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An animal model of oxaliplatin-induced cold allodynia reveals a crucial role for $Na_v1.6$ in peripheral pain pathways

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Abstract

Cold allodynia, pain in response to cooling, occurs during or within hours of oxaliplatin infusion and is thought to arise from a direct effect of oxaliplatin on peripheral sensory neurons. To characterize the pathophysiological mechanisms underlying acute oxaliplatin-induced cold allodynia, we established a new intraplantar oxaliplatin mouse model that rapidly developed long-lasting cold allodynia mediated entirely through tetrodotoxin-sensitive Na_v pathways. Using selective inhibitors and knockout animals, we found that $Na_v1.6$ was the key isoform involved, while thermosensitive transient receptor potential channels were not involved. Consistent with a crucial role for delayed-rectifier potassium channels in excitability in response to cold, intraplantar administration of the K^+-channel blocker 4-aminopyridine mimicked oxaliplatin-induced cold allodynia and was also inhibited by Na_vl.6 blockers. Intraplantar injection of the $Na_v1.6$-activator Cn2 elicited spontaneous pain, mechanical allodynia and enhanced 4-aminopyridine-induced cold allodynia. These findings provide behavioural evidence for a crucial role of $Na_v1.6$ in multiple peripheral pain pathways including cold allodynia.

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Conflict of interests

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

Introduction

Oxaliplatin, a third-generation platinum chemotherapeutic agent, is associated with acute dose-limiting neurotoxicity, which manifests as cooling-induced peripheral dysaesthesias and paraesthesias including cold allodynia [6; 12]. Acute oxaliplatin-induced cold allodynia is characterized by a rapid onset, with symptoms occurring during or shortly after infusion, and typically resolves within several days of treatment [5]. Many currently used animal models of oxaliplatin-induced neuropathy poorly reflect these characteristics, and often require multiple injections of oxaliplatin to elicit pain behaviours which develop slowly and are of prolonged duration [29; 39; 54]. Mechanistic studies in these animal models have attributed expressional changes and altered function of ion channels expressed on unmyelinated C-fiber nociceptors to the development of cold allodynia, such as the transient receptor potential (TRP) channels TRPM8, TRPA1 and the two-pore domain potassium (K^+) channels TREK1 and TRAAK [16; 21; 34; 58]. However, these findings are inconsistent with the clinical time course of acute oxaliplatin-induced cold allodynia and the predominant effects of oxaliplatin on myelinated A-fibers [2; 6; 26; 45; 46]. Thus, the pathophysiological mechanisms underlying acute oxaliplatin-induced cold allodynia remain unclear. While oxaliplatin-induced allodynia has been described as an axonal channelopathy resulting from modulation of neuronal Na_v channels [35], the contributions of the nine described isoforms ($Na_v1.1$ – $Na_v1.9$) have not been systematically assessed.

Dorsal root ganglion (DRG) neurons express several Na_v isoforms, including the tetrodotoxin (TTX) resistant isoforms $Na_v1.8$ and $Na_v1.9$, as well as the TTX-sensitive isoforms $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$ [40]. The TTX-resistant Na_v isoform $Na_v1.8$ in particular has been found to be crucial for pain evoked by noxious cold [59], while Na_vl.9 has been suggested to contribute to the pathogenesis of neuropathic pain [28]. In addition, $Na_v1.7$ is known to be crucial in pain pathways, as loss-of-function mutations in humans cause congenital insensitivity to pain [14], while gain-of-function mutations are associated with painful conditions such as erythromelalgia and paroxysmal extreme pain disorder [19]. In contrast, the functional roles of $Na_v1.1$ and $Na_v1.6$ in peripheral sensory neurons are less clear, and no evidence for involvement of these Na_v isoforms in pain phenotypes has been reported to date, as both homozygous $Scn1a^{-/-}$ and $Scn8a^{-/-}$ mice develop motor deficits and die around postnatal day 15 to 20, preventing assessment of behavioural effects in mature animals [9; 55].

We established an animal model of oxaliplatin that more closely mimics acute chemotherapy-induced peripheral neuropathy. We found that intraplantar oxaliplatin rapidly induced a long-lasting cold allodynia that was mediated entirely through TTX-sensitive Na_v isoform-dependent pathways. Surprisingly, $Na_v1.6$ was implicated as the key Na_v isoform involved, whereas thermosensitive TRP channels were not found to be involved. Consistent with reports of a crucial role for delayed-rectifier potassium channels in excitability in response to cold [52], intraplantar administration of the K^+ channel blocker 4-aminopyridine (4-AP) mimicked oxaliplatin-induced cold allodynia and was inhibited by Na_vl.6 blockers or potentiated by $Na_v1.6$ activators, supporting a crucial role for Na_vl.6 in chemically-mediated cold pain pathways.

Methods

Chemicals

Oxaliplatin and Dichloro(1,2-diaminocyclohexane)platinum(II) ($Pt(DACH)Cl_2$) were obtained from Sigma Aldrich (Castle Hill, New South Wales, Australia) and dissolved in 5% glucose/H_2O to a stock solution of 1 mg/mL to avoid spontaneous hydrolysis arising from the presence of Cl^- in physiological solutions. μ-Conotoxins GIIIA and TIIIA were a kind

gift from Professor Paul F. Alewood, The University of Queensland, Australia. Cn2 was isolated from the venom of the scorpion *Centruroides noxius* as previously described [43; 56]. M8-B (N-(2-aminoethyl)-N-(4-(benzyloxy)-3-methoxybenzyl)thiophene-2-carboxamide hydrochloride), a selective and potent antagonist of TRPM8), was synthesized and kindly provided by Amgen, Inc. [4]. The TRPM8 antagonist AMTB (N-(3-Aminopropy1)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide hydrochloride) and tetrodotoxin were from Tocris Bioscience (Bristol, United Kingdom). ProTxII was from Peptides International (Louisville, KY, USA). Peptides were routinely diluted in 0.1–0.3% albumin in phosphate-buffered saline to avoid adsorption to plastic surfaces. All other drugs and pharmacological modulators were diluted in phosphate-buffered saline. All other reagents were from Sigma Aldrich unless otherwise stated.

