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Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors

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Cold hypersensitivity is the hallmark of oxaliplatin-induced neuropathy, which develops in nearly all patients under this chemotherapy. To date, pain management strategies have failed to alleviate these symptoms, hence development of adapted analgesics is needed. Here, we report that oxaliplatin exaggerates cold perception in mice as well as in patients. These symptoms are mediated by primary afferent sensory neurons expressing the thermoreceptor TRPM8. Mechanistically, oxaliplatin promotes over-excitability by drastically lowering the expression of distinct potassium channels (TREK1, TRAAK) and by increasing the expression of pro-excitatory channels such as the hyperpolarization-activated channels (HCNs). These findings are corroborated by the analysis of TREK1-TRAAK null mice and use of the specific HCN inhibitor ivabradine, which abolishes the oxaliplatin-induced cold hypersensitivity. These results suggest that oxaliplatin exacerbates cold perception by modulating the transcription of distinct ionic conductances that together shape sensory neuron responses to cold. The translational and clinical implication of these findings would be that ivabradine may represent a tailored treatment for oxaliplatin-induced neuropathy.

INTRODUCTION

Chemotherapy-induced peripheral neuropathy is a common side effect of several anticancer agents including platinum analogues, vinca alkaloids, taxanes (Postma et al, 2005), and newer agents, such as epothilones, thalidomide, suramin, and

the proteasome inhibitor bortezomib (Richardson et al, 2003). This side effect may seriously compromise the patients' quality of life, limit dosage, and thus lead to changes in treatment to non-neurotoxic agents with the risk of limiting the effective clinical outcome. Among these compounds, oxaliplatin (used in the treatment of several solid tumours (Andre et al, 2004)) induces an acute neurotoxicity, which may appear as soon as after the first injection, and a chronic cumulative axonal sensory neuropathy (Stengel & Baron, 2009). Abnormal cold-triggered sensations, predominantly localized to hands and feet, are observed in most patients, and thermal hyperalgesia is a relevant clinical marker of early oxaliplatin neurotoxicity and may predict severe neuropathy (Attal et al, 2009).

Most chemotherapy-induced neuropathies improve after the drug is withdrawn, but long-term neuropathy can be found in a significant number of patients (van der Hoop et al, 1990). Unfortunately, while this complication is increasingly important, no very effective preventive or curative treatment is available. The usual symptomatic treatment of neuropathic pain

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or preventive treatment fails to improve patients (Wolf et al, 2008), thus there is a need to advance the understanding of the pathogenesis behind these neuropathies in order to propose effective therapeutic pain management.

Recent developments in preclinical models of oxaliplatin-induced cold hypersensitivity in rats (Ling et al, 2007a,b) may prove useful to gain insight into the pathophysiological mechanism of the oxaliplatin effect. Hypersensitivity to cold temperatures has been shown either after acute (Ling et al, 2007a) or repeated administration (Ling et al, 2007b) of oxaliplatin, which makes the model clinically relevant. In parallel, the molecular understanding of painful cold sensing in the primary afferent nociceptors has increased tremendously in the past few years (Bautista et al, 2007; Colburn et al, 2007; Dhaka et al, 2007; McKemy et al, 2002; Peier et al, 2002; Viana et al, 2002). In particular, identification of the transient receptor potential family of ion channels (TRPM8 and TRPA1), gated by cooling, was an important step in our understanding of how cold is detected. Moreover, the emerging picture is that cold-sensing neurons would express a particular set of ion channels that specifically determine their excitability at cold temperatures.

In this context we studied acute oxaliplatin-induced neurotoxicity in mice, in order to take advantage of strains that lack specific components involved in cold-sensing neuron excitability. We found that a single injection of oxaliplatin was followed by the rapid and reversible development of hypersensitivity to innocuous and noxious cold stimuli corresponding to the activation range of TRPM8 channels. In agreement, oxaliplatin did not induce cold hypersensitivity in TRPM8 knock out animals. No evidence of direct activation of TRPM8 channels by oxaliplatin was found, suggesting an effect on electrogenesis rather than on cold detection. Analysis of the expression of a set of ion channels previously identified as important tuners of cold perception (TREK1, TRAAK, $K_v1.1$, $Na_v1.8$ and HCN1) confirmed their involvement. Thus, our findings reveal that oxaliplatin promotes hyperexcitability by remodelling ion channel expression in cold-sensing nociceptors.

