sup> In a previous publication, we further validated the specificity of the custom anti-SUR1 antibody for immunohistochemistry by comparing immunolabeling of adjacent sections using our custom polyclonal antibody, a monoclonal antibody (S289-16; Novus Biologicals, Littleton, CO) and a commercial polyclonal antibody (sc-5789; Santa Cruz Biotechnology, Dallas, TX).<sup>47</sup>

### Immunohistochemistry, quantification of specific labeling

Under deep anesthesia, mice were euthanized, underwent trans-cardiac perfusion with normal saline (NS) (15 mL) followed by 10% neutral buffered formalin (15 mL). Spinal cord tissues at spinal segments L1–L5 were harvested and post-fixed. Tissues were cryoprotected with 30% sucrose, frozen in OCT and cryosectioned (10 μm).

Immunohistochemistry was performed as described in a non-blinded manner.<sup>35,36</sup> For SUR1 and TRPM4, sections were first processed for antigen retrieval in Epitope Retrieval Solution (cat# IW-1100; IHCWORLD, Woodstock, MD) using an Epitope Retrieval Steamer (cat# IW-1102; IHCWORLD) for 10–15 min, followed by cooling for 30 min, then washing in distilled H<sub>2</sub>O. For all immunolabelings, sections were incubated at 4 °C overnight with primary antibodies, including: goat anti-SUR1 (1:600; custom); chicken anti-TRPM4 (1:600; custom); goat anti-TRPM4 (1:200; G-20, cat# 27540; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-GFAP (1:300; conjugated Alexa Fluor 488, cat#53-9892-82; Invitrogen, Carlsbad, CA); rabbit anti-Iba1 (1:200; cat#019-19741; Wako, Osaka, Japan); rabbit anti-MBP (1:500; Ab40390; Abcam, Cambridge, UK); goat anti-IL-6 (1:100; cat# SC7920[H-183]; Santa Cruz Biotechnology); rabbit anti-CCL2 (1:200; cat# ab9669; Abcam); rabbit anti-CXCL1 (1:100; cat# PA1760; Boster, Pleasanton, CA). After several rinses in PBS, sections were incubated with species-appropriate fluorescent secondary antibodies (Alexa Fluor 488 and 555, Molecular Probes, Invitrogen) for 1 hour at room temperature. Controls for immunohistochemistry included the omission of primary antibodies.

Unbiased measurements of specific labeling within regions of interest (ROI) were obtained using NIS-Elements AR software (Nikon Instruments, Melville, NY) from sections (one section per mouse) immunolabeled as a single batch. All images for a given signal were captured using uniform parameters of magnification, area, exposure and gain. Segmentation analysis was performed by computing a histogram of pixel intensity for a particular ROI, and pixels were classified as having specific labeling based on signal intensity greater than

2× that of background. ROIs in coronal sections of the dorsal horn were defined by pixels with specific labeling for GFAP or Iba1 or MBP. Cell specific expression of the protein of interest within these ROIs was then determined as the fraction of pixels with specific labeling for the protein of interest (% ROI).

**Proximity ligation assay (PLA)** was used to detect SUR1-TRPM4 heteromers. Following antigen retrieval, as above, PLA was performed according to the manufacturer's protocol using the Duolink (Sigma Aldrich) assay, including in situ PLA probe anti-goat plus (DUO92003), in situ PLA probe anti-rabbit minus (DUO92005), in situ wash buffer, fluorescent (DUO82049) and fluorescent detection reagent (orange) (DUO92007). Controls for PLA included the omission of primary antibodies.

### Quantitative polymerase chain reaction

Tissues from the ipsilateral and contralateral dorsal quadrant of the spinal cord at L3–4 were processed using methods and primers as we described previously.<sup>32</sup>

### Statistics

Nominal data are presented as mean ± SE. Nominal data were analyzed using a t-test, ANOVA or repeated measures ANOVA, as appropriate, with post-hoc Fisher correction. The Kolmogorov-Smirnow test was used to assess normality of the data on mechanical sensitivity. Statistical tests were performed using Origin Pro (V8; OriginLab, North Hampton, MA). Significance was assumed if  $P < 0.05$ .

## Results

### PNI upregulates SUR1-TRPM4 in dorsal horn astrocytes of the spinal cord

WT mice underwent unilateral sciatic n. cuffing, a highly reproducible model of neuropathic pain.<sup>37,38</sup> On pod-3–14 after sciatic n. cuffing, spinal cord tissues from the dorsal horns at L3–4, which innervate the plantar surface of the hindpaw,<sup>48</sup> were harvested to assess the expression of SUR1 and TRPM4 in various cell types.

SUR1 was minimal in the dorsal horns of normal WT controls and in the contralateral dorsal horns of mice with sciatic n. cuffing but was prominent in the dorsal horn ipsilateral to the PNI (Figure 1(a) and (b)), consistent with a prior report.<sup>49</sup> Quantitative analysis of SUR1 immunopositivity confirmed a progressive increase in the ipsilateral dorsal horn over the course of 14 days (Figure 1(c)). Quantitative polymerase chain reaction (qPCR) confirmed upregulation of *Abcc8* mRNA in ipsilateral dorsal horn tissues (Figure 1(d)).

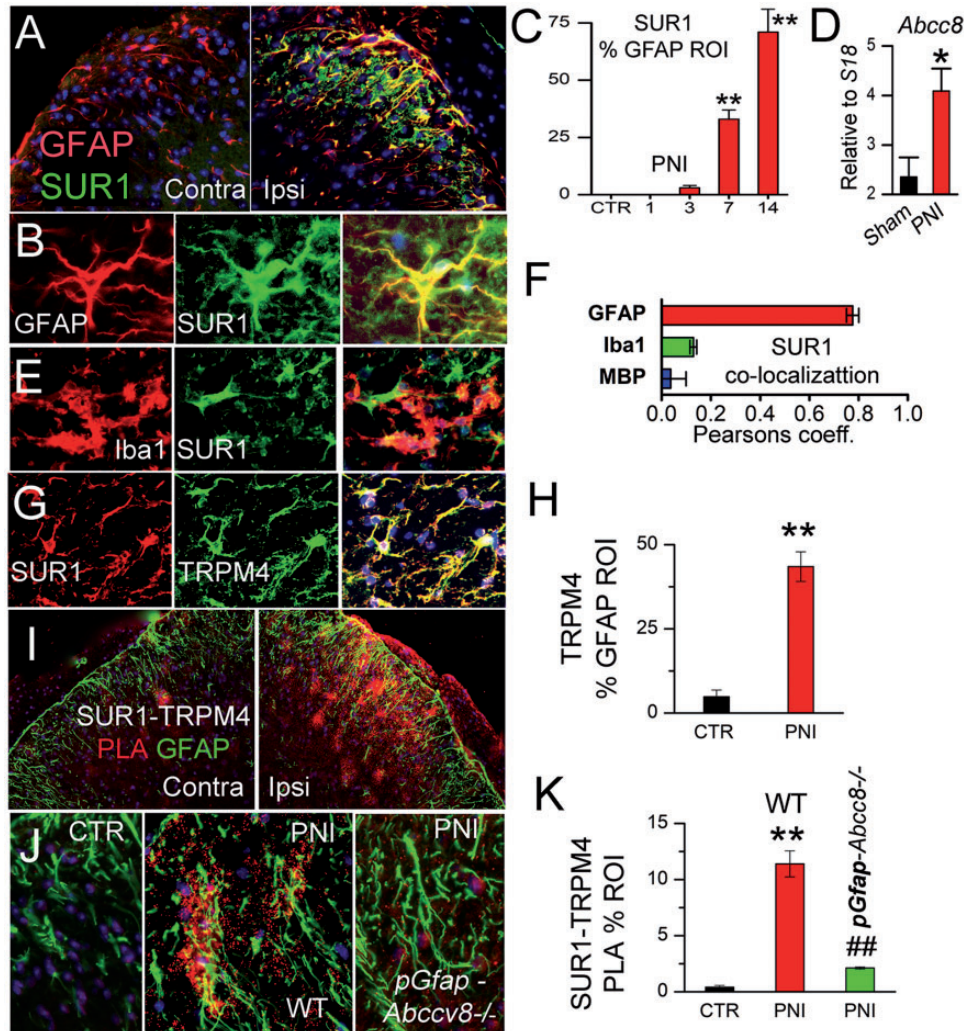
SUR1 expression was localized predominantly to cells with a stellate morphology that co-labeled for glial fibrillary acidic protein (GFAP), consistent with astrocytes (Figure 1(a) and (b)). Minimal SUR1 was observed in microglia/macrophages that co-labeled for ionized calcium binding adaptor molecule 1 (Iba1) (Figure 1(e)). Quantitative analysis of double immunolabeled sections revealed a high degree of co-localization of SUR1 with GFAP (Pearson's correlation coefficient [PCC], 0.80), minimal co-localization of SUR1 with Iba1 (PCC, 0.12) and minimal co-localization of SUR1 with oligodendrocyte myelin basic protein (MBP) (PCC, 0.03) (Figure 1(f)), consistent with most of the newly upregulated SUR1 being localized to astrocytes.