Animals

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

For behavioural assessment of oxaliplatin-induced neuropathy, we used adult male C57BL/6J mice, age 5–12 weeks. Age-matched controls were used for studies involving knockout animals, and all mouse strains were back-crossed for a minimum of 5 (5–9) generations on C57BL/6 background. Knockout animals were kindly provided by the following researchers: TRPA1$^{-/-}$ mice (D. Corey, Harvard Medical School, Boston, MA, USA), TRPM8$^{-/-}$ mice (A. Patapoutian, The Scripps Research Institute, La Jolla, CA, USA), global $Na_v1.8^{-/-}$, $Na_v1.9^{-/-}$, and $Na_v1.3^{-/-}$ mice (J. Wood, University College London, London, UK).

Induction of oxaliplatin-induced neuropathy and behavioural assessment

To characterize nociceptive effects in wild-type C57BL/6J and age-matched TRPA1$^{-/-}$, TRPM8$^{-/-}$, $Na_v1.8^{-/-}$, $Na_v1.9^{-/-}$ and $Na_v1.3^{-/-}$ mice, a single dose of oxaliplatin, oxalate, $Pt(DACH)Cl_2$, 4-AP, Cn2 or allyl isothiocyanate (AITC) was administered by shallow subcutaneous injection to the left hind paw in a volume of 40 μl (intraplantar injection, i.pl.) under light isoflurane anaesthesia. Quantification of spontaneous pain, cold and heat allodynia as well as mechanical allodynia was performed by a blinded observer unaware of the genotype and/or treatments received. Spontaneous nocifensive behaviour was quantified by counting the number of paw lifts, licks, shakes and flinches at room temperature (22–25°C) on a soft padded surface over a period of 5 min. Thermal allodynia was assessed by quantification of nocifensive behaviours over a 5 min period on a temperature-controlled Peltier plate (Hot/Cold Plate, Ugo Basile, Comerio, Italy). Mechanical allodynia was assessed by determining the paw withdrawal threshold to mechanical stimulation using an electronic von Frey apparatus (MouseMet Electronic von Frey, TopCat Metrology, Little Downham, United Kingdom). Briefly, mice were habituated in individual mouse runs for at least 10 min, and the paw withdrawal threshold was determined from the ipsilateral and controlateral paws in three separate trials, at least 5 min apart. The pressure applied through a soft-tipped probe was increased slowly over a pre-determined force rise rate (1 g/s). The force that elicited paw withdrawal was determined using the MouseMet Software and designated as the paw withdrawal threshold.

Where intraplantar injection elicited nocifensive behaviour at room temperature (Cn2, BAPTA, oxalate, 4-AP, AITC), thermal and mechanical allodynia was assessed after cessation of spontaneous pain (15 min to 1 h). To assess the effects of pharmacological modulators on the development of oxaliplatin-induced cold allodynia, compounds were

administered by intraplantar injection of appropriately concentrated solutions (HC030031, 100 μM; AMTB, 10 & mu;M; M8-B, 1 μM; TTX, 3 μM; A803467, 10 μM; ProTxII, 3 nM; GIIIA, 10 μM; TIIIA, 10 μM) 5–15 min prior to behavioural quantification. To assess the effect of pharmacological modulators on the nocifensive responses elicited by 4-AP, compounds were co-administered by intraplantar injection as appropriately concentrated solutions (Cn2, l nM; GIIIA, 10 μM; TTX, 3 μM; AMTB, 10 μM; HC030031, 100 μM) in a final volume of 40 μl. No systemic effects, including ataxia, altered gait or motor paralysis were apparent in any mice or after intraplantar injection of any pharmacological modulators. In addition, no sustained hind paw favouring, inflammation, swelling or ulceration of the oxaliplatin-injected paw was visible. Injection of equal volumes of 5% glucose/H_2O and phosphate-buffered saline with or without albumin did not elicit any nocifensive behaviour.

FLIPR Membrane Potential Assays

To verify the *in vitro* potency of compounds with activity Na_vl.6 channels, inhibition of veratridine-induced membrane potential responses were assessed using the FLIPR^{TETRA} (Molecular Devices, Sunnyvale, CA) plate reader. Na_v1.6-expressing CHO cells (EZcells, Chantest, Cleveland, OH) were loaded with Red Membrane Potential dye (Molecular Devices), and responses to stimulation with veratridine (50 μM) were assessed after 5 min pre-treatment with antagonists as previously described [50].

Data and statistical analysis

Fluorescence values from membrane potential imaging experiments were converted to response over baseline values using Screen Works 3.2.0.14 as previously described [50]. For concentration-response curves, maximum values from the response after addition of agonist were plotted against agonist concentration and a 4-parameter logistic Hill equation was fitted to the data using GraphPad Prism Version 5.03 (San Diego, CA). Statistical significance was defined as $p < 0.05$ and was determined using paired or unpaired Student's *t*-tests and one-way ANOVA analysis with Dunnett's post test as indicated. Statistical analysis was performed using GraphPad Prism Version 5.03.

Results

A mouse model of chemotherapy-induced cold allodynia based on intraplantar administration of oxaliplatin

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

This dose (1.6–2.0 mg/kg) is approximately equivalent to human therapeutic doses (2.5–3.5 mg/kg), and considerably lower than systemic doses previously reported to elicit acute cold allodynia in rodents (5 – 10 mg/kg) [58].

Strikingly, cold allodynia induced by a single dose of oxaliplatin became apparent within minutes of injection and persisted for several days, with significant pain behaviour evident for up to 7 days after injection of the highest dose (2.5 mM; 40 μg; Fig. 1b). The terminal elimination phase of platinum-containing metabolites is long, suggesting that the prolonged

effect of a single intraplantar injection of oxaliplatin could arise from the pharmacokinetics of these oxaliplatin metabolites. Alternatively, oxaliplatin, or platinum metabolites, may elicit irreversible changes in neuronal proteins which are involved in mediating increased excitability to cool stimuli.

