

1 **An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for Na<sub>v</sub>1.6**  
2 **in peripheral pain pathways**

3

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This is a post-print version of the following article: Deuis, Jennifer R., Zimmermann, Katharina, Romanovsky, Andrej A., Possani, Lourival D., Cabot, Peter J., Lewis, Richard J. and Vetter, Irina (2013) An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for Na(v)1.6 in peripheral pain pathways. *Pain*, 154 9: 1749-1757.

## 1 **Introduction**

2 Oxaliplatin, a third-generation platinum chemotherapeutic agent, is associated with acute  
3 dose-limiting neurotoxicity, which manifests as cooling-induced peripheral dysaesthesias and  
4 paraesthesias including cold allodynia [6; 12]. Acute oxaliplatin-induced cold allodynia is  
5 characterized by a rapid onset, with symptoms occurring during or shortly after infusion, and  
6 typically resolves within several days of treatment [5]. Many currently used animal models of  
7 oxaliplatin-induced neuropathy poorly reflect these characteristics, and often require multiple  
8 injections of oxaliplatin to elicit pain behaviours which develop slowly and are of prolonged  
9 duration [30; 40; 56]. Mechanistic studies in these animal models have attributed expressional  
10 changes and altered function of ion channels expressed on unmyelinated C-fiber nociceptors  
11 to the development of cold allodynia, such as the transient receptor potential (TRP) channels  
12 TRPM8, TRPA1 and the two-pore domain potassium ( $K^+$ ) channels TREK1 and TRAAK [16;  
13 21; 35; 60]. However, these findings are inconsistent with the clinical time course of acute  
14 oxaliplatin-induced cold allodynia and the predominant effects of oxaliplatin on myelinated  
15 A-fibers [2; 6; 27; 46; 47]. Thus, the pathophysiological mechanisms underlying acute  
16 oxaliplatin-induced cold allodynia remain unclear. While oxaliplatin-induced allodynia has  
17 been described as an axonal channelopathy resulting from modulation of neuronal  $Na_v$   
18 channels [36], the contributions of the nine described isoforms ( $Na_v1.1 - Na_v1.9$ ) have not  
19 been systematically assessed.

20 Dorsal root ganglion (DRG) neurons express several  $Na_v$  isoforms, including the tetrodotoxin  
21 (TTX) resistant isoforms  $Na_v1.8$  and  $Na_v1.9$ , as well as the TTX-sensitive isoforms  $Na_v1.1$ ,  
22  $Na_v1.2$ ,  $Na_v1.3$ ,  $Na_v1.6$  and  $Na_v1.7$  [41]. The TTX-resistant  $Na_v$  isoform  $Na_v1.8$  in particular  
23 has been found to be crucial for pain evoked by noxious cooling [61], while  $Na_v1.9$  has been  
24 suggested to contribute to the pathogenesis of neuropathic pain [29]. In addition,  $Na_v1.7$  is  
25 known to be crucial in pain pathways, as loss-of-function mutations in humans cause

1 congenital insensitivity to pain [14], while gain-of-function mutations are associated with  
2 painful conditions such as erythromelalgia and paroxysmal extreme pain disorder [19]. In  
3 contrast, the functional roles of  $\text{Na}_v1.1$  and  $\text{Na}_v1.6$  in peripheral sensory neurons are less clear,  
4 and no evidence for involvement of these  $\text{Na}_v$  isoforms in pain phenotypes has been reported  
5 to date, as both homozygous  $\text{Scn1a}^{-/-}$  and  $\text{Scn8a}^{-/-}$  mice develop motor deficits and die  
6 around postnatal day 15 to 20, preventing assessment of behavioural effects in mature animals  
7 [9; 57].

8 We established an animal model of oxaliplatin that more closely mimics acute chemotherapy-  
9 induced peripheral neuropathy. We found that intraplantar oxaliplatin rapidly induced a long-  
10 lasting cold allodynia that was mediated entirely through TTX-sensitive  $\text{Na}_v$  isoform-  
11 dependent pathways. Surprisingly,  $\text{Na}_v1.6$  was implicated as the key  $\text{Na}_v$  isoform involved,  
12 whereas thermosensitive TRP channels were not found to be involved. Consistent with reports  
13 of a crucial role for delayed-rectifier potassium channels in excitability in response to cold  
14 [53], intraplantar administration of the  $\text{K}^+$  channel blocker 4-aminopyridine (4-AP) mimicked  
15 oxaliplatin-induced cold allodynia and was inhibited by  $\text{Na}_v1.6$  blockers or potentiated by  
16  $\text{Na}_v1.6$  activators, supporting a crucial role for  $\text{Na}_v1.6$  in chemically-mediated cold pain  
17 pathways.

18

## 19 **Methods**

### 20 *Chemicals*

21 Oxaliplatin and Dichloro(1,2-diaminocyclohexane)platinum(II) ( $\text{Pt}(\text{DACH})\text{Cl}_2$ ) were  
22 obtained from Sigma Aldrich (Castle Hill, New South Wales, Australia) and dissolved in 5%  
23 glucose/ $\text{H}_2\text{O}$  to a stock solution of 1 mg/mL to avoid spontaneous hydrolysis arising from the  
24 presence of  $\text{Cl}^-$  in physiological solutions.  $\mu$ -Conotoxins GIIIA and TIIIA were a kind gift  
25 from Professor Paul F. Alewood, The University of Queensland, Australia. Cn2 was isolated

1 from the venom of the scorpion *Centruroides noxius* as previously described [44; 58]. M8-B  
2 (N-(2-aminoethyl)-N-(4-(benzyloxy)-3-methoxybenzyl)thiophene-2-carboxamide  
3 hydrochloride), a selective and potent antagonist of TRPM8), was synthesized and kindly  
4 provided by Amgen, Inc. [4]. The TRPM8 antagonist AMTB (N-(3-Aminopropyl)-2-[(3-  
5 methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide hydrochloride) and tetrodotoxin were  
6 from Tocris Bioscience (Bristol, United Kingdom). ProTxII was from Peptides International  
7 (Louisville, KY, USA). Peptides were routinely diluted in 0.1–0.3% albumin in phosphate-  
8 buffered saline to avoid adsorption to plastic surfaces. All other drugs and pharmacological  
9 modulators were diluted in phosphate-buffered saline. All other reagents were from Sigma  
10 Aldrich unless otherwise stated.

11

## 12 *Animals*

13 Ethical approval for *in vivo* experiments in animals was obtained from the local institutional  
14 animal ethics committee. Experiments involving animals were conducted in accordance with  
15 the Animal Care and Protection Act Qld (2002), the *Australian Code of Practice for the Care*  
16 *and Use of Animals for Scientific Purposes*, 7th edition (2004) and the *International*  
17 *Association for the Study of Pain Guidelines for the Use of Animals in Research*.

18

19 For behavioural assessment of oxaliplatin-induced neuropathy, we used adult male C57BL/6J  
20 mice, age 5–12 weeks. Age-matched controls were used for studies involving knockout  
21 animals, and all mouse strains were back-crossed for a minimum of 5 (5–9) generations on  
22 C57BL/6 background. Knockout animals were kindly provided by the following researchers:  
23 TRPA1<sup>-/-</sup> mice (D. Corey, Harvard Medical School, Boston, MA, USA), TRPM8<sup>-/-</sup> mice (A.  
24 Patapoutian, The Scripps Research Institute, La Jolla, CA, USA), global Na<sub>v</sub>1.8<sup>-/-</sup>, Na<sub>v</sub>1.9<sup>-/-</sup>,  
25 and Na<sub>v</sub>1.3<sup>-/-</sup> mice (J. Wood, University College London, London, UK).