SUR1 can assemble with TRPM4 to form SUR1-TRPM4 channels.<sup>29</sup> As with SUR1, TRPM4 was minimal in the dorsal horns of normal WT controls and in the contralateral dorsal horns of mice with sciatic n. cuffing but was prominent in the dorsal horn ipsilateral to the PNI (Figure 1(g)). Quantitative analysis of SUR1 immunopositivity confirmed a significant increase in the ipsilateral dorsal horn at 14 days (Figure 1(h)). Double immunolabeling for SUR1 and TRPM4 showed prominent co-localization of the two channel subunits in cells with a stellate morphology, consistent with astrocytes (Figure 1(g)).<sup>29</sup> Proximity ligation assay confirmed co-assembly of SUR1-TRPM4 heteromers in the ipsilateral dorsal horn (Figure 1(i) to (k)).

### Deletion of *Abcc8/SUR1*

Uninjured mice with global deletion of *Abcc8/SUR1* were previously reported to have a modest reduction (~25%) in the withdrawal threshold to mechanical, but not thermal, stimulation.<sup>17</sup> However, this genotype has not been studied after PNI. Here, mice with global deletion of *Abcc8/SUR1* and littermate WT controls underwent sciatic n. cuffing, which induces pain behaviors including mechanical allodynia and thermal hyperalgesia.<sup>37,38</sup> Von Frey filaments were used to assess ipsilateral and contralateral hindpaw withdrawal thresholds during the two weeks after PNI. In controls, sciatic n. cuffing gave rise to mechanical allodynia involving the ipsilateral hindpaw that developed over the course of 7 days and persisted on testing at pod-14 (Figure 2(a)). In mice with global deletion of *Abcc8/SUR1*, allodynia of the ipsilateral hindpaw to mechanical stimuli failed to develop, and withdrawal thresholds were not different from those of the contralateral uninjured hindpaw (Figure 2(a)).

The same mice were tested for thermal sensitivity using an automated Hargreaves apparatus. Compared to the contralateral hindpaw, the ipsilateral hindpaw of littermate controls exhibited significant thermal hyperalgesia (Figure 2(b)). By contrast, mice with global

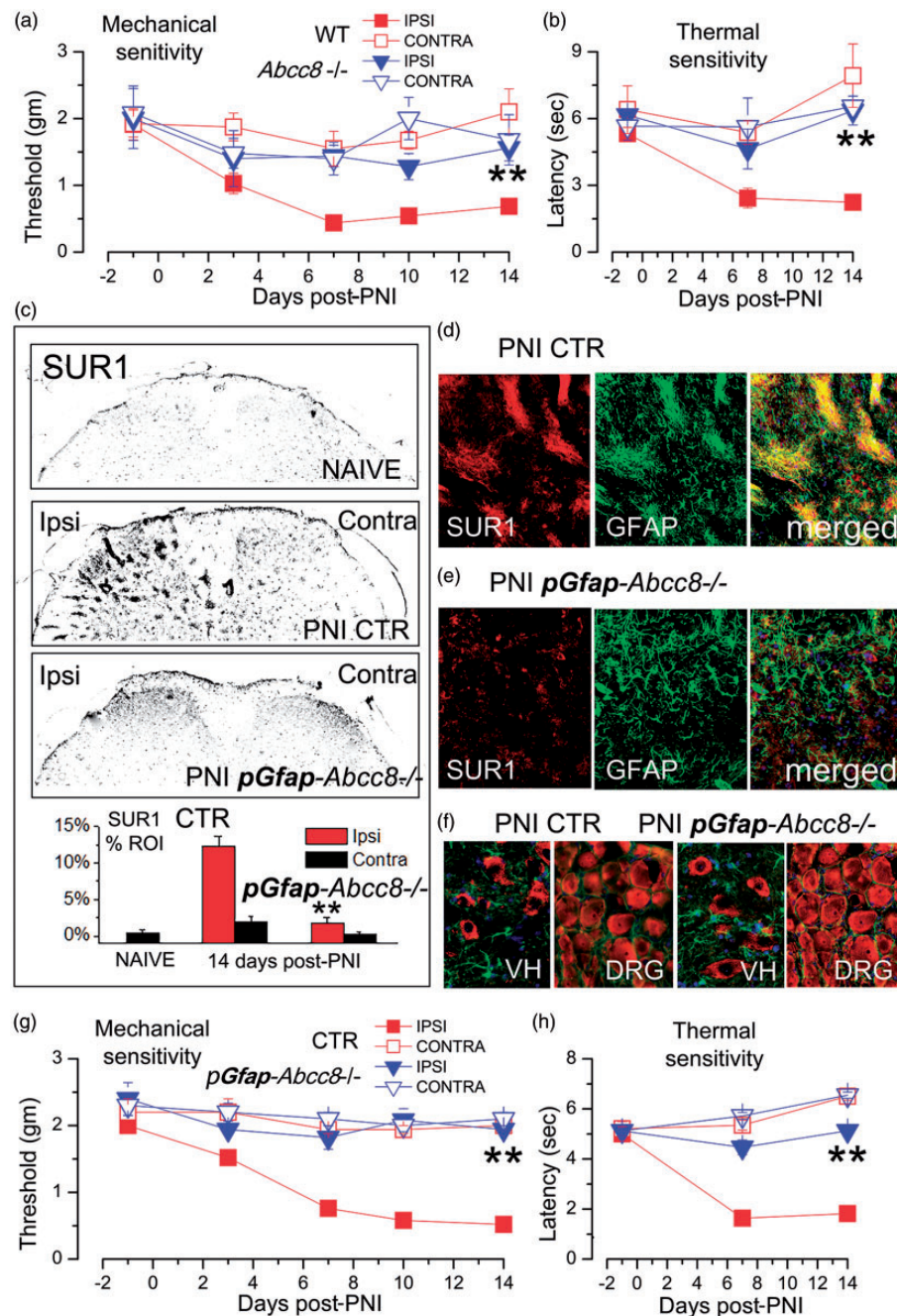


**Figure 1.** De novo upregulation of SUR1-TRPM4 in dorsal horn astrocytes at spinal segment L3-4 after PNI. (a) and (b) Double immunolabeling for GFAP (red) and SUR1 (green) at low (a) and high (b) magnification, ipsilateral (Ipsi) and contralateral (Contra) to sciatic n. cuffing; superimposed images in (a), right and (b), right. (c) Quantification of SUR1 in the ipsilateral GFAP-positive region of interest in control (CTR) and on pod-1–14 after sciatic n. cuffing; data from 4 CTR mice and 11 with PNI, 2–3 mice/time point; \*\*,  $P < 0.01$ . (d) *Abcc8* mRNA upregulation relative to *S18* shown by qPCR of ipsilateral dorsal horn (PNI) vs. sham; 3 mice/group; \*,  $P < 0.05$ . (e) Double immunolabeling for SUR1 and Iba1 shows minimal overlap. (f) Analysis of SUR1 co-localization with GFAP, Iba1 and MBP after PNI using Pearson's correlation coefficient, demonstrating a predominant astrocytic localization; 3 mice/group. (g) Double immunolabeling for SUR1 and TRPM4, ipsilateral to PNI; superimposed images are shown (right). (h) Quantification of TRPM4 in the ipsilateral GFAP-positive region of interest in control (CTR) and on pod-14 after sciatic n. cuffing; data from 4 CTR mice and 5 mice with PNI; \*\*,  $P < 0.01$ . (i, j) Proximity ligation assay (PLA) for SUR1 and TRPM4 (red) and GFAP immunolabeling (green), at low (i) and high (j) magnification, ipsilateral to sciatic n. cuffing in sham CTR, after PNI in WT mice, and after PNI in mice with *pGfap*-cre-driven *Abcc8* deletion. (k) Quantification of SUR1-TRPM4 PLA in the ipsilateral dorsal horn on pod-14 after sciatic n. cuffing in sham CTR, after PNI in WT mice, and after PNI in mice with *pGfap*-cre-driven *Abcc8* deletion; data from 4, 5 and 5 mice, respectively; \*\*,  $P < 0.01$  with respect to CTR; ###,  $P < 0.01$  with respect to PNI in WT.

deletion of *Abcc8*/SUR1 exhibited ipsilateral thermal sensitivity that was not different from the contralateral uninjured hindpaw (Figure 2(b)).