Nocifensive behaviour evoked by intraplantar injection of oxaliplatin became apparent at temperatures below 15°C (18.2 ± 6.5 flinches/5 min), but no heat allodynia was evident, with animals displaying little or no nocifensive behaviour at elevated temperatures up to 42°C (1.0 ± 0.7 flinches/5 min) (Fig. 1c). In addition, intraplantar injection of oxaliplatin also elicited mechanical allodynia, evidenced by decreased paw withdrawal threshold to mechanical stimulation (Fig. 1d; Control, 4.7± 0.3 g; oxaliplatin 2.5 ± 0.3 g). Therefore, this novel animal model of intraplantar oxaliplatin produces behavioural responses that parallel the human symptomatology of oxaliplatin-induced neuropathy, confirming a direct peripheral effect of oxaliplatin on sensory nerve endings as the basis of cold-evoked paraesthesias and dysaesthesias.

Oxaliplatin metabolites contribute to mechanical, but not cold allodynia

Oxaliplatin is rapidly hydrolyzed *in vivo* to bioactive derivatives through displacement of the oxalate group by H_2O and Cl^- to produce oxalate as well as reactive monochloro-, dichloro- and diaquo-diaminocyclohexane platinum metabolites [18; 22]. As these oxaliplatin metabolites have previously been suggested to contribute to oxaliplatin-induced neuropathy, we sought to characterize the contribution of oxalate and the oxaliplatin metabolite $Pt(DACH)Cl_2$ to oxaliplatin-induced cold allodynia in our model. We found that intraplantar injection of equivalent doses of $Pt(DACH)Cl_2$, (2.5 mM; 38 μg) or oxalate (2.5 mM; 14 μg) did not cause cold allodynia (Fig. 2a). However, injection of a higher dose of oxalate (150 mM; 810 μg/paw) caused short-lived (< 1 h) spontaneous nocifensive behaviour, evidenced by lifting, licking and shaking of the paw (Fig. 2b), as well as mechanical allodynia which remained apparent 24 h after a single intraplantar injection of oxalate (Fig. 2c; Control, 4.4 ± 0.5 g; oxalate 0.9± 0.3 g). Consistent with the ability of oxalate to chelate Ca^{2+} [23], both the spontaneous pain (29.1 ± 5.1 flinches/5 min) and mechanical allodynia (2.6 ± 0.3 g) were mimicked by intraplantar injection of the Ca^{2+} chelator BAPTA (10 mM; 191 μg) (Fig. 2b and 2c). In contrast, intraplantar injection of the cell membrane-permeable BAPTA-AM [48] (10 μM; 310 ng) had no effect on spontaneous nocifensive behaviour and did not elicit cold allodynia (Fig. 2a-c). The effect of oxalate and BAPTA on sensory nerve endings likely arises from the destabilizing effect of Ca^{2+} chelation on neuronal membranes which interferes with the surface screening charge. Thus, removal of extracellular Ca^{2+} results in increased excitability by decreasing the threshold potential and membrane resistance, and increasing Na^+ conductance [20]. These effects have been shown in *ex vivo* preparations to result in spontaneous action potential discharge and an increase in mean firing frequency [24; 42], corroborating the proalgesic effect of extracellular Ca^{2+} chelation we observed in our animal model.

Oxaliplatin-induced cold allodynia is mediated through $Na_v1.6$-expressing peripheral sensory fibers

Na_v channels are critical for the propagation of action potentials in excitable cells, including peripheral sensory nerves. The tetrodotoxin-resistant isoform $Na_v1.8$ in particular is crucial for neuronal excitability at cold temperatures and is essential for noxious cold pain [59]. Thus, we sought to elucidate the contribution of Na_vl.8 to oxaliplatin-induced cold allodynia. Surprisingly, the development of cold allodynia was unchanged in $Na_v1.8^{-/-}$ animals, and was also not affected by A803467, a $Na_v1.8$-selective small molecule inhibitor (Fig. 3a; $Na_v1.8^{-/-}$, 98 ± 15% of control; A803467 (10 μM), 90 ± 17% of control). Similarly, the tetrodotoxin-resistant Na_vl.9 has previously been suggested to contribute to

the pathogenesis of neuropathic pain and cold allodynia [28]. However, in our model the cold allodynia was unchanged in $Na_v1.9^{-/-}$ animals (Fig. 3a; 107 ± 8% of control), suggesting that TTX-sensitive Na_v isoforms are crucial for the development of oxaliplatin-induced cold allodynia. Indeed, intraplantar injection of low concentrations of TTX (3 μM) inhibited nocifensive responses upon exposure to a surface cooled to 10°C (Fig. 3a; 19 ± 4% of control). We thus assessed the contribution of $Na_v1.3$ and $Na_v1.7$ using knockout animals or subtype-selective inhibitors. Surprisingly, the development of cold allodynia was also unchanged in $Na_v1.3^{-/-}$ animals (103 ± 11% of control), or after intraplantar injection of the Na_vl.7-selective inhibitor ProTxII (3 nM; 115 ± 17% of control) (Fig. 3b), suggesting involvement of Na_v isoforms which have not yet been associated with any prominent function in pain pathways. In addition to $Na_v1.3$, $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, DRG neurons are known to express other tetrodotoxin-sensitive isoforms, including $Na_v1.1$, $Na_v1.2$ and $Na_v1.6$. Since knockout mouse models of these Na_v isoforms are lethal, we used a range of conotoxins with activity at Na_v isoforms that allowed dissection of the contribution of these isoforms to oxaliplatin-induced pain pathways. μ-Conotoxin TIIIA specifically inhibits Na_vl.2 and Na_vl.4 at low concentrations, while at high concentrations $Na_v1.1$, but not $Na_v1.6$, is also inhibited [53; 57] (Fig. 3c; $Na_v1.6$ pIC_{50} 6.0 ± 0.3). Intraplantar injection of both low (100 nM) or high (10 μM) concentrations of TIIIA did not significantly decrease oxaliplatin-induced cold allodynia (Fig. 3D), suggesting a crucial role for Na_vl.6 in cold pain pathways activated by oxaliplatin. Indeed, intraplantar injection of GIIIA (10 μM), which in addition to $Na_v1.1$ also inhibits $Na_v1.6$ at high concentrations, but has no effect on $Na_v1.3$ and $Na_v1.7$ [53], achieved near complete reversal of oxaliplatin-induced cold allodynia (14 ± 9% of control) (Fig. 3d). In contrast, nocifensive behavior elicited by intraplantar administration of the TRPA1 agonist AITC (5 mM) was not affected by GIIIA (30 μM; Fig 3e). Thus, this demonstrates for the first time a functional contribution of Na_vl.6 to cold pain pathways at the behavioural level.