1

2 *Induction of oxaliplatin-induced neuropathy and behavioural assessment*

3 To characterize nociceptive effects in wild-type C57BL/6J and age-matched TRPA1<sup>-/-</sup>,  
4 TRPM8<sup>-/-</sup>, Na<sub>v</sub>1.8<sup>-/-</sup>, Na<sub>v</sub>1.9<sup>-/-</sup> and Na<sub>v</sub>1.3<sup>-/-</sup> mice, a single dose of oxaliplatin, oxalate,  
5 Pt(DACH)Cl<sub>2</sub>, 4-AP or Cn2 was administered by shallow subcutaneous injection to the left  
6 hind paw in a volume of 40 µl (intraplantar injection, i.pl.) under light isoflurane anaesthesia.  
7 Quantification of spontaneous pain, cold and heat allodynia as well as mechanical allodynia  
8 was performed by a blinded observer unaware of the genotype and/or treatments received.  
9 Spontaneous nocifensive behaviour was quantified by counting the number of paw lifts, licks,  
10 shakes and flinches at room temperature (22–25°C) on a soft padded surface over a period of  
11 5 min. Thermal allodynia was assessed by quantification of nocifensive behaviours over a 5  
12 min period on a temperature-controlled Peltier plate (Hot/Cold Plate, Ugo Basile, Comerio,  
13 Italy). Mechanical allodynia was assessed by determining the paw withdrawal threshold to  
14 mechanical stimulation using an electronic von Frey apparatus (MouseMet Electronic von  
15 Frey, TopCat Metrology, Little Downham, United Kingdom). Briefly, mice were habituated  
16 in individual mouse runs for at least 10 min, and the paw withdrawal threshold was  
17 determined from the ipsilateral and controlateral paws in three separate trials, at least 5 min  
18 apart. The pressure applied through a soft-tipped probe was increased slowly over a pre-  
19 determined force rise rate (1 g/s). The force that elicited paw withdrawal was determined  
20 using the MouseMet Software and designated as the paw withdrawal threshold.  
21 Where intraplantar injection elicited nocifensive behaviour at room temperature (Cn2,  
22 BAPTA, oxalate, 4-AP), thermal and mechanical allodynia was assessed after cessation of  
23 spontaneous pain (15 min to 1 h). To assess the effects of pharmacological modulators on the  
24 development of oxaliplatin-induced cold allodynia, compounds were administered by  
25 intraplantar injection of appropriately concentrated solutions (HC030031, 100 µM; AMTB,

1 10  $\mu$ M; M8-B, 1  $\mu$ M; TTX, 3  $\mu$ M; A803467, 10  $\mu$ M; ProTxII, 3 nM; GIIIA, 10  $\mu$ M; TIIIA,  
2 10  $\mu$ M) 5-15 min prior to behavioural quantification. To assess the effect of pharmacological  
3 modulators on the nocifensive responses elicited by 4-AP, compounds were co-administered  
4 by intraplantar injection as appropriately concentrated solutions (Cn2, 1 nM; GIIIA, 10  $\mu$ M;  
5 TTX, 3  $\mu$ M; AMTB, 10  $\mu$ M; HC030031, 100  $\mu$ M) in a final volume of 40  $\mu$ l.  
6 No systemic effects, including ataxia, altered gait or motor paralysis were apparent in any  
7 mice or after intraplantar injection of any pharmacological modulators. In addition, no  
8 sustained hind paw favouring, inflammation, swelling or ulceration of the oxaliplatin-injected  
9 paw was visible. Injection of equal volumes of 5% glucose/H<sub>2</sub>O and phosphate-buffered  
10 saline with or without albumin did not elicit any nocifensive behaviour.

11

#### 12 *FLIPR Membrane Potential Assays*

13 To verify the *in vitro* potency of compounds with activity Na<sub>v</sub>1.6 channels, inhibition of  
14 veratridine-induced membrane potential responses were assessed using the FLIPR<sup>TETRA</sup>  
15 (Molecular Devices, Sunnyvale, CA) plate reader. Na<sub>v</sub>1.6-expressing CHO cells (EZcells,  
16 Chantest, Cleveland, OH) were loaded with Red Membrane Potential dye (Molecular  
17 Devices), and responses to stimulation with veratridine (50  $\mu$ M) were assessed after 5 min  
18 pre-treatment with antagonists as previously described [51].

19

#### 20 *Data and statistical analysis*

21 Fluorescence values from membrane potential imaging experiments were converted to  
22 response over baseline values using Screen Works 3.2.0.14 as previously described [51]. For  
23 concentration-response curves, maximum values from the response after addition of agonist  
24 were plotted against agonist concentration and a 4-parameter logistic Hill equation was fitted  
25 to the data using GraphPad Prism Version 5.03 (San Diego, CA). Statistical significance was

1 defined as  $p < 0.05$  and was determined using paired or unpaired Student's *t*-tests and one-  
2 way ANOVA analysis with Dunnett's post test as indicated. Statistical analysis was performed  
3 using GraphPad Prism Version 5.03.

4

## 5 **Results**

### 6 *A mouse model of chemotherapy-induced cold allodynia based on intraplantar administration* 7 *of oxaliplatin*

8 In humans, cold allodynia generally occurs during or within hours of oxaliplatin infusion and  
9 is characterized by pain in response to normally innocuous cooling, presumably resulting  
10 from a direct effect of oxaliplatin on peripheral sensory neurons. To isolate the actions of  
11 oxaliplatin on peripheral sensory neurons, we established a novel mouse model of oxaliplatin-  
12 induced cold allodynia based on the administration of oxaliplatin by shallow subcutaneous  
13 (intraplantar, i.pl.) injection into the hind paw of C57/BL6J mice. Intraplantar injection of  
14 oxaliplatin (4–40  $\mu\text{g}$ ) elicited rapid, dose-dependent development of cold allodynia,  
15 evidenced by flinching, lifting, licking and shaking of the affected hind paw upon exposure to  
16 a cooled surface (10°C) (Fig. 1a).

17 This dose (1.6-2.0 mg/kg) is approximately equivalent to human therapeutic doses (2.5-3.5  
18 mg/kg), and considerably lower than systemic doses previously reported to elicit acute cold  
19 allodynia in rodents (5 – 10 mg/kg) [60].

20 Strikingly, cold allodynia induced by a single dose of oxaliplatin became apparent within  
21 minutes of injection and persisted for several days, with significant pain behaviour evident for  
22 up to 7 days after injection of the highest dose (2.5 mM; 40  $\mu\text{g}$ ; Fig. 1b). The terminal  
23 elimination phase of platinum-containing metabolites is long, suggesting that the prolonged  
24 effect of a single intraplantar injection of oxaliplatin could arise from the pharmacokinetics of  
25 these oxaliplatin metabolites. Alternatively, oxaliplatin, or platinum metabolites, may elicit

1 irreversible changes in neuronal proteins which are involved in mediating increased  
2 excitability to cool stimuli.  
3 Nocifensive behaviour evoked by intraplantar injection of oxaliplatin became apparent at  
4 temperatures below 15°C ( $18.2 \pm 6.5$  flinches/5 min), but no heat allodynia was evident, with  
5 animals displaying little or no nocifensive behaviour at elevated temperatures up to 42°C ( $1.0$   
6  $\pm 0.7$  flinches/5 min) (Fig. 1c). In addition, intraplantar injection of oxaliplatin also elicited  
7 mechanical allodynia, evidenced by decreased paw withdrawal threshold to mechanical  
8 stimulation (Fig. 1d; Control,  $4.7 \pm 0.3$  g; oxaliplatin  $2.5 \pm 0.3$  g). Therefore, this novel animal  
9 model of intraplantar oxaliplatin produces behavioural responses that parallel the human  
10 symptomatology of oxaliplatin-induced neuropathy, confirming a direct peripheral effect of  
11 oxaliplatin on sensory nerve endings as the basis of cold-evoked paraesthesias and  
12 dysaesthesias.