We further explored the role of *Abcc8*/SUR1 specifically in astrocytes, since SUR1 is expressed not only in activated astrocytes, as shown above (Figure 1), but in other cell types within the CNS, including DRG<sup>15,16</sup> and other neurons,<sup>50</sup> Schwann cells<sup>16</sup> and activated

microglia.<sup>32,51</sup> Mice with *pGfap*-cre-driven *Abcc8* deletion and littermate controls underwent sciatic n. cuffing. Compared to uninjured mice, littermate controls with sciatic n. cuffing showed prominent upregulation of SUR1 in ipsilateral dorsal horn that co-localized with the astrocyte marker, GFAP (Figure 2(c) and (d)). By contrast, in mice with *pGfap*-cre-driven *Abcc8* deletion, sciatic n. cuffing resulted in minimal SUR1 upregulation



**Figure 2.** Both global and *pGfap*-cre-driven *Abcc8* deletion impedes the development of pain behaviors after PNI. A,B: Mice with global *Abcc8* deletion and WT littermate controls (WT) underwent sciatic n. cuffing on pod-0 and were tested for mechanical sensitivity using von Frey filaments (a) and for thermal sensitivity using the automated Hargreaves test (b) on the days denoted on the abscissas; ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws were tested in both WT controls (squares) and with gene deletion (triangles); 5 mice/group; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral hindpaws of WT vs. gene deletion. (c) Immunolabeling for SUR1 in the dorsal horn at spinal segment L3-4 from an uninjured mouse (naive) and, following sciatic n. cuffing (PNI), in a littermate control mouse (CTR) and a mouse with *pGfap*-cre-driven *Abcc8* deletion; a black signal denotes positive immunolabeling for SUR1; bar graph: quantification of SUR1 in ipsilateral and contralateral dorsal horns from the 3 experimental conditions: naive (bar at left), PNI in CTR (middle bars), and PNI with *pGfap*-cre-driven *Abcc8* deletion (bars at right); 5 mice/group; \*\*,  $P < 0.01$ . (d)–(f) High magnification views of spinal cord sections immunolabeled for SUR1 ((d)–(f)) and double labeled for GFAP showing astrocyte expression of SUR in dorsal horn astrocytes from a control mouse (d), but not from a mouse with *pGfap*-cre-driven *Abcc8* deletion (e); the ventral horns (VH) and DRG of the same mice show SUR1 expression in neurons of both genotypes (f). (g) and (h) Mice with *pGfap*-cre-driven *Abcc8* deletion and littermate controls (CTR) underwent sciatic n. cuffing on pod-0 and were tested for mechanical sensitivity using von Frey filaments (g) and for thermal sensitivity using the automated Hargreaves test (h) on the days denoted on the abscissas; ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws were tested in both controls (squares) and with gene deletion (triangles); 10 and 5 mice/group in (g) and (h), respectively; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral hindpaws of control vs. gene deletion.

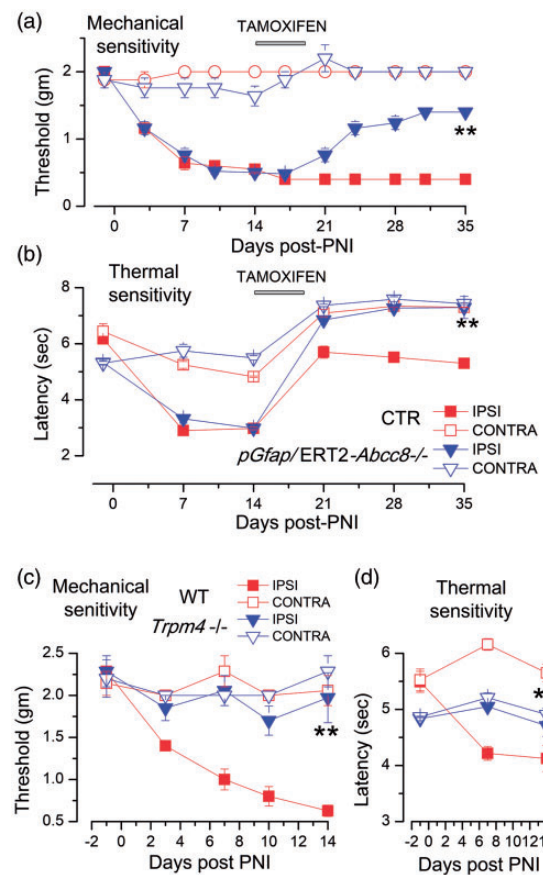
(Figure 2(c) and (e)) and minimal PLA signal for SUR1-TRPM4 (Figure 1(k)) in the dorsal horn, whereas SUR1 expression in ventral horn neurons and DRG neurons was unaffected (Figure 2(f)).

In littermate controls, sciatic n. cuffing gave rise to mechanical allodynia involving the ipsilateral hindpaw that developed over the course of 7 days and persisted on testing at pod-14 (Figure 2(g)). In mice with *pGfap*-cre-driven *Abcc8* deletion, allodynia of the ipsilateral hindpaw to mechanical stimuli failed to develop, and withdrawal thresholds were not different from those of the contralateral uninjured hindpaw (Figure 2(g)). On testing for thermal sensitivity, the ipsilateral hindpaw of controls exhibited significant thermal hyperalgesia (Figure 2(h)), whereas mice with *pGfap*-cre-driven *Abcc8* deletion exhibited ipsilateral thermal sensitivity that was not different from the contralateral uninjured hindpaw (Figure 2(h)).

We also examined whether delayed silencing of *Abcc8* in astrocytes could ameliorate pain behaviors after those behaviors had been established. Mice with *Abcc8* deletion regulated by *pGFAP*-cre/ERT2 and littermate controls underwent sciatic n. cuffing and were tested for mechanical sensitivity. In all mice, mechanical allodynia developed in the ipsilateral hindpaw over the first 7 days and persisted through to pod-14 (Figure 3(a)). All mice were administered tamoxifen once daily on pod-14–18. Littermate controls continued to exhibit ipsilateral mechanical allodynia unabated until pod-35. By contrast, the ipsilateral hindpaw of mice with *pGFAP*-cre/ERT2-driven *Abcc8* deletion slowly regained a significantly more normal mechanical sensitivity, although ipsilateral sensitivity did not reach that in the contralateral hindpaw (Figure 3(a)). On testing for thermal sensitivity, all mice demonstrated marked hyperalgesia through to pod-14 (Figure 3(b)). Subsequently, littermate controls showed partial loss of thermal hyperalgesia, as may occur over time with nerve constriction in mice<sup>52</sup> or which may have been due to tamoxifen.<sup>53</sup> By contrast, mice with *pGFAP*-cre/ERT2-driven *Abcc8* deletion showed complete reversion to the normal thermal sensitivity exhibited by the contralateral hindpaw (Figure 3(b)).

### Deletion of *Trpm4*/TRPM4

As shown above, sciatic n. cuffing was associated with upregulation of SUR1-TRPM4 in ipsilateral dorsal horn astrocytes. To assess the possible involvement of TRPM4 in neuropathic pain, mice with global deletion of *Trpm4*/TRPM4 and littermate WT controls underwent sciatic n. cuffing. In littermate controls, sciatic n. cuffing gave rise to mechanical allodynia involving the ipsilateral hindpaw that developed over the course of 7 days and persisted on testing at pod-14 (Figure 3(c)).