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

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

In some sensory and central neurons, cooling elicits enhanced excitability and increased firing frequency as a result of cold-induced closure of background potassium channels [3; 15; 33; 38; 52]. This effect appears to be opposed by continued activity of K_vl channels which act as an excitability break and regulate cold sensitivity in trigeminal neurons in concert with TRPM8 [32; 52]. Since it is known that oxaliplatin inhibits potassium channels [26] in addition to sodium channels [2; 8; 23; 27; 46], we assessed if the oxaliplatin-induced effects could be replicated by inhibition of delayed rectifier potassium channels in sensory nerve endings. Indeed, intraplantar injection of 4-AP (1 mM) elicited cold allodynia (35.2 ± 8.6 flinches/5 min) which was not affected by intraplantar injection of the TRPA1 inhibitor HC030031 (100 μM; 37.4 ± 13.4 flinches/5 min) or the TRPM8 inhibitor AMTB (10 μM; 46.6 ± 10.5 flinches/5 min) (Fig. 5a). Like oxaliplatin-induced cold allodynia, enhanced nocifensive responses to cold elicited by 4-AP were inhibited by the Na_vl.6 inhibitor GIIIA

(8.6 ± 4.4 flinches/5 min), confirming an important role for $Na_v1.6$ in cold pain pathways (Fig. 5A).

Pain behaviors induced by selective $Na_v1.6$ activation

To further characterize the role of $Na_v1.6$ in pain pathways, we also assessed spontaneous pain behaviours, thermal allodynia and mechanical allodynia after intraplantar injection of the $Na_v1.6$-selective activator Cn2. Cn2 is a β-scorpion toxin isolated from the venom of the scorpion *Centruroides noxius* that specifically enhances activity of $Na_v1.6$ with an EC_{50} of 39 nM, causing a leftward shift of the voltage-dependence of activation and a transient resurgent current [43]. Intraplantar injection of Cn2 elicited dose-dependent spontaneous pain characterized by licking, lifting and vigorous shaking of the injected paw that was transient for lower concentrations (1 nM, < 15 min). At the highest concentration tested (30 nM; Fig. 5b), the frequency and severity of these responses rapidly diminished after injection, although some nocifensive behaviours remained evident for > 4 h after intraplantar administration. Thus, for subsequent experiments, low concentrations of Cn2 were utilized.

Intraplantar Cn2 (1 nM) did not elicit thermal allodynia, with little or no nocifensive behaviour evident at 10°C or 42°C (Fig. 5c) but caused significant ($p < 0.01$) mechanical allodynia, evidenced by decreased paw withdrawal thresholds to mechanical stimulation (Fig. 5d; Control, 5.4 ± 0.4 g; Cn2 (1 nM), 2.8 ± 0.6 g). To examine the contribution of potassium channels to $Na_v1.6$-dependent cold-pain, we co-administered Cn2 (10 nM) with 4-AP (500 μM) by intraplantar injection. As opposed to Cn2 alone, this combination potentiated the cold allodynia produced by 4-AP (Fig. 5e; 4-AP, 12.0 ± 3.1 flinches/5 min; 4-AP + Cn2, 38.0 ± 8.2 flinches/5 min), providing evidence that activation of $Na_v1.6$ *per se* produced only spontaneous pain and mechanical allodynia, but when combined with inhibition of delayed rectifier potassium channels could enhance cold allodynia.

Discussion

Acute oxaliplatin-induced neuropathy occurs in almost all patients and manifests as circumoral and distal sensory and/or motor disturbances including paraesthesias and dysaesthesias and muscle fasciculations. These symptoms are triggered by exposure to cold and are associated with a significant reduction in the cold pain threshold [6]. However, the pathophysiological basis of acute oxaliplatin-induced neuropathy, in particular cold allodynia, is poorly understood. This is in part due to a paucity of animal models that accurately reflect the neuropathic symptomatology encountered clinically and, specifically, the rapid onset of cold allodynia. To better understand chemically-induced cold allodynia, we established an animal model of chemotherapy-induced peripheral neuropathy based on the intraplantar injection of oxaliplatin. This model supports a direct excitatory action of oxaliplatin on peripheral sensory nerve endings as the causative mechanism underlying oxaliplatin-induced neuropathy, with cold allodynia becoming evident within minutes and persisting for several days after a single local injection of oxaliplatin. We were able to show that inhibition of potassium channels leads to increased neuronal excitability at low temperatures independent of activation of TRP channels, and that peripheral $Na_v1.6$ was crucial for the propagation of these signals and the appearance of cold allodynia.