13

#### 14 *Oxaliplatin metabolites contribute to mechanical, but not cold allodynia*

15 Oxaliplatin is rapidly hydrolyzed *in vivo* to bioactive derivatives through displacement of the  
16 oxalate group by H<sub>2</sub>O and Cl<sup>-</sup> to produce oxalate as well as reactive monochloro-, dichloro-  
17 and diaquo-diaminocyclohexane platinum metabolites [18; 22]. As these oxaliplatin  
18 metabolites have previously been suggested to contribute to oxaliplatin-induced neuropathy,  
19 we sought to characterize the contribution of oxalate and the oxaliplatin metabolite  
20 Pt(DACH)Cl<sub>2</sub> to oxaliplatin-induced cold allodynia in our model. We found that intraplantar  
21 injection of equivalent doses of Pt(DACH)Cl<sub>2</sub>, (2.5 mM; 38 µg) or oxalate (2.5 mM; 14 µg)  
22 did not cause cold allodynia (Fig. 2a). However, injection of a higher dose of oxalate (150  
23 mM; 810 µg/paw) caused short-lived (< 1 h) spontaneous nocifensive behaviour, evidenced  
24 by lifting, licking and shaking of the paw (Fig. 2b), as well as mechanical allodynia which  
25 remained apparent 24 h after a single intraplantar injection of oxalate (Fig. 2c; Control,  $4.4 \pm$



1 0.5 g; oxalate  $0.9 \pm 0.3$  g). Consistent with the ability of oxalate to chelate  $\text{Ca}^{2+}$  [24], both the  
2 spontaneous pain ( $29.1 \pm 5.1$  flinches/5 min) and mechanical allodynia ( $2.6 \pm 0.3$  g) were  
3 mimicked by intraplantar injection of the  $\text{Ca}^{2+}$  chelator BAPTA (10 mM; 191  $\mu\text{g}$ ) (Fig. 2b  
4 and 2c). In contrast, intraplantar injection of the cell membrane-permeable BAPTA-AM [49]  
5 (10  $\mu\text{M}$ ; 310 ng) had no effect on spontaneous nocifensive behaviour and did not elicit cold  
6 allodynia (Fig. 2a-c). The effect of oxalate and BAPTA on sensory nerve endings likely arises  
7 from the destabilizing effect of  $\text{Ca}^{2+}$  chelation on neuronal membranes, with removal of  
8 extracellular  $\text{Ca}^{2+}$  leading to increased excitability by decreasing the threshold potential and  
9 membrane resistance, and increasing  $\text{Na}^+$  conductance [20]. These effects have been shown  
10 in *ex vivo* preparations to result in spontaneous action potential discharge and an increase in  
11 mean firing frequency [25; 43], corroborating the proalgesic effect of extracellular  $\text{Ca}^{2+}$   
12 chelation we observed in our animal model.

13

#### 14 *Oxaliplatin-induced cold allodynia is mediated through $\text{Na}_v1.6$ -expressing peripheral sensory* 15 *fibers*

16  $\text{Na}_v$  channels are critical for the propagation of action potentials in excitable cells, including  
17 peripheral sensory nerves. The tetrodotoxin-resistant isoform  $\text{Na}_v1.8$  in particular is crucial  
18 for neuronal excitability at cold temperatures and is essential for noxious cold pain [61]. Thus,  
19 we sought to elucidate the contribution of  $\text{Na}_v1.8$  to oxaliplatin-induced cold allodynia.  
20 Surprisingly, the development of cold allodynia was unchanged in  $\text{Na}_v1.8^{-/-}$  animals, and was  
21 also not affected by A803467, a  $\text{Na}_v1.8$ -selective small molecule inhibitor (Fig. 3a;  $\text{Na}_v1.8^{-/-}$ ,  
22  $98 \pm 15\%$  of control; A803467 (10  $\mu\text{M}$ ),  $90 \pm 17\%$  of control). Similarly, the tetrodotoxin-  
23 resistant  $\text{Na}_v1.9$  has previously been suggested to contribute to the pathogenesis of  
24 neuropathic pain and cold allodynia [29]. However, in our model the cold allodynia was  
25 unchanged in  $\text{Na}_v1.9^{-/-}$  animals (Fig. 3a;  $107 \pm 8\%$  of control), suggesting that TTX-sensitive

1  $\text{Na}_v$  isoforms are crucial for the development of oxaliplatin-induced cold allodynia. Indeed,  
2 intraplantar injection of low concentrations of TTX (3  $\mu\text{M}$ ) inhibited nocifensive responses  
3 upon exposure to a surface cooled to 10°C (Fig. 3a;  $19 \pm 4\%$  of control). We thus assessed the  
4 contribution of  $\text{Na}_v1.3$  and  $\text{Na}_v1.7$  using knockout animals or subtype-selective inhibitors.  
5 Surprisingly, the development of cold allodynia was also unchanged in  $\text{Na}_v1.3^{-/-}$  animals ( $103$   
6  $\pm 11\%$  of control), or after intraplantar injection of the  $\text{Na}_v1.7$ -selective inhibitor ProTxII (3  
7 nM;  $115 \pm 17\%$  of control) (Fig. 3b), suggesting involvement of  $\text{Na}_v$  isoforms not typically  
8 attributed behavioural roles in pain pathways. In addition to  $\text{Na}_v1.3$ ,  $\text{Na}_v1.7$ ,  $\text{Na}_v1.8$  and  
9  $\text{Na}_v1.9$ , DRG neurons are known to express other tetrodotoxin-sensitive isoforms, including  
10  $\text{Na}_v1.1$ ,  $\text{Na}_v1.2$  and  $\text{Na}_v1.6$ . Since knockout mouse models of these  $\text{Na}_v$  isoforms are lethal,  
11 we used a range of conotoxins with activity at  $\text{Na}_v$  isoforms that allowed dissection of the  
12 contribution of these isoforms to oxaliplatin-induced pain pathways.  $\mu$ -Conotoxin TIIIA  
13 specifically inhibits  $\text{Na}_v1.2$  and  $\text{Na}_v1.4$  at low concentrations, while at high concentrations  
14  $\text{Na}_v1.1$ , but not  $\text{Na}_v1.6$ , is also inhibited [55; 59] (Fig. 3c;  $\text{Na}_v1.6$   $\text{pIC}_{50}$   $6.0 \pm 0.3$ ). Intraplantar  
15 injection of both low (100 nM) or high (10  $\mu\text{M}$ ) concentrations of TIIIA did not significantly  
16 decrease oxaliplatin-induced cold allodynia (Fig. 3 D), suggesting a crucial role for  $\text{Na}_v1.6$  in  
17 cold pain pathways activated by oxaliplatin. Indeed, intraplantar injection of GIIIA (10  $\mu\text{M}$ ),  
18 which in addition to  $\text{Na}_v1.1$  also inhibits  $\text{Na}_v1.6$  at high concentrations, but has no effect on  
19  $\text{Na}_v1.3$  and  $\text{Na}_v1.7$  [55], achieved near complete reversal of oxaliplatin-induced cold allodynia  
20 ( $14 \pm 9\%$  of control) (Fig. 3d). In contrast, nocifensive behavior elicited by intraplantar  
21 administration of the TRPA1 agonist AITC (allyl isothiocyanate, 5mM) was not affected by  
22 GIIIA (30  $\mu\text{M}$ ; Fig 3e). Thus, this demonstrates for the first time a functional contribution of  
23  $\text{Na}_v1.6$  to cold pain pathways at the behavioural level.