RESULTS

Cold hyperalgesia and cool allodynia in oxaliplatin treated mice

To assess cold sensitivity in mice, we first measured acute tail withdrawal response to a noxious cold stimulation (Fig 1A). Vehicle-treated mice showed stable thresholds through the duration of the experiments (one daily test for 1 week). In contrast, oxaliplatin-treated animals exhibited altered cold sensitivity. Oxaliplatin induced a clear dose-dependent and transient reduction of withdrawal thresholds that peaked 90 h post injection and reversed towards control values thereafter (Fig 1A). At 6 mg/kg (therapeutic dose), the cold hypersensitivity was manifested by a 50% threshold decrease. The tail immersion test is mainly supported by a spinal reflex arc, thus, in order to have a more integrated behaviour, we challenged the mice on a dynamic cold plate (Yalcin et al, 2009). This test entails the slow lowering of temperature of the test arena floor

from warm to cold and quantifying spontaneous nocifensive behaviour to ascertain the tolerance threshold to noxious cold. Vehicle-treated animals manifested escape behaviour at approximately 5°C, whilst oxaliplatin-treated mice presented the same escape behaviour at a much more elevated temperature (~15°C), reflecting a clear cold hypersensitivity (Fig 1B). To discriminate allodynic effects, we performed the tail immersion test at an innocuous temperature (21°C). This temperature does not elicit any withdrawal in vehicle-treated animals, whilst it induced withdrawals in oxaliplatin-treated mice, with the same dose dependency as for cold hyperalgesia (Fig 1C). Spontaneous allodynia was assessed in these animals through their ability to discriminate between warm and cool surfaces. Mice were allowed to explore adjacent surfaces, with one held at 25°C and the other ranging from 25 to 15°C, a temperature range considered to be innocuously cool (Rainville et al, 1999) (Fig 1D). When both sides were at the same temperature (both 25°C), neither vehicle- nor oxaliplatin-treated mice displayed any preference. As the variable plate was cooled, vehicle-treated mice started to show a preference for the warm side when the variable side was below 19°C. With oxaliplatin treatment, the preference of the mice for the warm side developed as soon as the variable side was set to 23°C, demonstrating clear allodynic behaviour to cool temperatures (Fig 1D). In parallel, we assessed sensitivity of the mice to noxious heat through their response to tail immersion at 46°C (Supporting Fig 1A). Vehicle- or oxaliplatin-treated mice at all doses showed indistinguishable thresholds during the entire duration of the experiments (one daily test for 1 week), reflecting an unaltered response to heat.

Mechanical hypersensitivity in oxaliplatin-treated mice

Along with this alteration of cold perception, we investigated whether oxaliplatin modified the mechanical tactile/pain perception. We used three von Frey filaments corresponding to innocuous, intermediate, and noxious stimulations (0.07, 0.6, and 1.4 g, respectively). Pain threshold was considered to be reached for two withdrawals out of five consecutive filament applications. Oxaliplatin treatment resulted in the development of a dose-dependent increase in nociceptive scores (Fig 2A), reflecting a mechanical allodynia (0.07 g stimulus), and a mechanical hyperalgesia (0.6 and 1.4 g).

To verify that the painful signs observed were purely sensitive, we evaluated whether oxaliplatin would affect muscle strength or motor coordination (Supporting Fig 1B and C) and found that these parameters were not affected.