The metabolism of oxaliplatin is complex and involves rapid hydrolysis ($t_{½\alpha}$ ~ 14 min) to oxalate and various bioactive platinum compounds, which in turn form adducts with DNA, proteins, peptides and amino acids that undergo slow, triphasic elimination through predominantly renal routes, with a long terminal half-life for platinum of up to 11 days [18; 22]. It is difficult to estimate the concentration of oxaliplatin that sensory neurons are exposed to in human patients. While the plasma concentration of free oxaliplatin after intravenous administration is relatively low [18; 22], consistent with an apparent large

volume of distribution, oxaliplatin is likely to accumulate in sensory neurons through active transport by copper transporters and the L-carnitine transporter OCTN1 [25; 30].

Given the rapid onset and prolonged nature of the sensory disturbances associated with oxaliplatin infusion [5], contribution of various oxaliplatin metabolites to the development of peripheral neuropathy has been suggested. In DRG explants, $Pt(DACH)Cl_2$ was more neurotoxic than oxaliplatin [31], while after repeated intraperitoneal administration, oxalate elicited cold hyperalgesia and increased paw withdrawal responses to application of acetone, but not mechanical allodynia [41]. We thus assessed the effect of equimolar doses of oxalate and $Pt(DACH)Cl_2$, two major oxaliplatin metabolites, on the development of cold and mechanical allodynia after intraplantar injection. However, while neither metabolite elicited cold allodynia after a single local injection, Ca^{2+} chelation by oxalate elicited spontaneous nocifensive behaviour as well as prolonged mechanical allodynia. This effect was mimicked by intraplantar injection of BAPTA and can be attributed to the effects of extracellular Ca^{2+} removal on membrane properties, including decreased threshold potential and membrane resistance, as well as increased Na^+ conductance [20].

Acute oxaliplatin-induced neuropathy has been postulated to involve the modulation of axonal Na_v channels, based on the observation that oxaliplatin infusion elicited changes in Na_v-dependent variables in humans [27; 35]. Similarly, in rat DRG neurons, oxaliplatin resulted in increased Na^+ currents and a shift of the voltage-response relationship towards more negative potentials, [2] with similar effects observed in cockroach neurons and frog myelinated axons [8; 23].

The lack of contribution of $Na_v1.8$ to oxaliplatin-induced cold allodynia, which we demonstrated in both $Na_v1.8$ knockout animals and after intraplantar administration of the $Na_v1.8$-selective inhibitor A803467, was surprising given the crucial role of this Na_v isoform in cold pain. Specifically, $Na_v1.8$ in nociceptive peripheral sensory neurons has previously been demonstrated to be critical for the development of pain evoked by noxious cold [59], and in mice with diphtheria toxin-mediated ablation of $Na_v1.8$-expressing nociceptors, noxious cold responses are virtually abolished [1]. Oxaliplatin induces repetitive firing, broadening of the repolarization phase and after-hyperpolarization in myelinated A-fibers, while non-myelinated C-fibers remain largely unaffected [2; 26; 45; 46]. In contrast, although $Na_v1.8$ is widely expressed in peripheral sensory neurons, including a subpopulation of myelinated A-fibers [44], its contribution to cold-evoked pain behavior arises mainly from nociceptive C-fibers [1; 51; 59]. Thus, our finding that $Na_v1.8$ does not contribute to oxaliplatin-induced cold allodynia can be explained by the differential expression of Na_v isoforms in peripheral sensory nerve fibers that contribute to the pathophysiology of oxaliplatin-induced cold allodynia. The role of $Na_v1.8$ in pathological cold pain appears to be different from its role in physiological cold pain [59], and in addition differs to other models of chemically-induced cold allodynia, such as ciguatoxin-induced cold pain, where $Na_v1.8$ still contributes significantly to pain behaviours [51].

Consistent with a major role for $Na_v1.6$ in the propagation of action potentials in myelinated A-fibers [53; 57], we found no contribution of $Na_v1.3$, $Na_v1.7$ and $Na_v1.9$ to oxaliplatin-induced cold allodynia, while pharmacological inhibition of $Na_v1.6$ virtually abolished pain behaviour. Supporting a crucial role for $Na_v1.6$ in oxaliplatin-induced cold allodynia is the observation that *in vitro*, oxaliplatin elicits $Na_v1.6$-mediated resurgent currents and that oxaliplatin-induced A-fiber effects were abolished in $Na_v1.6$ knockout animals [46]. Similarly, we observed significant potentiation of veratridine-induced $Na_v1.6$ responses in our membrane potential assay (data not shown), consistent with the previously reported effect of oxaliplatin on fast inactivation [46].

Thus, we have obtained the first evidence that Na_vl.6 expressed in peripheral sensory neurons contributes to cold pain behaviours. However, since Na_v1.6 is highly expressed at nodes of Ranvier in both peripheral sensory and motor axons, as well as nodes in the central nervous system [10], Na_v1.6 would be difficult to target therapeutically. Indeed, mice with loss-of-function mutations in Scn8a, the gene encoding for Na_vl.6, are characterized by early onset progressive paralysis of the hind limbs, leading to juvenile lethality at approximately postnatal day 20 [9]. Thus, the results presented here support a role for Na_v1.6 in pathological pain states. Future experiments in sensory fiber-specific knockout model would be valuable to further dissect the role of Na_vl.6 in pain pathways.

Thermosensitive TRP channels, in particular TRPM8, TRPA1 and TRPC5, are expressed in peripheral sensory neurons and are activated by cooling [36; 47; 60]. Chronic administration of oxaliplatin in animal models has been shown to elicit changes in TRP channel expression, and both TRPM8 and TRPA1 have been causally implied in the development of chemotherapy-induced cold allodynia [21; 34; 58]. However, the rapid onset of cold allodynia both clinically and in our novel model of acute oxaliplatin-induced cold allodynia suggests that changes in the expression level of TRP channels are unlikely to contribute to the observed symptomatology. Indeed, we found no significant effect of TRPA1, TRPM8 or TRPC5 to oxaliplatin-induced cold allodynia using both genetically modified animals and pharmacological modulators where possible. This finding is consistent with the predominantly A-fiber-mediated origin of oxaliplatin-induced cold allodynia, as cold-sensitive TRP channels are expressed predominantly on peptidergic and isolectin B4-positive C-fibers [17; 47; 60].