**Figure 3.** Delayed silencing of *Abcc8* in astrocytes or constitutive global silencing of *Trpm4* reduces pain behaviors after PNI. (a) and (b) Mice with *pGFAP*-cre/ERT2-driven *Abcc8* deletion and littermate controls underwent sciatic n. cuffing on pod-0 and were tested for mechanical sensitivity using von Frey filaments (a) and for thermal sensitivity using the automated Hargreaves test (b) on the days denoted on the abscissas; all mice were administered tamoxifen once daily on pod-14–18; ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws were tested in both controls (squares) and with gene deletion (triangles); 5 mice/group; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral hindpaws of control vs. gene deletion. (c) and (d) Mice with global *Trpm4* deletion and WT littermate controls (WT) underwent sciatic n. cuffing on pod-0 and were tested for mechanical sensitivity using von Frey filaments (c) and for thermal sensitivity using the automated Hargreaves test (d) on the days denoted on the abscissas; ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws were tested in both WT controls (squares) and with gene deletion (triangles); 7 mice/group; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral vs. contralateral hindpaws of WT.

In mice with global deletion of *Trpm4*/TRPM4, allodynia of the ipsilateral hindpaw to mechanical stimuli failed to develop, and withdrawal thresholds were not different from those of the contralateral uninjured hindpaw (Figure 3(c)). On testing for thermal sensitivity, the ipsilateral hindpaw of controls exhibited significant thermal hyperalgesia (Figure 3(d)), whereas mice with global



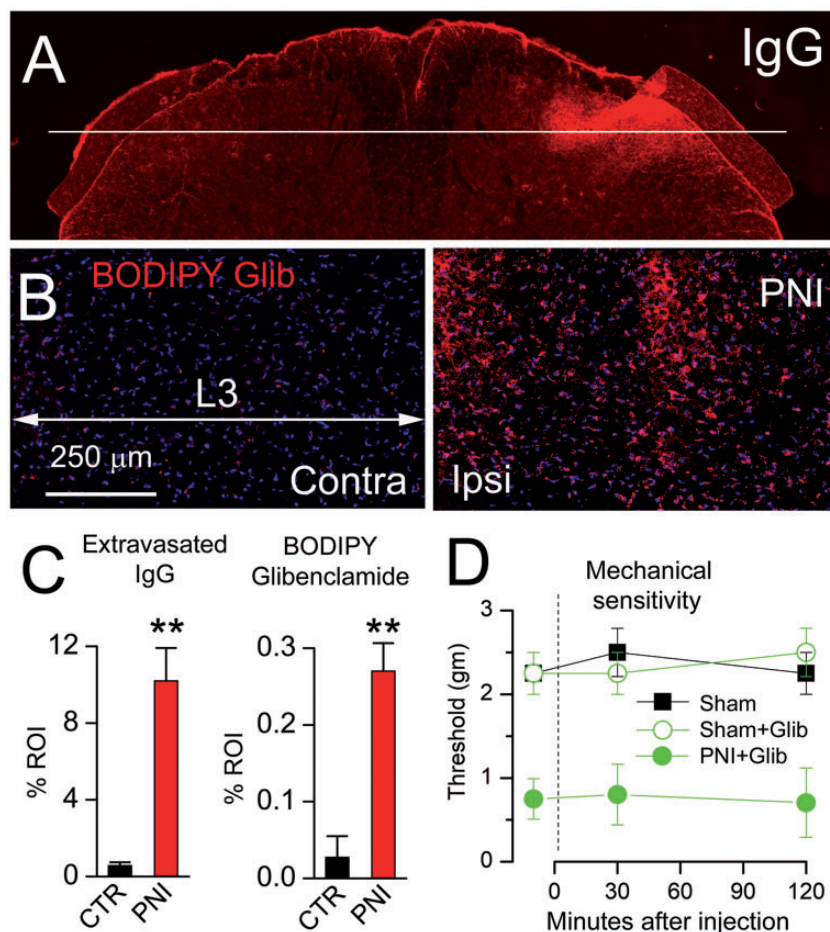
deletion of *Trpm4*/TRPM4 exhibited ipsilateral thermal sensitivity that was not different from the contralateral uninjured hindpaw (Figure 3(d)).

### Glibenclamide—Prophylactic treatment

The salutary effects of genetic deletion of *Abcc8* suggested that pharmacological inhibition of SUR1 by glibenclamide might be beneficial. Moreover, glibenclamide is a potent blocker of SUR1-TRPM4 channels.<sup>29</sup> Normally the blood-brain/blood-spinal barrier (BSB) is not permeable to glibenclamide.<sup>54</sup> However, PNI leads to neuroinflammation in the dorsal horn<sup>12,13</sup> that is characterized by a BSB that is leaky, which allows the extravasation of serum proteins that persists for 4–8 weeks<sup>6,7</sup> and may facilitate the targeted entry of therapeutic drugs. Here, we confirmed that sciatic

n. cuffing leads to BSB opening in the dorsal horn 2 weeks post-PNI, as shown by leakage of circulating IgG, and that the entry of glibenclamide into the involved tissues was facilitated (Figure 4(a) to (c)).

Glibenclamide, when tested as a single-dose injection in the acute setting, has been used in neuropathic pain models with nerve injury to explore involvement of  $K_{ATP}$  channels in the mechanism of action of analgesics.<sup>18–28</sup> In these studies, single-dose glibenclamide had no effect on pain thresholds in the absence of analgesic, a finding that we confirmed here. Uninjured WT mice and WT mice with mechanical allodynia due to sciatic n. cuffing were administered a single dose of glibenclamide and were tested acutely using von Frey filaments. No effect of glibenclamide was detected following single-dose drug injection (Figure 4(d)).



**Figure 4.** Single-dose glibenclamide enters the dorsal horn after PNI, but does not affect pain behaviors. (a) After sciatic n. cuffing, BSB leakage is shown by IgG extravasation in the ipsilateral dorsal horn (red). (b) Fluorescent bodipy-glibenclamide enters the ipsilateral (PNI) but not contralateral (Contra) dorsal horn at the level indicated by line in (a). (c) Quantification for extravasated IgG and bodipy-glibenclamide in the dorsal horn; 3 mice/group; \*\*,  $P < 0.01$ . (d) Mechanical sensitivity tested using von Frey filaments in normal uninjured WT mice before and after administering vehicle (black filled square) or single-dose glibenclamide (green empty circles), and in the ipsilateral hindpaw of mice with established mechanical allodynia due to sciatic n. cuffing after administering single-dose glibenclamide (green filled circle); 3 mice/group, uninjured; 5 mice/group, mechanical allodynia.

In contrast to single-dose administration, a regimen of repeated daily dosing of glibenclamide over the course of many days is required to reduce neuroinflammation linked to astrocyte upregulation of SUR1 in the spinal cord.<sup>35,36</sup> Here, we assessed the effect of glibenclamide on neuropathic pain behaviors when drug was administered prophylactically, beginning on the day of PNI and repeated daily until the end of the experiment.

WT mice underwent sciatic n. cuffing and were randomly assigned to receive either vehicle or glibenclamide (10  $\mu$ g IP) daily. In controls, sciatic n. cuffing gave rise to mechanical allodynia involving the ipsilateral hindpaw that developed during the first 7 days and persisted on testing at pod-14 (Figure 5(a)). In mice treated daily with glibenclamide, allodynia of the ipsilateral hindpaw to mechanical stimuli failed to develop, and withdrawal thresholds were not different from those of the contralateral uninjured hindpaw (Figure 5(a)).

The same mice were tested for thermal sensitivity. Compared to the contralateral hindpaw, the ipsilateral hindpaw of both vehicle controls and glibenclamide-treated animals exhibited significant thermal hyperalgesia on pod-7 (Figure 5(b)). However, by pod-14 glibenclamide-treated mice showed normal thermal sensitivity that was not different from the contralateral uninjured hindpaw, whereas vehicle-treated mice continued to show ipsilateral thermal hyperalgesia (Figure 5(b)).

### Glibenclamide and neuroinflammation

After PNI, dorsal horn astrocytes exhibit a chronic activation state characterized by secretion of numerous pro-inflammatory cytokines, chemokines and other factors, including IL-6,<sup>55–57</sup> CCL2<sup>58–60</sup> and CXCL1.<sup>61–64</sup> Blockade of each of these individually results in significant attenuation of pain behaviors.