Oxaliplatin alters cold-sensitive neurons temperature thresholds

To investigate the cold sensitivity of dorsal root ganglion (DRG) neurons in culture, we measured fluctuations of intracellular calcium in response to cooling. As previously shown (Madrid et al, 2009; Noel et al, 2009), the thresholds of cold-sensitive DRG neurons varied over a large range (35–15°C) as demonstrated by the simultaneous recordings of four cold-sensitive neurons from vehicle-treated mice (Fig 3A). The frequency distribution of threshold temperatures (Fig 3B) shows that cold-sensitive DRGs

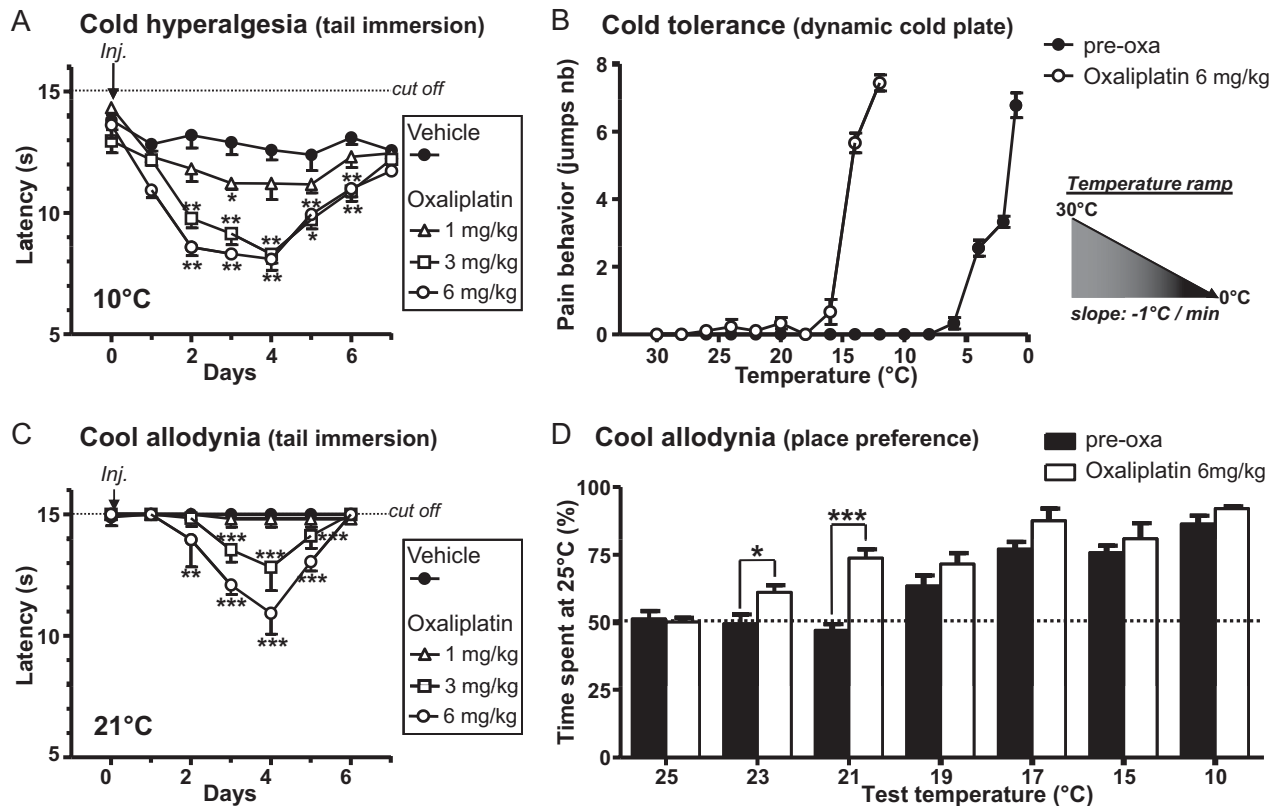


Figure 1. Oxaliplatin effects on cold/cool perception of mice.

- A.** Withdrawal thresholds to tail immersion at 10°C measured daily for 6 days before treatment (day 0) and after single i.p. injection with vehicle (filled circles, $n = 10$) or 1, 3 or 6 mg/kg of oxaliplatin (open triangle, open square and open circle, respectively; $n = 10$ per group). The dotted line at 15 s represents the test cut off value.
- B.** Dynamic cold plate test performed 90 h after vehicle/oxaliplatin injection. The number of nocifensive reactions (jumps) was measured from 30 to 1°C (vehicle: filled circles; oxaliplatin 6 mg/kg open circles; $n = 8$ per group).
- C.** Withdrawal thresholds to tail immersion at 21°C measured daily for 6 days in mice before (day 0) and after single i.p. injection with vehicle (filled circles, $n = 10$) or 1, 3 or 6 mg/kg of oxaliplatin (open triangle, open square and open circle, respectively; $n = 10$ per group).
- D.** Thermic place preference at 90 h post vehicle/oxaliplatin injection. Mice were allowed to choose between adjacent surfaces set to 25°C versus a range of temperatures as shown. The percentage of time spent at 25°C over a 3 min period is shown. Filled and open bars represent the vehicle and the oxaliplatin (6 mg/kg) groups, respectively ($n = 10$ mice per group).