24

25 *Oxaliplatin-induced cold allodynia develops independently of cold-sensitive TRP channels*

1 In peripheral sensory neurons, cold stimuli are transformed to electrical signals through  
2 activation of thermosensitive TRP channels, notably TRPM8, TRPA1 and TRPC5. We thus  
3 sought to elucidate the contribution of cold-sensitive TRP channels to the development of  
4 cold allodynia in our novel model of acute oxaliplatin-induced neuropathy. Surprisingly,  
5 oxaliplatin-induced cold allodynia was unaffected in TRPM8<sup>-/-</sup> animals (128 ± 17% of  
6 control) or by the TRPM8-selective inhibitors AMTB (10 μM; 107 ± 13% of control) and  
7 M8-B (1 μM; 108 ± 13% of control) (Fig. 4). Cold allodynia was also not significantly  
8 decreased in TRPA1<sup>-/-</sup> animals (115 ± 18% of control) or after treatment with the TRPA1  
9 antagonist HC030031 (100 μM; 76 ± 14% of control), and developed normally in TRPC5<sup>-/-</sup>  
10 animals (data not shown) (Fig. 4). Thus, alternative mechanisms to transform a cool stimulus  
11 to an electrical signal are likely to contribute to oxaliplatin-induced cold allodynia.

12  
13 It is known that cooling alters the membrane properties of excitable cells, leading to increased  
14 input resistance which in turn brings cells closer to the spiking threshold [23; 54]. In addition,  
15 in some sensory and central neurons, cooling elicits enhanced excitability and increased and  
16 increased firing frequency as a result of cold-induced closure of background potassium  
17 channels [3; 15; 34; 39; 53]. This effect appears to be opposed by continued activity of K<sub>v</sub>1  
18 channels which act as an excitability break and regulate cold sensitivity in trigeminal neurons  
19 in concert with TRPM8 [33; 53].

20 Since it is known that oxaliplatin inhibits potassium channels [27] in addition to sodium  
21 channels [2; 8; 24; 28; 47], we assessed if the oxaliplatin-induced effects could be replicated  
22 by inhibition of delayed rectifier potassium channels in sensory nerve endings. Indeed,  
23 intraplantar injection of 4-AP (1 mM) elicited cold allodynia (35.2 ± 8.6 flinches/5 min)  
24 which was not affected by intraplantar injection of the TRPA1 inhibitor HC030031 (100 μM;  
25 37.4 ± 13.4 flinches/5 min) or the TRPM8 inhibitor AMTB (10 μM; 46.6 ± 10.5 flinches/5

1 min) (Fig. 5a). Like oxaliplatin-induced cold allodynia, enhanced nocifensive responses to  
2 cold elicited by 4-AP were inhibited by the  $\text{Na}_v1.6$  inhibitor GIIIA ( $8.6 \pm 4.4$  flinches/5 min),  
3 confirming an important role for  $\text{Na}_v1.6$  in cold pain pathways (Fig. 5 A).

4

#### 5 *Pain behaviors induced by selective $\text{Na}_v1.6$ activation*

6 To further characterize the role of  $\text{Na}_v1.6$  in pain pathways, we also assessed spontaneous  
7 pain behaviours, thermal allodynia and mechanical allodynia after intraplantar injection of the  
8  $\text{Na}_v1.6$ -selective activator Cn2. Cn2 is a  $\beta$ -scorpion toxin isolated from the venom of the  
9 scorpion *Centruroides noxius* that specifically enhances activity of  $\text{Na}_v1.6$  with an  $\text{EC}_{50}$  of 39  
10 nM, causing a leftward shift of the voltage-dependence of activation and a transient resurgent  
11 current [44]. Intraplantar injection of Cn2 elicited dose-dependent spontaneous pain  
12 characterized by licking, lifting and vigorous shaking of the injected paw that was transient  
13 for lower concentrations (1 nM, < 15 min). At the highest concentration tested (30 nM; Fig.  
14 5b), the frequency and severity of these responses rapidly diminished after injection, although  
15 some nocifensive behaviours remained evident for > 4 h after intraplantar administration.  
16 Thus, for subsequent experiments, low concentrations of Cn2 were utilized.

17 Intraplantar Cn2 (1 nM) did not elicit thermal allodynia, with little or no nocifensive  
18 behaviour evident at 10°C or 42°C (Fig. 5c) but caused significant ( $p < 0.01$ ) mechanical  
19 allodynia, evidenced by decreased paw withdrawal thresholds to mechanical stimulation (Fig.  
20 5d; Control,  $5.4 \pm 0.4$  g; Cn2 (1 nM),  $2.8 \pm 0.6$  g). To examine the contribution of potassium  
21 channels to  $\text{Na}_v1.6$ -dependent cold-pain, we co-administered Cn2 (10 nM) with 4-AP (500  
22  $\mu\text{M}$ ) by intraplantar injection. As opposed to Cn2 alone, this combination potentiated the cold  
23 allodynia produced by 4-AP (Fig. 5e; 4-AP,  $12.0 \pm 3.1$  flinches/5 min; 4-AP + Cn2,  $38.0 \pm$   
24  $8.2$  flinches/5 min), providing evidence that activation of  $\text{Na}_v1.6$  *per se* produced only

1 spontaneous pain and mechanical allodynia, but when combined with inhibition of delayed  
2 rectifier potassium channels could enhance cold allodynia.

3

#### 4 **Discussion**

5 Acute oxaliplatin-induced neuropathy occurs in almost all patients and manifests as  
6 circumoral and distal sensory and/or motor disturbances including paraesthesias and  
7 dysaesthesias and muscle fasciculations. These symptoms are triggered by exposure to cold  
8 and are associated with a significant reduction in the cold pain threshold [6]. However, the  
9 pathophysiological basis of acute oxaliplatin-induced neuropathy, in particular cold allodynia,  
10 is poorly understood. This is in part due to a paucity of animal models that accurately reflect  
11 the neuropathic symptomatology encountered clinically and, specifically, the rapid onset of  
12 cold allodynia. To better understand chemically-induced cold allodynia, we established an  
13 animal model of chemotherapy-induced peripheral neuropathy based on the intraplantar  
14 injection of oxaliplatin. This model supports a direct excitatory action of oxaliplatin on  
15 peripheral sensory nerve endings as the causative mechanism underlying oxaliplatin-induced  
16 neuropathy, with cold allodynia becoming evident within minutes and persisting for several  
17 days after a single local injection of oxaliplatin. We were able to show that inhibition of  
18 potassium channels leads to increased neuronal excitability at low temperatures independent  
19 of activation of TRP channels, and that peripheral  $\text{Na}_v1.6$  was crucial for the propagation of  
20 these signals and the appearance of cold allodynia.

21

22 The metabolism of oxaliplatin is complex and involves rapid hydrolysis ( $t_{1/2\alpha} \sim 14$  min) to  
23 oxalate and various bioactive platinum compounds, which in turn form adducts with DNA,  
24 proteins, peptides and amino acids that undergo slow, triphasic elimination through  
25 predominantly renal routes, with a long terminal half-life for platinum of up to 11 days [18];

1 22]. It is difficult to estimate the concentration of oxaliplatin that sensory neurons are exposed  
2 to in human patients. While the plasma concentration of free oxaliplatin after intravenous  
3 administration is relatively low [18; 22], consistent with an apparent large volume of  
4 distribution, oxaliplatin is likely to accumulate in sensory neurons through active transport by  
5 copper transporters and the L-carnitine transporter OCTN1 [26; 31].