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

An alternative mechanism of inducing cold sensitivity is based on inhibition of potassium channels. In addition to modulation of Na_v, oxaliplatin also inhibits neuronal potassium channels [8; 26]. In peripheral myelinated fibers, the effects of oxaliplatin on compound action potentials were similar to those of 4-AP [26]. 4-AP inhibits delayed-rectifier channels, including K_v1.1 and K_v1.2 which have been shown to be highly expressed in cold-insensitive neurons and contribute to lack of cold-sensitivity in trigeminal neurons [32; 49; 52]. Indeed, intraplantar injection of 4-AP caused behavioural responses similar to oxaliplatin, and elicited cold allodynia that was not modulated by inhibition of TRPM8 or TRPA1, but was decreased by pharmacological inhibition of Na_v1.6. These findings are consistent with K_v1.1 and K_v1.2 being expressed predominantly in large DRG neurons which give rise to myelinated A-fibers [37], and corroborate an important role for K_v channels in cold sensing and cold allodynia.

Indeed, activation of Na_v1.6 alone was not sufficient to elicit cold allodynia, with the Na_v1.6-specific scorpion toxin Cn2 eliciting spontaneous pain and mechanical allodynia but not cold allodynia. However, when combined with inhibition of K_v channels, Cn2 produced profound enhancement of 4-AP-induced cold allodynia. Thus Cn2 not only confirms the pivotal role played by Na_v1.6 in chemically-induced cold pain but reveals a role for Na_v1.6

in spontaneous pain and mechanical allodynia. In conclusion, the new animal model of oxaliplatin-induced cold allodynia described here reveals an important role for $Na_v1.6$ in pain pathways, with chemically-induced cold allodynia mediated through inhibition of potassium channels on Na_vl.6-expressing peripheral sensory fibers.

Acknowledgments

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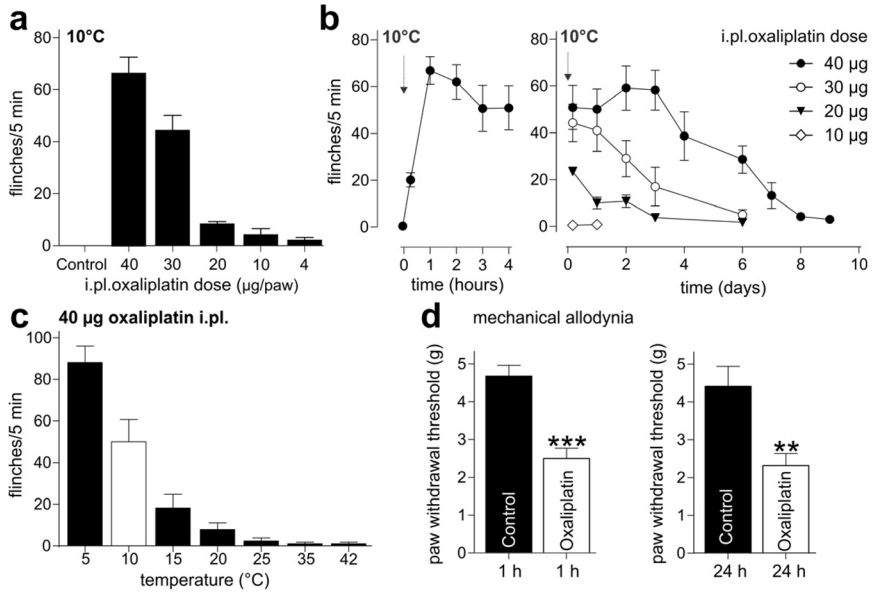

Figure 1. A novel animal model of chemotherapy-induced neuropathy based on the intraplantar injection of oxaliplatin

(a) Intraplantar injection of oxaliplatin (4 – 40 μg/paw) rapidly elicits cold allodynia, with increased paw lifting, licking, shaking and flinching evident 1 h after injection upon exposure to a temperature-controlled surface maintained at 10°C. Injection of vehicle (Control; 5% glucose/H_2O) did not elicit any nocifensive responses. **(b)** Oxaliplatin-induced cold allodynia has a rapid onset (left panel, 0–4 h post-injection), with nocifensive responses upon exposure of the injected hind paw to cool temperatures becoming apparent within minutes after injection (arrow). Cold allodynia after a single injection persists for several days (right panel, 4 h - 9 days post-injection) after intraplantar injection (arrow). **(c)** Nocifensive responses evoked by intraplantar injection of oxaliplatin (40 μg/paw) are temperature-dependent, with significant paw withdrawals elicited upon exposure to temperatures below 15°C (24 h after injection). No withdrawal responses were evident at elevated temperatures up to 42°C. White bar; for all subsequent experiments, cold allodynia was assessed 24 h after injection of 40 μg oxaliplatin/paw by quantifying paw withdrawal responses at 10°C. **(d)** Intraplantar injection of oxaliplatin (40 μg/paw) elicited mild mechanical allodynia, with a significant decrease in paw withdrawal threshold to mechanical stimulation compared to control (5% glucose/H_2O). Left panel, decreased mechanical threshold was apparent at 1 h after injection and persisted 24 h after injection (right panel). Statistical significance was determined using an unpaired Student's t-test; ***, $p < 0.001$; **, $p < 0.01$ compared to vehicle. Data are presented as mean ± SEM (n = 5- 12 animals/ group).