We studied tissues from mice from the foregoing experiment administered daily glibenclamide vs. vehicle. The involved spinal cord segment was immunolabeled for GFAP and co-labeled for either IL-6, CCL2 or CXCL1. After sciatic n. cuffing, ipsilateral but not contralateral dorsal horn astrocytes, identified by GFAP immunolabeling, showed prominent upregulation of IL-6, CCL2 and CXCL1 (Figure 5(c) to (e)), as reported.<sup>55,56,60–62,64</sup> Co-labeling with Iba1 showed that cytokine and chemokine expression was absent in microglia/macrophages (not shown). The dorsal horns of mice administered glibenclamide showed less prominent immunolabeling for IL-6, CCL2 and CXCL1. Quantification of IL-6, CCL2 and CXCL1 immunoreactivity showed significant increases in vehicle-treated mice with sciatic n. cuffing compared to uninjured controls, and significantly smaller increases in mice with sciatic n. cuffing treated daily with glibenclamide (Figure 5(f)).

### Glibenclamide—Therapeutic treatment

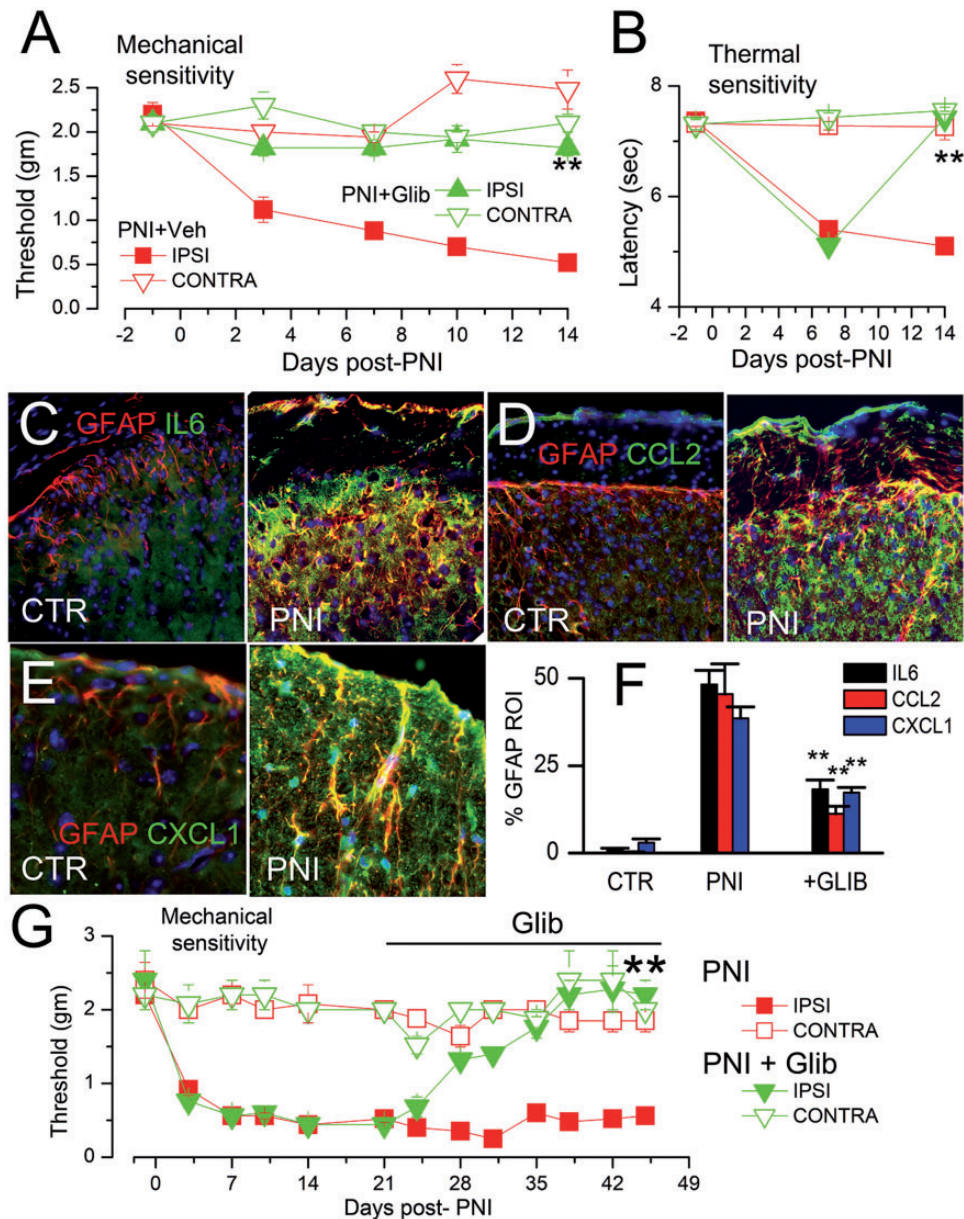
To examine a clinically relevant scenario, we studied the effect of glibenclamide when administered beginning after mechanical allodynia was established. WT mice underwent sciatic n. cuffing, following which mechanical allodynia involving the ipsilateral hindpaw developed over the course of 7 days and persisted unabated through to pod-21 (Figure 5(g)). Mice then were assigned randomly to receive either vehicle or glibenclamide (10  $\mu$ g IP), beginning on pod-21 and repeated daily until the end of the experiment on pod-45. Vehicle-treated mice showed no extinction, continuing to exhibit mechanical allodynia for the full 45 days of the experiment. However, over the course of the next 14 days, mice receiving glibenclamide daily gradually reverted to mechanical sensitivity similar to the contralateral hindpaw (Figure 5(g)).

### Discussion

The principal findings of the present study are that: (i) both global and *pGfap*-cre-driven *Abcc8* deletion, as well as early, repeated pharmacological inhibition of SUR1 by daily administration of glibenclamide, are effective in preventing the development of neuropathic pain behaviors in the murine sciatic n. cuff model; (ii) both delayed silencing of *Abcc8* and delayed inhibition of SUR1 by repeated administration of glibenclamide ameliorate pain behaviors after they have been established, underscoring the potential for translation to the pain clinic; (iii) after PNI, glibenclamide enters the dorsal horn of the affected spinal segment due to BSB dysfunction linked to neuroinflammation; (iv) the salutary effects of daily glibenclamide on neuropathic pain behaviors correlate with reduced neuroinflammation in the spinal cord; (v) in the absence of nerve injury, daily glibenclamide does not influence normal mechanical or thermal sensation; (vi) the effect of astrocyte deletion of *Abcc8*/SUR1 is replicated by global deletion of *Trpm4*/TRPM4, consistent with involvement of SUR1-TRPM4 channels.

### Glibenclamide vs. KATP in pain models

The effects of glibenclamide on sensory thresholds and pain are complex and context dependent. A considerable body of work has implicated the L-arginine/nitric oxide/cyclic GMP/K<sub>ATP</sub> channel pathway in the mechanism of action of numerous analgesics, with glibenclamide and other SUR agonists and antagonists being used to implicate K<sub>ATP</sub> channels. In models with neuropathic pain induced by chronic sciatic n. constriction or L5-L6 spinal n. ligation, single-dose administration of glibenclamide invariably blunted the anti-allodynic effects of numerous analgesics, including: gabapentin,<sup>18</sup> R-PIA,<sup>19</sup>



**Figure 5.** Repeated-dose glibenclamide, administered either prophylactically or therapeutically, impedes or reverses the development of pain behaviors after PNI. (a) and (b) WT mice underwent sciatic n. cuffing on pod-0 and were tested for mechanical sensitivity using von Frey filaments (a) and for thermal sensitivity using the automated Hargreaves test (b) on the days denoted on the abscissas; the mice received daily administration of vehicle (red squares) or glibenclamide (green triangles); ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws were tested in both vehicle- (squares) and glibenclamide- (triangles) treated animals; 5 mice/group; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral hindpaws of vehicle vs. glibenclamide. (c) and (e) Double immunolabeling for GFAP (red) plus IL-6 (c), CCL2 (d) or CXCL1 (e), ipsilateral (PNI) and contralateral (CTR) to PNI, in untreated WT mice. (f) Quantification in the GFAP-positive region of interest for IL-6, CCL2 and CXCL1 in untreated WT (PNI) mice and mice treated daily with glibenclamide (GLIB) ipsilateral to PNI; tissues were from the experiment in Panels A,B; 5 mice/group; \*\*,  $P < 0.01$ . (g) WT mice underwent sciatic n. cuffing on pod-0 and on pod-21 began daily administration of vehicle (red squares) or glibenclamide (green triangles); mechanical sensitivity of ipsilateral (filled symbols) and contralateral (empty symbols) hindpaws was tested using von Frey filaments on the days denoted on the abscissa; 5 mice/group; \*\*,  $P < 0.01$  by repeated measures ANOVA for ipsilateral hindpaws of vehicle vs. glibenclamide.