6 Given the rapid onset and prolonged nature of the sensory disturbances associated with  
7 oxaliplatin infusion [5], contribution of various oxaliplatin metabolites to the development of  
8 peripheral neuropathy has been suggested. In DRG explants, Pt(DACH)Cl<sub>2</sub> was more  
9 neurotoxic than oxaliplatin [32], while after repeated intraperitoneal administration, oxalate  
10 elicited cold hyperalgesia and increased paw withdrawal responses to application of acetone,  
11 but not mechanical allodynia [42]. We thus assessed the effect of equimolar doses of oxalate  
12 and Pt(DACH)Cl<sub>2</sub>, two major oxaliplatin metabolites, on the development of cold and  
13 mechanical allodynia after intraplantar injection. However, while neither metabolite elicited  
14 cold allodynia after a single local injection, Ca<sup>2+</sup> chelation by oxalate elicited spontaneous  
15 nocifensive behaviour as well as prolonged mechanical allodynia. This effect was mimicked  
16 by intraplantar injection of BAPTA and can be attributed to the effects of extracellular Ca<sup>2+</sup>  
17 removal on membrane properties, including decreased threshold potential and membrane  
18 resistance, as well as increased Na<sup>+</sup> conductance [20].

19

20 Acute oxaliplatin-induced neuropathy has been postulated to involve the modulation of  
21 axonal Na<sub>v</sub> channels, based on the observation that oxaliplatin infusion elicited changes in  
22 Na<sub>v</sub>-dependent variables in humans [28; 36]. Similarly, in rat DRG neurons, oxaliplatin  
23 resulted in increased Na<sup>+</sup> currents and a shift of the voltage-response relationship towards  
24 more negative potentials, [2] with similar effects observed in cockroach neurons and frog  
25 myelinated axons [8; 24].

1  
2 The lack of contribution of Na<sub>v</sub>1.8 to oxaliplatin-induced cold allodynia, which we  
3 demonstrated in both Na<sub>v</sub>1.8 knockout animals and after intraplantar administration of the  
4 Na<sub>v</sub>1.8-selective inhibitor A803467, was surprising given the crucial role of this Na<sub>v</sub> isoform  
5 in cold pain. Specifically, Na<sub>v</sub>1.8 in nociceptive peripheral sensory neurons has previously  
6 been demonstrated to be critical for the development of pain evoked by noxious cold [61], and  
7 in mice with diphtheria toxin-mediated ablation of Na<sub>v</sub>1.8-expressing nociceptors, noxious  
8 cold responses are virtually abolished [1]. Oxaliplatin induces repetitive firing, broadening of  
9 the repolarization phase and after-hyperpolarization in myelinated A-fibers, while non-  
10 myelinated C-fibers remain largely unaffected [2; 27; 46; 47]. In contrast, although Na<sub>v</sub>1.8 is  
11 widely expressed in peripheral sensory neurons, including a subpopulation of myelinated A-  
12 fibers [45], its contribution to cold-evoked pain behavior arises mainly from nociceptive C-  
13 fibers [1; 52; 61]. Thus, our finding that Na<sub>v</sub>1.8 does not contribute to oxaliplatin-induced  
14 cold allodynia can be explained by the differential expression of Na<sub>v</sub> isoforms in peripheral  
15 sensory nerve fibers that contribute to the pathophysiology of oxaliplatin-induced cold  
16 allodynia. The role of Na<sub>v</sub>1.8 in pathological cold pain appears to be different from its role in  
17 physiological cold pain [61], and in addition differs to other models of chemically-induced  
18 cold allodynia, such as ciguatoxin-induced cold pain, where Na<sub>v</sub>1.8 still contributes  
19 significantly to pain behaviours [52].

20  
21 Consistent with a major role for Na<sub>v</sub>1.6 in the propagation of action potentials in myelinated  
22 A-fibers [55; 59], we found no contribution of Na<sub>v</sub>1.3, Na<sub>v</sub>1.7 and Na<sub>v</sub>1.9 to oxaliplatin-  
23 induced cold allodynia, while pharmacological inhibition of Na<sub>v</sub>1.6 virtually abolished pain  
24 behaviour. Supporting a crucial role for Na<sub>v</sub>1.6 in oxaliplatin-induced cold allodynia is the  
25 observation that *in vitro*, oxaliplatin elicits Na<sub>v</sub>1.6-mediated resurgent currents and that

1 oxaliplatin-induced A-fiber effects were abolished in Na<sub>v</sub>1.6 knockout animals [47]. Similarly,  
2 we observed significant potentiation of veratridine-induced Na<sub>v</sub>1.6 responses in our  
3 membrane potential assay (data not shown), consistent with the previously reported effect of  
4 oxaliplatin on fast inactivation [47].

5  
6 Thus, we have obtained the first evidence that Na<sub>v</sub>1.6 expressed in peripheral sensory neurons  
7 contributes to cold pain behaviours. However, since Na<sub>v</sub>1.6 is highly expressed at nodes of  
8 Ranvier in both peripheral sensory and motor axons, as well as nodes in the central nervous  
9 system [10], Na<sub>v</sub>1.6 would be difficult to target therapeutically. Indeed, mice with loss-of-  
10 function mutations in Scn8a, the gene encoding for Na<sub>v</sub>1.6, are characterized by early onset  
11 progressive paralysis of the hind limbs, leading to juvenile lethality at approximately  
12 postnatal day 20 [9]. Thus, the results presented here support a role for Na<sub>v</sub>1.6 in pathological  
13 pain states. Future experiments in sensory fiber-specific knockout model would be valuable to  
14 further dissect the role of Na<sub>v</sub>1.6 in pain pathways.

15  
16 Thermosensitive TRP channels, in particular TRPM8, TRPA1 and TRPC5, are expressed in  
17 peripheral sensory neurons and are activated by cooling [37; 48; 62]. Chronic administration  
18 of oxaliplatin in animal models has been shown to elicit changes in TRP channel expression,  
19 and both TRPM8 and TRPA1 have been causally implied in the development of  
20 chemotherapy-induced cold allodynia [21; 35; 60]. However, the rapid onset of cold  
21 allodynia both clinically and in our novel model of acute oxaliplatin-induced cold allodynia  
22 suggests that changes in the expression level of TRP channels are unlikely to contribute to the  
23 observed symptomatology. Indeed, we found no significant effect of TRPA1, TRPM8 or  
24 TRPC5 to oxaliplatin-induced cold allodynia using both genetically modified animals and  
25 pharmacological modulators where possible. This finding is consistent with the



1 predominantly A-fiber-mediated origin of oxaliplatin-induced cold allodynia, as cold-  
2 sensitive TRP channels are expressed predominantly on peptidergic and isolectin B4-positive  
3 C-fibers [17; 48; 62].

4 Activation of TRPM8 by cooling has been demonstrated in peripheral sensory neurons,  
5 trigeminal neurons and corneal neurons [7; 11; 17] . In addition, heterologously expressed  
6 TRPM8 is also activated by cooling, and a role for TRPM8 has been demonstrated in  
7 environmental cold sensing as well as noxious cold pain in several behavioural studies [7; 13].  
8 We found a significant response to acetone in naïve C57BL/6 mice, consisting of vigorous  
9 shaking, licking and aversive behaviours, and for this reason chose to assess cold pain  
10 behaviour by exposure to a temperature-controlled plate. Previous studies have also reported  
11 sensitivity to acetone in naïve animals, which was decreased in TRPM8 knockout animals  
12 [13]. This observation could account for the effect of TRPM8 on oxaliplatin-induced cold  
13 allodynia previously reported.