from vehicle-treated mice can be separated in two subpopulations with high and low thresholds with a limit between the two groups around 25°C. In contrast, the same analysis with cold-sensitive neurons from oxaliplatin-treated mice shows that the vast majority of neurons responds mainly with a low threshold (between 35 and 25°C). Furthermore, we observed in some of these neurons from oxaliplatin-treated mice, episodes of spontaneous intracellular calcium oscillations even before cooling (not shown). In addition, the proportion of cold-sensitive neurons in the culture is doubled by oxaliplatin (Fig 3C) consistent with a state of hyperexcitability of these nociceptors induced by chemotherapy.

TRPM8-expressing nociceptors mediate oxaliplatin-induced increase of cool/cold perception

Pharmacological characterization of cold-sensitive neurons *in vitro* using chemical agonists showed that these cells from both

vehicle- and oxaliplatin-treated mice similarly use TRPM8 as the major cold transduction mechanism (Supporting Fig 2). Moreover, cool allodynia develops in the range of temperatures activating the thermoreceptor TRPM8 (McKemy et al, 2002; Peier et al, 2002). Thus, we evaluated whether the effects of oxaliplatin would be abolished in mice deficient for this channel. As presented in Fig 4A, in the cold tolerance paradigm used, TRPM8-null mice did not elicit nocifensive behaviour to noxious cold either before or 90 h after oxaliplatin injection. Similarly, in the thermal preference test (Fig 4B), oxaliplatin failed to induce cool allodynia in TRPM8 null mice in contrast to wild type animals (Fig 1D). However, the mechanical pain symptoms still developed in these knock out (KO) mice (Fig 4C). Collectively, these results indicate that oxaliplatin mediates a cold hypersensitivity (both hyperalgesia to noxious cold, and allodynia to innocuous cool) via TRPM8 afferent fibres, but the mechanism remains to be determined.

Mechanical stimuli (von Frey)

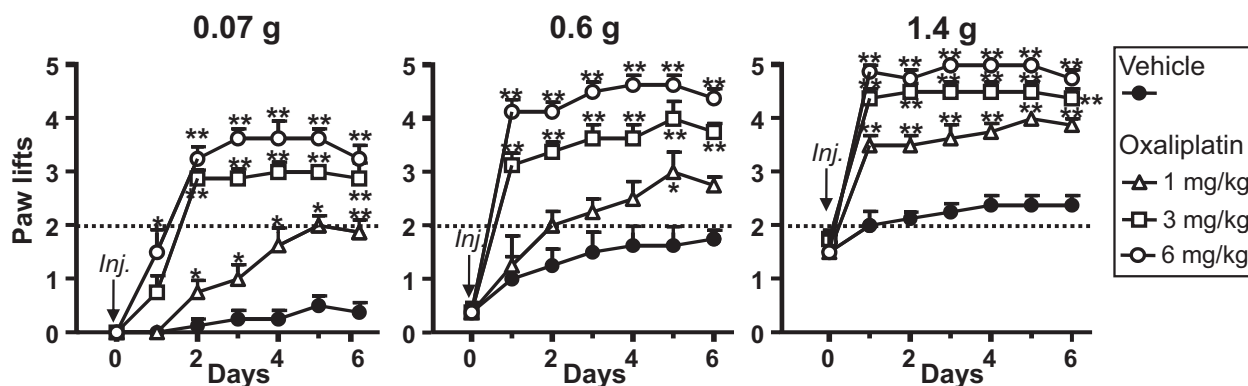


Figure 2. Effect of oxaliplatin on mechanical perception in wild type mice. Number of paw lifts out of five mechanical stimulations using von Frey filaments corresponding to innocuous (0.07 g), intermediate (0.6 g), and noxious (1.4 g) bending forces. The pain threshold is obtained for two lifts (dotted line). The measurements were done daily before treatment (day 0) and after single i.p. injection of vehicle (filled circles, $n = 10$) or 1, 3 or 6 mg/kg of oxaliplatin (open triangle, open square and open circle, respectively; $n = 10$ per group).