mangiferin,<sup>20</sup> Angeli's salt,<sup>21</sup> nefopam<sup>22</sup> diosmin,<sup>23</sup> zerumbone,<sup>24</sup> [6]-gingerol,<sup>25</sup> curcumin,<sup>26</sup> JM-20<sup>27</sup> and astragaloside IV.<sup>28</sup> However, in nearly all of these studies, when controls were performed without analgesic, it

was found that pain thresholds were not affected by single-dose glibenclamide administered either intraperitoneally or intrathecally. Similarly, in a study not involving analgesics, whereas intraplantar or intrathecal

injection of SUR1-subtype  $K_{ATP}$  channel agonists (diazoxide and NN414) reduced mechanical sensitivity after PNI, glibenclamide, as well as other  $K_{ATP}$  antagonists (tolbutamide, gliclazide) had no significant effect on mechanical or thermal thresholds.<sup>17</sup> In accordance with the foregoing, here we showed that a single dose of glibenclamide (10  $\mu$ g IP;  $\sim$ 0.3 mg/kg) had no effect on acute pain behaviors in either uninjured mice or mice with allodynia. Together, these findings indicate that, while  $K_{ATP}$  channels are important for the analgesic effect of many compounds, and may contribute to mechanical sensory thresholds,  $K_{ATP}$  channels may not be dominant under basal conditions of neuropathic pain.

### Chronically activated, pro-inflammatory secretory phenotype

Neuroinflammation linked to glial cell activation is increasingly recognized to play a prominent role in the initiation and maintenance of pain hypersensitivity.<sup>9–13</sup> Microglia, astrocytes, and oligodendrocytes modulate CNS inflammation triggered by PNI.<sup>65</sup> After PNI, dorsal horn astrocytes exhibit a chronically activated, pro-inflammatory secretory (CAPS) phenotype that is induced by activated microglia and damaged neurons.<sup>9</sup> The astrocytic CAPS phenotype is characterized by chronic activation and secretion of pro-inflammatory cytokines (TNF, IL-1 $\beta$ , IL-6), chemokines (CCL2, CCL7, CXCL1) and other factors.<sup>66,67</sup> Among the many factors secreted by astrocytes, several stand out including IL-6,<sup>55–57</sup> CCL2<sup>58–60</sup> and CXCL1.<sup>61–64</sup> In neuropathic pain models, blockade or gene deletion of each of these individually results in significant attenuation of pain behaviors. A major consequence of the astrocytic CAPS phenotype is neuroinflammation and neuronal hyperactivation. Chemokines such as CCL2 and CXCL1 are best known for recruiting leukocytes, but they also act on their cognate receptors, CCR2 and CXCR2, expressed on dorsal horn neurons (astrocyte-to-neuron signaling), resulting in synaptic hyperexcitability in the dorsal horn that leads to hypersensitivity following PNI.<sup>9,68</sup>

### Repeated dosing with glibenclamide

In different mouse models with neuroinflammation involving spinal cord astrocytes, neuroinflammation and its associated neurofunctional phenotype may be gradually extinguished by repeated daily dosing with glibenclamide. In a murine model of multiple sclerosis (MS) – experimental allergic encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG)<sub>35–55</sub> – repeated daily administration of glibenclamide beginning 24 days after MOG<sub>35–55</sub> immunization, well after clinical symptoms had plateaued, improved

clinical motor function and reduced the expression of the proinflammatory factors TNF, BAFF, CCL2 and NOS2 in GFAP-positive astrocytes in the spinal cord.<sup>35,36</sup> Here we show that, in the sciatic n. cuff model of neuropathic pain, repeated daily administration of glibenclamide beginning 21 days after PNI, well after mechanical allodynia had developed, improved pain behaviors and, when used prophylactically, reduced the expression of the proinflammatory factors IL-6, CCL2 and CXCL1 in GFAP-positive astrocytes in the spinal cord. In both the EAE and PNI models, the effects of repeated prophylactic dosing with glibenclamide on GFAP-linked neuroinflammation and the associated neurofunctional phenotype were replicated by global deletion of *Abcc8* and, as shown here, by *pGfap*-cre-driven *Abcc8* deletion. These observations underscore the important pathological consequences of astrocytes that exhibit the CAPS phenotype, and the importance of SUR1 in sustaining this pathological astrocytic phenotype.

We did not examine the effect of sex in our experiments. Notably, sex differences in  $K_{ATP}$  subunit expression and sex-specific responses to  $K_{ATP}$  agonists have been reported in the trigeminal ganglion.<sup>69</sup> However, previous work indicates that spinal microglia, not spinal astrocytes, are the dominant cell type in sex-dependent glial signaling in pathological pain, with treatments that target astrocytes showing similar efficacy in both sexes.<sup>70</sup> The present work on neuroinflammation linked to spinal cord astrocytes in PNI, all carried out in males, and the previous work on neuroinflammation linked to spinal cord astrocytes in EAE, all carried out in females,<sup>35,36</sup> show that *Abcc8*/SUR1 is a druggable target in chronically activated astrocytes of both sexes.

The mechanism by which repeated dosing of glibenclamide and *Abcc8* suppression reduce the expression of pro-inflammatory cytokines/chemokines by astrocytes *in vivo* remains to be elucidated, but may involve transcription that is regulated by intracellular calcium, such as nuclear factor of activated T-cells.<sup>32</sup> A transcriptional mechanism was previously implicated for glibenclamide inhibition of cytokine/chemokine expression by reactive astrocytes *in vitro*.<sup>36</sup>

### Conclusion

Neuropathic pain following peripheral nerve injury remains a major public health problem that is magnified by the prevalence of opioid use disorder. Its pathogenesis remains incompletely understood and major challenges remain to discover novel drugs with therapeutic efficacy that will be well tolerated, have minimal side effects and be devoid of addictive potential. Here, we report that glibenclamide is highly effective in reducing neuropathic pain behaviors in a murine sciatic n. injury

model when a low dose of drug is administered daily, both prophylactically, before the onset of neuropathic pain, as well as later, after symptoms have fully developed. Attenuation of pain behaviors by glibenclamide correlated with reduced neuroinflammation in the spinal cord. Unlike opioids, glibenclamide appears to have minimal effect on normal mechanical or thermal sensitivity but, with repeated dosing, exerts anti-hypersensitivity effects primarily in sensitizing conditions. Glibenclamide may be an attractive candidate drug for the treatment of some forms neuropathic pain such as that due to peripheral nerve injury.

### Author Contributions

Study conceptualization: JMS; study design and oversight: VG, AK, and JMS; surgeries: OT; data collection for neuro-functional tests: OT, SA, and TKM; immunohistochemistry: SI and VG; transgenic mice: YN and JB; funding procurement: JMS and CAS; first draft of the manuscript: JMS; critical contributions to the manuscript: OT, CAS, AK, VG, and JMS. All authors have read and approved the final manuscript.

### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: JMS and VG have filed US Patent Application Number 16/976,414, filed 8/27/20, and European Patent Application Number 19760644.5, filed 9/25/20, "Composition and methods for treating pain." All other authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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### References