14  
15 An alternative mechanism of inducing cold sensitivity is based on inhibition of potassium  
16 channels. In addition to modulation of  $Na_v$ , oxaliplatin also inhibits neuronal potassium  
17 channels [8; 27]. In peripheral myelinated fibers, the effects of oxaliplatin on compound  
18 action potentials were similar to those of 4-AP [27]. 4-AP inhibits delayed-rectifier channels,  
19 including  $K_v1.1$  and  $K_v1.2$  which have been shown to be highly expressed in cold-insensitive  
20 neurons and contribute to lack of cold-sensitivity in trigeminal neurons [33; 50; 53]. Indeed,  
21 intraplantar injection of 4-AP caused behavioural responses similar to oxaliplatin, and elicited  
22 cold allodynia that was not modulated by inhibition of TRPM8 or TRPA1, but was decreased  
23 by pharmacological inhibition of  $Na_v1.6$ . These findings are consistent with  $K_v1.1$  and  $K_v1.2$   
24 being expressed predominantly in large DRG neurons which give rise to myelinated A-fibers  
25 [38], and corroborate an important role for  $K_v$  channels in cold sensing and cold allodynia.

1 Indeed, activation of Na<sub>v</sub>1.6 alone was not sufficient to elicit cold allodynia, with the Na<sub>v</sub>1.6-  
2 specific scorpion toxin Cn2 eliciting spontaneous pain and mechanical allodynia but not cold  
3 allodynia. However, when combined with inhibition of K<sub>v</sub> channels, Cn2 produced profound  
4 enhancement of 4-AP-induced cold allodynia. Thus Cn2 not only confirms the pivotal role  
5 played by Na<sub>v</sub>1.6 in chemically-induced cold pain but reveals a role for Na<sub>v</sub>1.6 in spontaneous  
6 pain and mechanical allodynia. In conclusion, the new animal model of oxaliplatin-induced  
7 cold allodynia described here reveals an important role for Na<sub>v</sub>1.6 in pain pathways, with  
8 chemically-induced cold allodynia mediated through inhibition of potassium channels on  
9 Na<sub>v</sub>1.6-expressing peripheral sensory fibers.

10

#### 11 **Acknowledgements**

12 This work was supported by an NHMRC Australian Biomedical Postdoctoral Fellowship  
13 (569918, IV), NHMRC Fellowship (APP1019761, RJL) and Australian Postgraduate Award  
14 (JRD). Funding for this project was obtained through an Australian Research Council LIEF  
15 grant for the FLIPR<sup>TETRA</sup>, a Cancer Council Research Grant (IV), an NHMRC project grant  
16 (IV, KZ), an NHMRC Program Grant (RJL) and a National Institutes of Health grant  
17 R01NS41233 (AAR).

18

#### 19 **Conflict of interests**

20 AAR has consulted for TRP programs at several pharmaceutical companies, and his TRP-  
21 related research has been supported by Amgen, Inc., Abbott Laboratories, and AbbVie.

22

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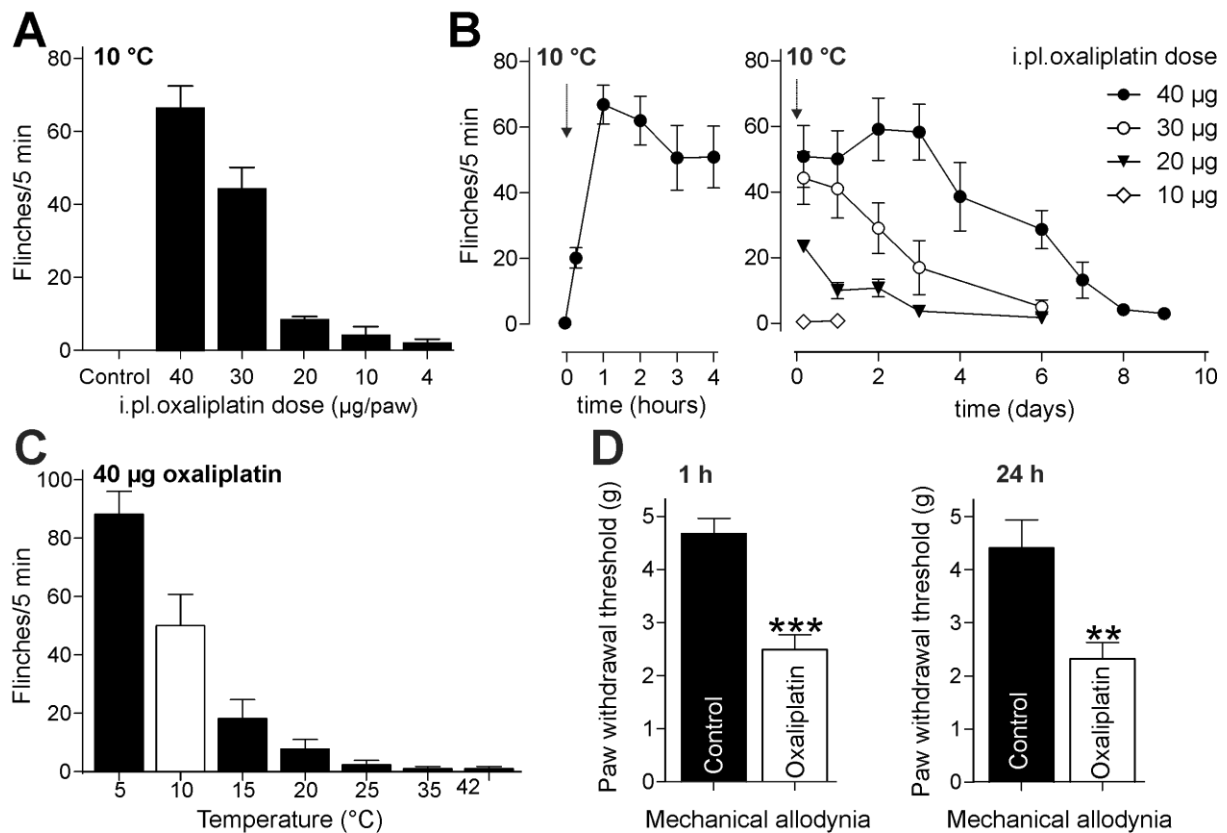
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23

1 **Figure captions**

**Figure 1**



2 **Figure 1. A novel animal model of chemotherapy-induced neuropathy based on the**

3 **intraplantar injection of oxaliplatin. (a)** Intraplantar injection of oxaliplatin (4 – 40 µg/paw)

4 rapidly elicits cold allodynia, with increased paw lifting, licking, shaking and flinching

5 evident 1 h after injection upon exposure to a temperature-controlled surface maintained at

6 10°C. Injection of vehicle (Control; 5% glucose/H<sub>2</sub>O) did not elicit any nocifensive responses.

7 **(b)** Oxaliplatin-induced cold allodynia has a rapid onset (left panel, 0-4 h post-injection), with

8 nocifensive responses upon exposure of the injected hind paw to cool temperatures becoming

9 apparent within minutes after injection (arrow). Cold allodynia after a single injection persists

10 for several days (right panel, 4 h - 9 days post-injection) after intraplantar injection (arrow).