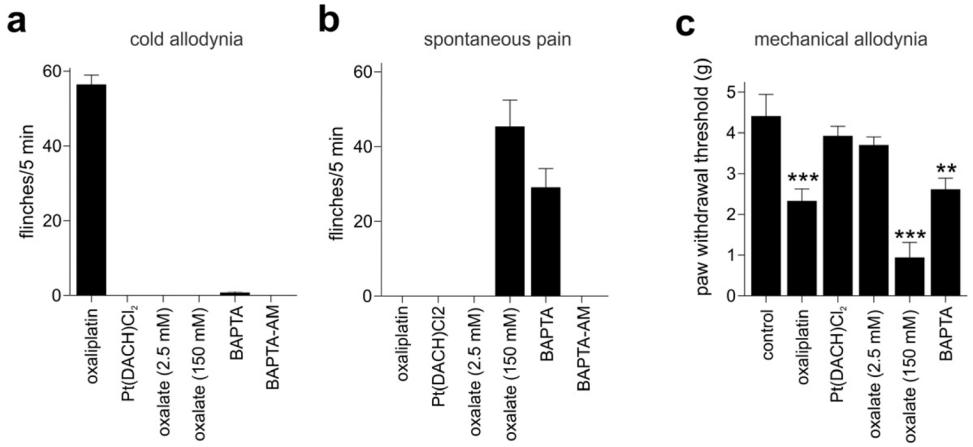

Figure 2. Oxaliplatin metabolites contribute to mechanical, but not cold allodynia

a) Intraplantar injection of equimolar doses of the oxaliplatin metabolites $Pt(DACH)Cl_2$ (2.5 mM; 38 μg/paw) and oxalate (2.5 mM; 14 μg/paw) did not elicit paw withdrawal responses at 10°C, compared to the pronounced cold allodynia elicited by oxaliplatin (2.5 mM; 40 μg/paw). Cold allodynia was also not elicited by intraplantar injection of the Ca^{2+} chelator BAPTA (10 mM; 191 μg/paw) or the membrane-permeable BAPTA-AM (10 μM; 310 ng/paw). **(b)** Intraplantar injection of high doses of oxalate (150 mM; 810 μg/paw) and BAPTA (10 mM; 191 μg/paw), but not low doses of oxalate, oxaliplatin, BAPTA-AM or $Pt(DACH)Cl_2$, elicited short-lasting (< 1 h) spontaneous nocifensive behaviour (increased number of paw lifts, licks, shakes and flinches) evident at room temperature. **(c)** Intraplantar injection of high doses of oxalate (150 mM; 810 μg/paw), BAPTA (10 mM; 191 μg/paw) and oxaliplatin (2.5 mM; 40 μg/paw) caused a significant decrease in the paw withdrawal threshold to mechanical stimulation, while mechanical responses were unchanged after intraplantar injection of equimolar doses of $Pt(DACH)Cl_2$, and oxalate. Statistical significance was determined using a one-way ANOVA with Dunnett's post test. ***, $p < 0.001$; **, $p < 0.01$ compared to Control (vehicle). Data are presented as mean ± SEM (n = 3 – 5) animals/group.

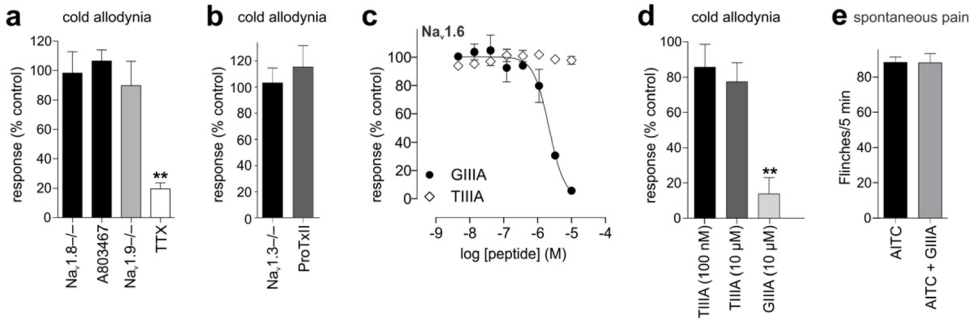

Figure 3. Na_V isoforms involved in the development of cold allodynia after intraplantar injection of oxaliplatin

(a) Cold allodynia induced by intraplantar injection of oxaliplatin (24 h after injection of 2.5 mM oxaliplatin; 40 μg/paw) was not significantly different from control in $Na_V1.8^{-/-}$ animals, or after intraplantar injection of the $Na_V1.8$ inhibitor A803467 (10 μM). Paw flinches were also not significantly different from control in $Na_V1.9^{-/-}$ animals, but were significantly ($p < 0.01$) inhibited by intraplantar injection of TTX (3 μM). **(b)** Oxaliplatin-induced cold allodynia was not significantly different from control in $Na_V1.3^{-/-}$ animals, or after intraplantar injection of the $Na_V1.7$ inhibitor ProTxII (3 nM). **(c)** μ-Conotoxin GIIIA concentration-dependently (pIC_{50} 6.0 ± 0.3) inhibits $Na_V1.6$, while TIIIA does not affect $Na_V1.6$-mediated responses. Effect of μ-conotoxins on veratridine (50 μM)-induced $Na_V1.6$ responses was assessed using a FLIPR membrane-potential assay in HEK cells heterologously expressing $Na_V1.6$. **(d)** Intraplantar injection of TIIIA at concentrations which inhibit $Na_V1.2$ (100 nM), or $Na_V1.1$ but not $Na_V1.6$ (10 μM) did not significantly decrease oxaliplatin-induced cold allodynia. In contrast, GIIIA at a concentration which fully inhibits $Na_V1.6$ (10 μM) caused near complete inhibition of cold allodynia. **(e)** GIIIA (30 μM) had no effect on nocifensive behaviours elicited by intraplantar administration of the TRPA1 agonist AITC (5 mM). Data is presented relative to vehicle-injected wi1d-type animals or age-matched litter controls. Statistical significance was determined using a one-way ANOVA with Dunnett's post test. **, $p < 0.01$ compared to Control (vehicle or age-matched litter controls). Data are presented as mean± SEM (n = 4 – 8 animals/group).