- Robinson LR. Traumatic injury to peripheral nerves. *Muscle Nerve* 2000; 23: 863–873.
- Haanpaa M, Attal N, Backonja M, Baron R, Bennett M, Bouhassira D, Cruccu G, Hansson P, Haythornthwaite JA, Iannetti GD, Jensen TS, Kauppila T, Nurmikko TJ, Rice AS, Rowbotham M, Serra J, Sommer C, Smith BH, Treede RD. NeuPSIG guidelines on neuropathic pain assessment. *Pain* 2011; 152: 14–27.
- Beltran MJ, Burns TC, Eckel TT, Potter BK, Wenke JC, Hsu JR, and Skeletal Trauma Research C. Fate of combat nerve injury. *J Orthop Trauma* 2012; 26: e198–e203.
- Birch R, Misra P, Stewart MP, Eardley WG, Ramasamy A, Brown K, Shenoy R, Anand P, Clasper J, Dunn R, Etherington J. Nerve injuries sustained during warfare: part I—epidemiology. *J Bone Joint Surg Br* 2012; 94: 523–528.
- Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. *Annu Rev Neurosci* 2009; 32: 1–32.
- Gordh T, Sharma HS. Chronic spinal nerve ligation induces microvascular permeability disturbances, astrocytic reaction, and structural changes in the rat spinal cord. *Acta Neurochir Suppl* 2006; 96: 335–340.
- Echeverry S, Shi XQ, Rivest S, Zhang J. Peripheral nerve injury alters blood-spinal cord barrier functional and molecular integrity through a selective inflammatory pathway. *J Neurosci* 2011; 31: 10819–10828.
- Old EA, Clark AK, Malcangio M. The role of glia in the spinal cord in neuropathic and inflammatory pain. *Handb Exp Pharmacol* 2015; 227: 145–170.
- Zhang ZJ, Jiang BC, Gao YJ. Chemokines in neuron-glia cell interaction and pathogenesis of neuropathic pain. *Cell Mol Life Sci* 2017; 74: 3275–3291.
- Vallejo R, Tilley DM, Vogel L, Benyamin R. The role of glia and the immune system in the development and maintenance of neuropathic pain. *Pain Pract* 2010; 10: 167–184.
- Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. *Science* 2016; 354: 572–577.
- Ji RR, Berta T, Nedergaard M. Glia and pain: is chronic pain a gliopathy? *Pain* 2013; 154 Suppl 1: S10–S28.
- Cairns BE, Arendt-Nielsen L, Sacerdote P. Perspectives in pain research 2014: Neuroinflammation and glial cell activation: the cause of transition from acute to chronic pain? *Scand J Pain* 2015; 6: 3–6.
- Bryan J, Munoz A, Zhang X, Dufer M, Drews G, Krippeit-Drews P, Aguilar-Bryan L. ABCC8 and ABCC9: ABC transporters that regulate K<sup>+</sup> channels. *Pflugers Arch* 2007; 453: 703–718.
- Kawano T, Zoga V, McCallum JB, Wu HE, Gemes G, Liang MY, Abram S, Kwok WM, Hogan QH, Sarantopoulos CD. ATP-sensitive potassium currents in rat primary afferent neurons: biophysical, pharmacological properties, and alterations by painful nerve injury. *Neuroscience* 2009; 162: 431–443.
- Zoga V, Kawano T, Liang MY, Bienengraeber M, Weihrauch D, McCallum B, Gemes G, Hogan Q, Sarantopoulos C. KATP channel subunits in rat dorsal root ganglia: alterations by painful axotomy. *Mol Pain* 2010; 6: 1744–8069.
- Luu W, Bjork J, Salo E, Entenmann N, Jurgenson T, Fisher C, Klein AH. Modulation of SUR1 KATP channel subunit activity in the peripheral nervous system reduces mechanical hyperalgesia after nerve injury in mice. *Int J Mol Sci* 2019; 20: 2251–2210.
- Mixcoatl-Zecuatl T, Flores-Murrieta FJ, Granados-Soto V. The nitric oxide-cyclic GMP-protein kinase G-K<sup>+</sup> channel pathway participates in the antiallodynic effect of spinal gabapentin. *Eur J Pharmacol* 2006; 531: 87–95.
- Song JG, Hahm KD, Kim YK, Leem JG, Lee C, Jeong SM, Park PH, Shin JW. Adenosine triphosphate-sensitive

- potassium channel blockers attenuate the antiallodynic effect of R-PIA in neuropathic rats. *Anesthes Analges* 2011; 112: 1494–1499.
20. de Los Monteros-Zuñiga AE, Izquierdo T, Quiñonez-Bastidas GN, Rocha-González HI, Godínez-Chaparro B. Anti-allodynic effect of mangiferin in neuropathic rats: Involvement of nitric oxide-cyclic GMP-ATP sensitive K (+) channels pathway and serotonergic system. *Pharmacol Biochem Behav* 2016; 150–151: 190–197.
  21. Longhi-Balbinot DT, Rossaneis AC, Pinho-Ribeiro FA, Bertozzi MM, Cunha FQ, Alves-Filho JC, Cunha TM, Peron JP, Miranda KM, Casagrande R, Verri WA Jr. The nitroxyl donor, angeli's salt, reduces chronic constriction injury-induced neuropathic pain. *Chem Biol Interact* 2016; 256: 1–8.
  22. Koh WU, Shin JW, Bang JY, Kim SG, Song JG. The antiallodynic effects of nefopam are mediated by the adenosine Triphosphate-Sensitive potassium channel in a neuropathic pain model. *Anesthes Analges* 2016; 123: 762–770.
  23. Bertozzi MM, Rossaneis AC, Fattori V, Longhi-Balbinot DT, Freitas A, Cunha FQ, Alves-Filho JC, Cunha TM, Casagrande R, Verri WA Jr. Diosmin reduces chronic constriction injury-induced neuropathic pain in mice. *Chem Biol Interact* 2017; 273: 180–189.
  24. Zulazmi NA, Gopalsamy B, Min JC, Farouk AA, Sulaiman MR, Bharatham BH, Perimal EK. Zerumbone alleviates neuropathic pain through the involvement of l-arginine-nitric oxide-cGMP-K(+) ATP channel pathways in chronic constriction injury in mice model. *Molecules* 2017; 22: 555.
  25. Mata-Bermudez A, Izquierdo T, de Los Monteros-Zuñiga E, Coen A, Godínez-Chaparro B. Antiallodynic effect induced by [6]-gingerol in neuropathic rats is mediated by activation of the serotonergic system and the nitric oxide-cyclic guanosine monophosphate-adenosine triphosphate-sensitive K(+) channel pathway. *Phytother Res* 2018; 32: 2520–2530.
  26. Pastrana-Quintos T, Salgado-Moreno G, Pérez-Ramos J, Coen A, Godínez-Chaparro B. Anti-allodynic effect induced by curcumin in neuropathic rat is mediated through the NO-cyclic-GMP-ATP sensitive K(+) channels pathway. *BMC Complement Med Ther* 2020; 20: 1–7.
  27. Garrido-Suárez BB, Garrido G, Castro-Labrada M, Merino N, Valdés O, Pardo Z, Ochoa-Rodríguez E, Verdecia-Reyes Y, Delgado-Hernández R, Godoy-Figueiredo J, Ferreira SH. Anti-hypernociceptive and anti-inflammatory effects of JM-20: a novel hybrid neuroprotective compound. *Brain Res Bull* 2020; 165: 185–197.
  28. Dai WL, Zhang L, Han L, Yang X, Hu L, Miao C, Song L, Xiao H, Liu JH, Liu WT. Regulation of the KATP-JNK gap junction signaling pathway by immunomodulator astragaloside IV attenuates neuropathic pain. *Reg Anesth Pain Med* 2020; 45: 955–963.
  29. Woo SK, Kwon MS, Ivanov A, Gerzanich V, Simard JM. The sulfonylurea receptor 1 (Sur1)-transient receptor potential melastatin 4 (Trpm4) channel. *J Biol Chem* 2013; 288: 3655–3667.
  30. Simard JM, Geng Z, Woo SK, Ivanova S, Tosun C, Melnichenko L, Gerzanich V. Glibenclamide reduces inflammation, vasogenic edema, and caspase-3 activation after subarachnoid hemorrhage. *J Cereb Blood Flow Metab* 2009; 29: 317–330.
  31. Tosun C, Kurland DB, Mehta R, Castellani RJ, deJong JL, Kwon MS, Woo SK, Gerzanich V, Simard JM. Inhibition of the Sur1-Trpm4 channel reduces neuroinflammation and cognitive impairment in subarachnoid hemorrhage. *Stroke* 2013; 44: 3522–3528.
  32. Kurland DB, Gerzanich V, Karimy JK, Woo SK, Vennekens R, Freichel M, Nilius B, Bryan J, Simard JM. The Sur1-Trpm4 channel regulates NOS2 transcription in TLR4-activated microglia. *J Neuroinflammation* 2016; 13: 130.
  33. Jiang B, Li L, Chen Q, Tao Y, Yang L, Zhang B, Zhang JH, Feng H, Chen Z, Tang J, Zhu G. Role of glibenclamide in brain injury after intracerebral hemorrhage. *Transl Stroke Res* 2017; 8: 183–193.
  34. Xu F, Shen G, Su Z, He Z, Yuan L. Glibenclamide ameliorates the disrupted blood-brain barrier in experimental intracerebral hemorrhage by inhibiting the activation of NLRP3 inflammasome. *Brain Behav* 2019; 9: e01254.
  35. Makar TK, Gerzanich V, Nimmagadda VK, Jain R, Lam K, Mubariz F, Trisler D, Ivanova S, Woo SK, Kwon MS, Bryan J, Bever CT, Simard JM. Silencing of Abcc8 or inhibition of newly upregulated Sur1-Trpm4 reduce inflammation and disease progression in experimental autoimmune encephalomyelitis. *J Neuroinflammation* 2015; 12: 210.
  36. Gerzanich V, Makar TK, Guda PR, Kwon MS, Stokum JA, Woo SK, Ivanova S, Ivanov A, Mehta RI, Morris AB, Bryan J, Bever CT, Simard JM. Effects of glibenclamide during the chronic phase of murine experimental autoimmune encephalomyelitis. *J Neuroinflammation* 2017; 14: 177.
  37. Benbouzid M, Pallage V, Rajalu M, Waltisperger E, Doridot S, Poisbeau P, Freund-Mercier MJ, Barrot M. Sciatic nerve cuffing in mice: a model of sustained neuropathic pain. *Eur J Pain* 2008; 12: 591–599.
  38. Yalcin I, Megat S, Barthas F, Waltisperger E, Kremer M, Salvat E, Barrot M. The sciatic nerve cuffing model of neuropathic pain in mice. *JoVE* 2014.
  39. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010; 8: e1000412.
  40. Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J. Sur1 knockout mice. A model for K(ATP) channel-independent regulation of insulin secretion. *J Biol Chem* 2000; 275: 9270–9277.
  41. Nakamura Y, Bryan J. Targeting SUR1/Abcc8-type neuroendocrine KATP channels in pancreatic islet cells. *PLoS One* 2014; 9: e91525.
  42. Vennekens R, Olausson J, Meissner M, Bloch W, Mathar I, Philipp SE, Schmitz F, Weissgerber P, Nilius B, Flockerzi V, Freichel M. Increased IgE-dependent mast cell activation and anaphylactic responses in mice lacking the calcium-activated nonselective cation channel TRPM4. *Nat Immunol* 2007; 8: 312–320.
  43. Tsybalyuk S, Smith M, Gore C, Tsybalyuk O, Ivanova S, Sansur C, Gerzanich V, Simard JM. Brivaracetam