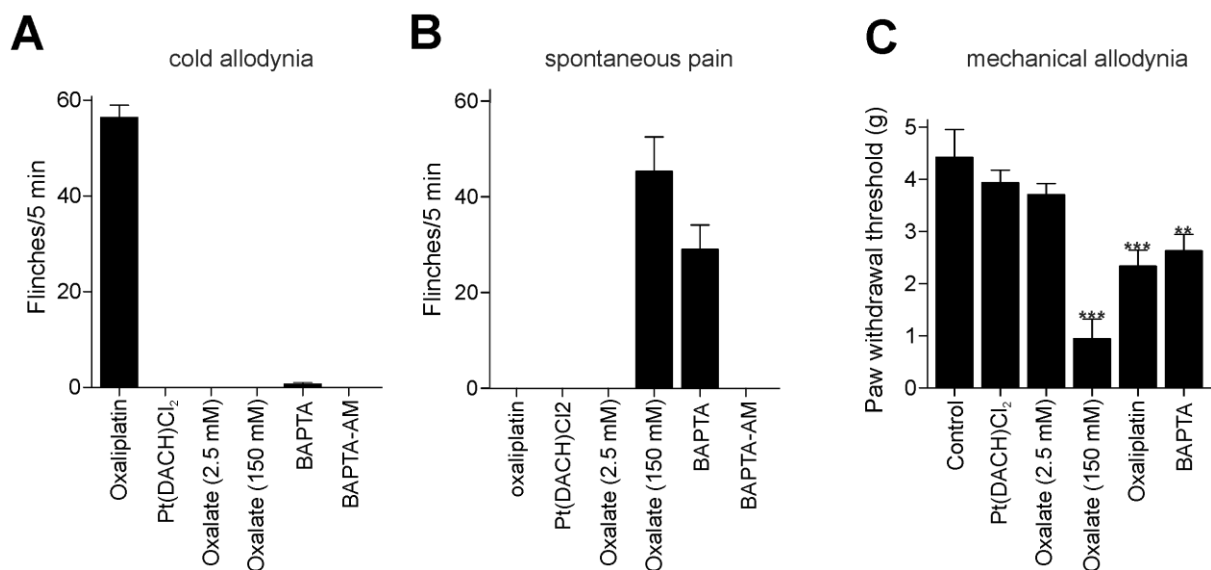
11 **(c)** Nocifensive responses evoked by intraplantar injection of oxaliplatin (40 µg/paw) are

12 temperature-dependent, with significant paw withdrawals elicited upon exposure to

13 temperatures below 15°C (24 h after injection). No withdrawal responses were evident at

1 elevated temperatures up to 42°C. White bar; for all subsequent experiments, cold allodynia  
 2 was assessed 24 h after injection of 40 µg oxaliplatin/paw by quantifying paw withdrawal  
 3 responses at 10°C. (d) Intraplantar injection of oxaliplatin (40 µg/paw) elicited mild  
 4 mechanical allodynia, with a significant decrease in paw withdrawal threshold to mechanical  
 5 stimulation compared to control (5% glucose/H<sub>2</sub>O). Left panel, decreased mechanical  
 6 threshold was apparent at 1 h after injection and persisted 24 h after injection (right panel).  
 7 Statistical significance was determined using an unpaired Student's t-test; \*\*\*, *p* < 0.001; \*\*,   
 8 *p* < 0.01 compared to vehicle. Data are presented as mean ± SEM (n = 5- 12 animals/group).  
 9

## Figure 2



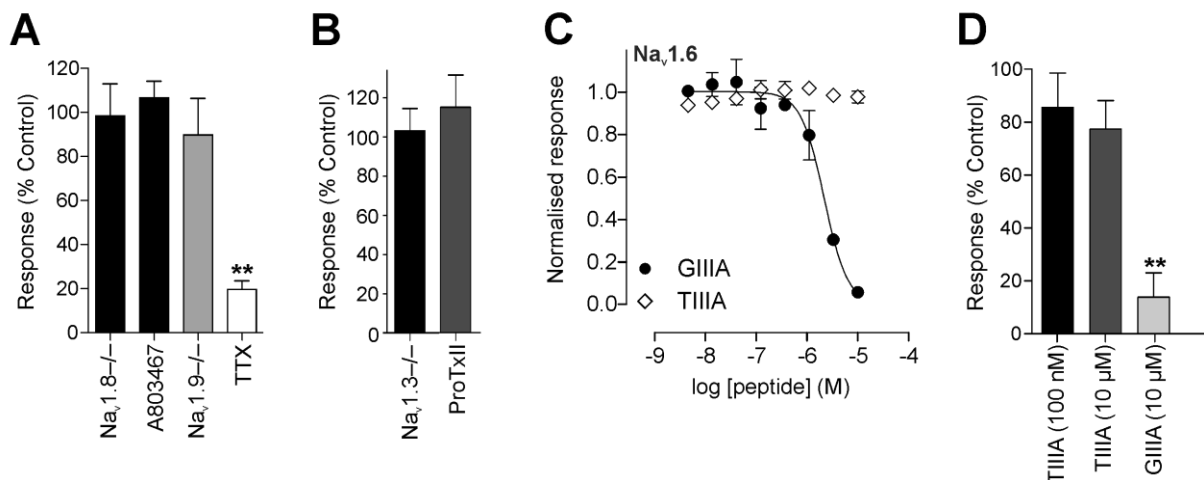
10  
 11 **Figure 2. Oxaliplatin metabolites contribute to mechanical, but not cold allodynia. (a)**

12 Intraplantar injection of equimolar doses of the oxaliplatin metabolites Pt(DACH)Cl<sub>2</sub> (2.5  
 13 mM; 38 µg/paw) and oxalate (2.5 mM; 14 µg/paw) did not elicit paw withdrawal responses at  
 14 10°C, compared to the pronounced cold allodynia elicited by oxaliplatin (2.5 mM; 40  
 15 µg/paw). Cold allodynia was also not elicited by intraplantar injection of the Ca<sup>2+</sup> chelator  
 16 BAPTA (10 mM; 191 µg/paw) or the membrane-permeable BAPTA-AM (10 µM; 310  
 17 ng/paw). (b) Intraplantar injection of high doses of oxalate (150 mM; 810 µg/paw) and

1 BAPTA (10 mM; 191  $\mu\text{g/paw}$ ), but not low doses of oxalate, oxaliplatin, BAPTA-AM or  
 2  $\text{Pt(DACH)Cl}_2$ , elicited short-lasting ( $< 1$  h) spontaneous nocifensive behaviour (increased  
 3 number of paw lifts, licks, shakes and flinches) evident at room temperature. (c) Intraplantar  
 4 injection of high doses of oxalate (150 mM; 810  $\mu\text{g/paw}$ ), BAPTA (10 mM; 191  $\mu\text{g/paw}$ ) and  
 5 oxaliplatin (2.5 mM; 40  $\mu\text{g/paw}$ ) caused a significant decrease in the paw withdrawal  
 6 threshold to mechanical stimulation, while mechanical responses were unchanged after  
 7 intraplantar injection of equimolar doses of  $\text{Pt(DACH)Cl}_2$ , and oxalate. Statistical  
 8 significance was determined using a one-way ANOVA with Dunnett's post test. \*\*\*,  $p <$   
 9 0.001; \*\*,  $p < 0.01$  compared to Control (vehicle). Data are presented as mean  $\pm$  SEM (n = 3  
 10 – 5) animals/group.