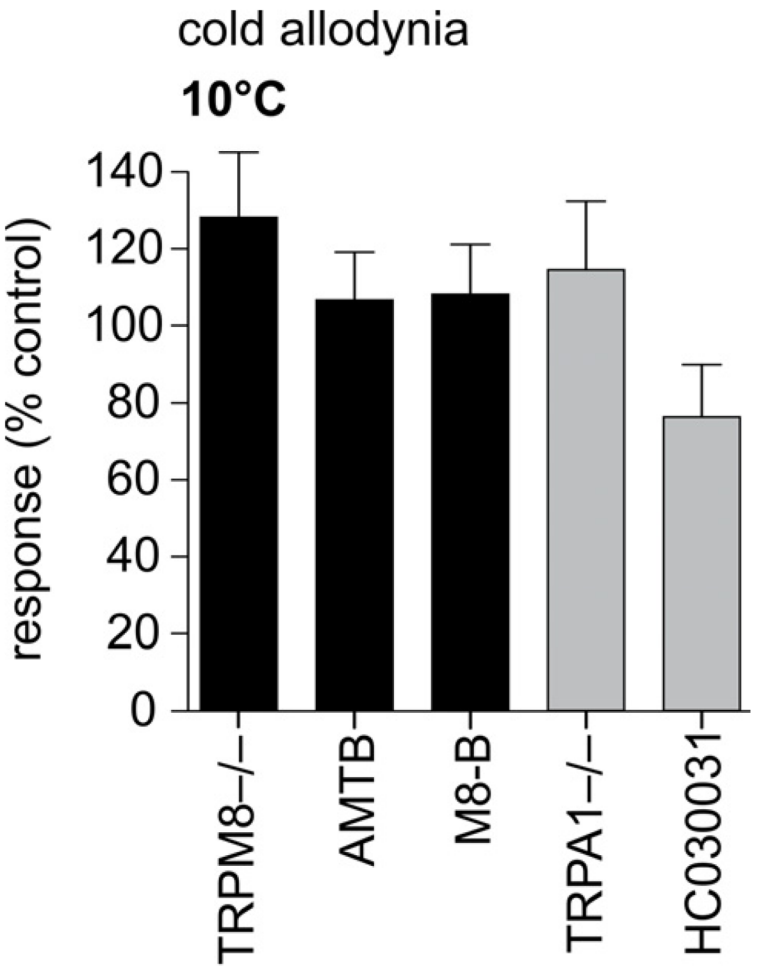

Figure 4. Oxaliplatin-induced cold allodynia develops independently of cold-sensitive TRP channels

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

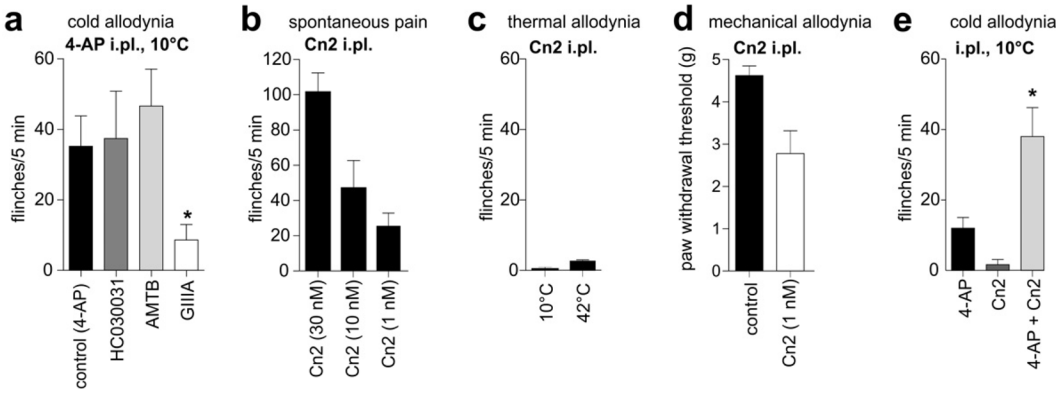

Figure 5. Inhibition of potassium channels on $Na_V1.6$-expressing pain pathways elicits cold allodynia

(a) Intraplantar injection of the potassium channel inhibitor 4-AP (1 mM) elicited cold allodynia, evidenced by an increased number of paw lifts, licks, shakes and flinches on exposure to a temperature-controlled plate maintained at 10°C. Cold allodynia induced by 4-AP was not significantly inhibited by concomitant intraplantar injection of the TRPM8 antagonist AMTB (10 μM) or the TRPA1 antagonist HC030031 (100 μM), but was significantly ($p < 0.05$) inhibited by intraplantar μ-conotoxin GIIIA (10 μM). **(b)** Intraplantar injection of Cn2 (1 nM – 30 nM) elicited dose-dependent spontaneous pain. Responses were quantified by counting the number of behaviours immediately after injection **(c)** Activation of peripheral $Na_V1.6$ by Cn2 (1 nM) did not elicit cold (10°C) or heat (42°C) allodynia. **(d)** Intraplantar Cn2 (1 nM) caused significant ($p < 0.01$) mechanical allodynia, with the paw withdrawal threshold to mechanical stimulation decreased from 5.4 ± 0.4 g (control) to 2.8 ± 0.6 g (Cn2, 1 nM). **(e)** Intraplantar injection of the $Na_V1.6$ activator Cn2 (10 nM) alone did not elicit significant cold allodynia (1.7 ± 1.5 flinches/5 min at 10°C), but significantly ($p < 0.05$) potentiated cold allodynia elicited by intraplantar injection of 4-AP (500 μM; 12.0 ± 3.1 flinches/5 min at 10°C) when co-administered (4-AP + Cn2; 38.0 ± 8.2 flinches/5 min). Statistical significance was determined using a one-way ANOVA with Dunnett's post test. *, $p < 0.05$; **, $p < 0.01$ compared to control. Data are presented as mean ± SEM (n = 4 – 7 animals/group).