- attenuates pain behaviors in a murine model of neuropathic pain. *Mol Pain* 2019; 15: 1744806919886503.
44. Barrot M. Tests and models of nociception and pain in rodents. *Neuroscience* 2012; 211: 39–50.
  45. Deuis JR, Dvorakova LS, Vetter I. Methods used to evaluate pain behaviors in rodents. *Front Mol Neurosci* 2017; 10: 284–209.
  46. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T. A proposal for validation of antibodies. *Nat Methods* 2016; 13: 823–827.
  47. Gerzanich V, Stokum JA, Ivanova S, Woo SK, Tsymbalyuk O, Sharma A, Akkentli F, Imran Z, Aarabi B, Sahuquillo J, Simard JM. Sulfonylurea receptor 1, transient receptor potential cation channel subfamily M member 4, and KIR6.2: Role in hemorrhagic progression of contusion. *J Neurotrauma* 2019; 36: 1060–1079.
  48. Rigaud M, Gemes G, Barabas ME, Chernoff DI, Abram SE, Stucky CL, Hogan QH. Species and strain differences in rodent sciatic nerve anatomy: implications for studies of neuropathic pain. *Pain* 2008; 136: 188–201.
  49. Wu XF, Liu WT, Liu YP, Huang ZJ, Zhang YK, Song XJ. Reopening of ATP-sensitive potassium channels reduces neuropathic pain and regulates astroglial gap junctions in the rat spinal cord. *Pain* 2011; 152: 2605–2615.
  50. Tinker A, Aziz Q, Li Y, Specterman M. ATP-Sensitive potassium channels and their physiological and pathophysiological roles. *Compr Physiol* 2018; 8: 1463–1511.
  51. Ortega FJ, Vukovic J, Rodriguez MJ, Bartlett PF. Blockade of microglial KATP -channel abrogates suppression of inflammatory-mediated inhibition of neural precursor cells. *Glia* 2014; 62: 247–258.
  52. Vogel C, Mossner R, Gerlach M, Heinemann T, Murphy DL, Riederer P, Lesch KP, Sommer C. Absence of thermal hyperalgesia in serotonin transporter-deficient mice. *J Neurosci* 2003; 23: 708–715.
  53. Tsubaki M, Takeda T, Matsumoto M, Kato N, Yasuhara S, Koumoto YI, Imano M, Satou T, Nishida S. Tamoxifen suppresses paclitaxel-, vincristine-, and bortezomib-induced neuropathy via inhibition of the protein kinase C/extracellular signal-regulated kinase pathway. *Tumour Biol* 2018; 40: 1010428318808670.
  54. Lahmann C, Kramer HB, Ashcroft FM. Systemic administration of glibenclamide fails to achieve therapeutic levels in the brain and cerebrospinal fluid of rodents. *PLoS One* 2015; 10: e0134476.
  55. DeLeo JA, Colburn RW, Nichols M, Malhotra A. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res* 1996; 16: 695–700.
  56. Wei XH, Na XD, Liao GJ, Chen QY, Cui Y, Chen FY, Li YY, Zang Y, Liu XG. The up-regulation of IL-6 in DRG and spinal dorsal horn contributes to neuropathic pain following L5 ventral root transection. *Exp Neurol* 2013; 241: 159–168.
  57. Zhou YQ, Liu Z, Liu ZH, Chen SP, Li M, Shahveranov A, Ye DW, Tian YK. Interleukin-6: an emerging regulator of pathological pain. *J Neuroinflammation* 2016; 13: 141.
  58. Menetski J, Mistry S, Lu M, Mudgett JS, Ransohoff RM, Demartino JA, Macintyre DE, Abbadie C. Mice overexpressing chemokine ligand 2 (CCL2) in astrocytes display enhanced nociceptive responses. *Neuroscience* 2007; 149: 706–714.
  59. Gao YJ, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu ZZ, Park JY, Lind AL, Ma Q, Ji RR. JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. *J Neurosci* 2009; 29: 4096–4108.
  60. Thacker MA, Clark AK, Bishop T, Grist J, Yip PK, Moon LD, Thompson SW, Marchand F, McMahon SB. CCL2 is a key mediator of microglia activation in neuropathic pain states. *Eur J Pain* 2009; 13: 263–272.
  61. Zhang ZJ, Cao DL, Zhang X, Ji RR, Gao YJ. Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *Pain* 2013; 154: 2185–2197.
  62. Cao DL, Zhang ZJ, Xie RG, Jiang BC, Ji RR, Gao YJ. Chemokine CXCL1 enhances inflammatory pain and increases NMDA receptor activity and COX-2 expression in spinal cord neurons via activation of CXCR2. *Exp Neurol* 2014; 261: 328–336.
  63. Cao DL, Qian B, Zhang ZJ, Gao YJ, Wu XB. Chemokine receptor CXCR2 in dorsal root ganglion contributes to the maintenance of inflammatory pain. *Brain Res Bull* 2016; 127: 219–225.
  64. Yang LH, Xu GM, Wang Y. Up-regulation of CXCL1 and CXCR2 contributes to remifentanyl-induced hypernociception via modulating spinal NMDA receptor expression and phosphorylation in rats. *Neurosci Lett* 2016; 626: 135–141.
  65. Machelska H, Celik MO. Recent advances in understanding neuropathic pain: glia, sex differences, and epigenetics. *F1000Res* 2016; 5: 2743.
  66. Ji RR, Xu ZZ, Gao YJ. Emerging targets in neuroinflammation-driven chronic pain. *Nat Rev Drug Discov* 2014; 13: 533–548.
  67. Alfonso Romero-Sandoval E, Sweitzer S. Nonneuronal central mechanisms of pain: glia and immune response. *Prog Mol Biol Transl Sci* 2015; 131: 325–358.
  68. Tiwari V, Guan Y, Raja SN. Modulating the delicate glial-neuronal interactions in neuropathic pain: promises and potential caveats. *Neurosci Biobehav Rev* 2014; 45: 19–27.
  69. Niu K, Saloman JL, Zhang Y, Ro JY. Sex differences in the contribution of ATP-sensitive K<sup>+</sup> channels in trigeminal ganglia under an acute muscle pain condition. *Neuroscience* 2011; 180: 344–352.
  70. Chen G, Luo X, Qadri MY, Berta T, Ji RR. Sex-dependent glial signaling in pathological pain: distinct roles of spinal microglia and astrocytes. *Neurosci Bull* 2018; 34: 98–108.