11

Figure 3



12

13 **Figure 3. Na<sub>v</sub> isoforms involved in the development of cold allodynia after intraplantar**

14 **injection of oxaliplatin. (a)** Cold allodynia induced by intraplantar injection of oxaliplatin

15 (24 h after injection of 2.5 mM oxaliplatin; 40  $\mu\text{g/paw}$ ) was not significantly different from

16 control in Na<sub>v</sub>1.8<sup>-/-</sup> animals, or after intraplantar injection of the Na<sub>v</sub>1.8 inhibitor A803467 (10

17  $\mu\text{M}$ ). Paw flinches were also not significantly different from control in Na<sub>v</sub>1.9<sup>-/-</sup> animals, but

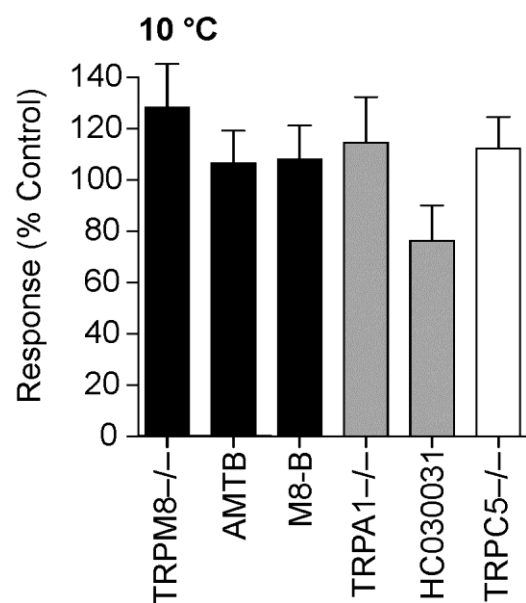
18 were significantly ( $p < 0.01$ ) inhibited by intraplantar injection of TTX (3  $\mu\text{M}$ ). (b)

19 Oxaliplatin-induced cold allodynia was not significantly different from control in Na<sub>v</sub>1.3<sup>-/-</sup>

1 animals, or after intraplantar injection of the Na<sub>v</sub>1.7 inhibitor ProTxII (3 nM). (c) μ-Conotoxin  
2 GIIIA concentration-dependently (pIC<sub>50</sub> 6.0 ± 0.3) inhibits Na<sub>v</sub>1.6, while TIIIA does not affect  
3 Na<sub>v</sub>1.6-mediated responses. Effect of μ-conotoxins on veratridine (50 μM)-induced Na<sub>v</sub>1.6  
4 responses was assessed using a FLIPR membrane-potential assay in HEK cells heterologously  
5 expressing Na<sub>v</sub>1.6. (d) Intraplantar injection of TIIIA at concentrations which inhibit Na<sub>v</sub>1.2  
6 (100 nM), or Na<sub>v</sub>1.1 but not Na<sub>v</sub>1.6 (10 μM) did not significantly decrease oxaliplatin-induced  
7 cold allodynia. In contrast, GIIIA at a concentration which fully inhibits Na<sub>v</sub>1.6 (10 μM)  
8 caused near complete inhibition of cold allodynia. (e) GIIIA (30 μM) had no effect on  
9 nocifensive behaviours elicited by intraplantar administration of the TRPA1 agonist AITC (5  
10 mM). Data is presented relative to vehicle-injected wild-type animals or age-matched litter  
11 controls. Statistical significance was determined using a one-way ANOVA with Dunnett's  
12 post test. \*\*, *p* < 0.01 compared to Control (vehicle or age-matched litter controls). Data are  
13 presented as mean ± SEM (n = 4 – 8 animals/group).

14

## Figure 4



15

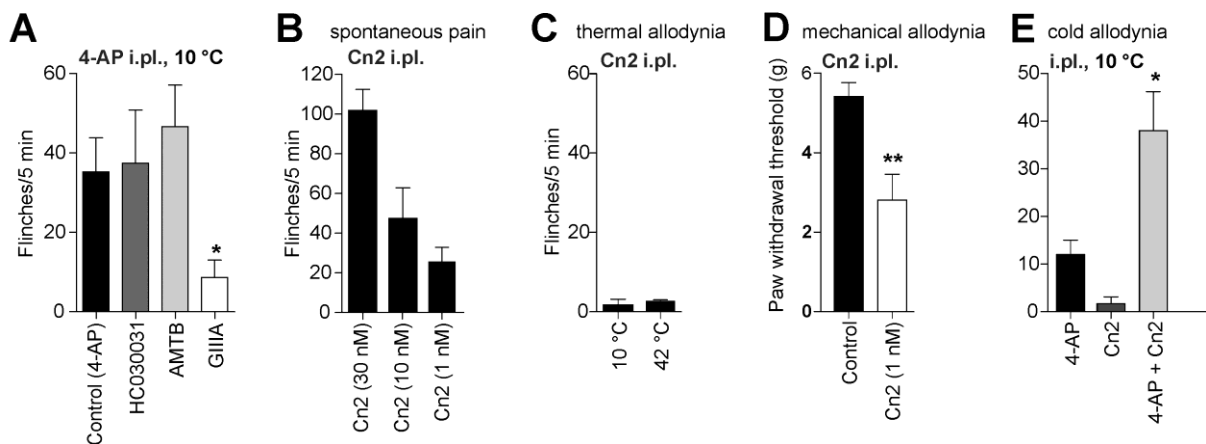
Figure 4. Oxaliplatin-induced cold allodynia

16

develops independently of cold-sensitive TRP channels. Oxaliplatin-induced cold allodynia

1 was not changed in TRPM8<sup>-/-</sup> animals or after intraplantar injection of the TRPM8  
 2 antagonists AMTB (10 μM) and M8-B (1 μM). Similarly, no significant difference in the  
 3 number of paw flinches was observed in TRPA1<sup>-/-</sup> animals or after intraplantar injection of  
 4 the TRPA1 antagonist HC030031 (100 μM). Statistical significance was determined using a  
 5 one-way ANOVA with Dunnett's post test. Data are presented as mean ± SEM (n = 5–10  
 6 animals/group).

7  
 8  
 Figure 5



9  
 10 **Figure 5. Inhibition of potassium channels on Na<sub>v</sub>1.6-expressing pain pathways elicits**

11 **cold allodynia.** (a) Intraplantar injection of the potassium channel inhibitor 4-AP (1 mM)  
 12 elicited cold allodynia, evidenced by an increased number of paw lifts, licks, shakes and  
 13 flinches on exposure to a temperature-controlled plate maintained at 10°C. Cold allodynia  
 14 induced by 4-AP was not significantly inhibited by concomitant intraplantar injection of the  
 15 TRPM8 antagonist AMTB (10 μM) or the TRPA1 antagonist HC030031 (100 μM), but was  
 16 significantly (*p* < 0.05) inhibited by intraplantar μ-conotoxin GIIIA (10 μM). (b) Intraplantar  
 17 injection of Cn2 (1 nM – 30 nM) elicited dose-dependent spontaneous pain. Responses were  
 18 quantified by counting the number of behaviours immediately after injection (c) Activation of  
 19 peripheral Na<sub>v</sub>1.6 by Cn2 (1 nM) did not elicit cold (10°C) or heat (42°C) allodynia. (d)

1 Intraplantar Cn2 (1 nM) caused significant ( $p < 0.01$ ) mechanical allodynia, with the paw  
2 withdrawal threshold to mechanical stimulation decreased from  $5.4 \pm 0.4$  g (control) to  $2.8 \pm$   
3  $0.6$  g (Cn2, 1 nM). (e) Intraplantar injection of the  $\text{Na}_v1.6$  activator Cn2 (10 nM) alone did not  
4 elicit significant cold allodynia ( $1.7 \pm 1.5$  flinches/5 min at  $10^\circ\text{C}$ ), but significantly ( $p < 0.05$ )  
5 potentiated cold allodynia elicited by intraplantar injection of 4-AP ( $500 \mu\text{M}$ ;  $12.0 \pm 3.1$   
6 flinches/5 min at  $10^\circ\text{C}$ ) when co-administered (4-AP + Cn2;  $38.0 \pm 8.2$  flinches/5 min).  
7 Statistical significance was determined using a one-way ANOVA with Dunnett's post test. \*,  
8  $p < 0.05$ ; \*\*,  $p < 0.01$  compared to control. Data are presented as mean  $\pm$  SEM (n = 4 – 7  
9 animals/group).

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