Oxaliplatin transcriptionally regulates a set of ion channels important for cold sensing

Does oxaliplatin directly alter the activity of TRPM8 or does it induce downstream changes from this class of ion channels able to explain this hypersensitivity? When tested directly on recombinant TRPM8 channels, neither oxaliplatin nor its two metabolites were able either to shift channel activation threshold towards warmer temperatures or to potentiate the amplitude of TRPM8 activity (Supporting Fig 3). The timing of painful effects of oxaliplatin (dozen of hours) suggests that they could result from a transcriptional modification within the nociceptors specialized in cold detection. Therefore, we performed quantitative PCR analysis to detect potential changes in the expression of candidate genes coding for ion channels known to be involved in cold-sensing nociceptor excitability comprised of the cold thermosensors TRPM8 and TRPA1; the cold-sensitive potassium channels TREK1, TRAAK, the $K_v1.1$ and $K_v1.2$ potassium channels; the $Na_v1.8$ sodium channel; and the hyperpolarization-activated channels (HCN1-4). Total RNA was obtained from lumbar L1-6 DRG 90 h post vehicle or oxaliplatin injection (10 mice per condition). Expression levels were normalized to the expression of two invariant housekeeping genes (HKGs) in the four RNA samples analysed (Fig 5, Supporting methods). For most of the analysed transcripts, several sets of primers were selected in individual exons. Amongst the two thermoreceptors analysed, oxaliplatin did not modify TRPM8 expression. Moreover, as previously reported, TRPM8 was found to be more abundantly expressed in trigeminal ganglion compared to DRG (not shown). The expression of TRPA1 was found to be slightly enhanced in DRG but at the limit of statistical significance. In contrast, the two-pore potassium channels TREK1 and TRAAK were potently down-regulated by oxaliplatin treatment in DRG. The slowly inactivating voltage-gated potassium channel $K_v1.1$ was also found down-regulated in DRG samples albeit to a lesser extent

(by ~20%) compared to that of TREK1 and TRAAK (~70%). With respect to the pro-excitatory channels analysed, the sodium channel $Na_v1.8$ transcript was slightly increased. Concerning transcripts coding for hyperpolarization activated currents (Ih), we found that among the four HCNs, only the HCN1 and two subtypes were expressed in DRG as previously demonstrated. Oxaliplatin treatment resulted in highly significant increase of HCN1. Collectively, this transcriptome analysis reveals that oxaliplatin induces a global remodelling of the candidate ion channel expression in DRGs.

TRPA1 channels are important for oxaliplatin-mediated mechanical hypersensitivity

Expression analysis revealed that TRPM8 and TRPA1 channels were minimally affected, although TRPA1 was found to be slightly increased. In addition to its role in detecting irritant chemicals, TRPA1 has been controversially implicated in noxious cold and mechanical sensation; therefore, we used the selective TRPA1 antagonist HC-030031 to evaluate its effects on oxaliplatin-induced neuropathy. As presented in Fig 6A, oxaliplatin-mediated cold hyperalgesic animals were treated intraperitoneal (i.p.) with HC030031 at 100 mg/kg (an *in vivo* active concentration in rodents (Eid et al, 2008)) or its vehicle. Thirty minutes after treatment, mice were subjected to the cold tolerance test. HC-030031 treatment had no effect on the oxaliplatin-induced cold hyperalgesia. Interestingly, in vehicle-treated animals that show intolerance to noxious cold at much colder values (~5°C), HC030031 attenuated the nocifensive behaviour of the mice. In contrast, the mechanical hyperalgesia was completely corrected by HC030031 (Fig 6B), corroborating the notion that TRPA1 channels play an important role in the mechanisms responsible for mechanical hypersensitivity in neuropathic condition (Eid et al, 2008). However, acute mechanical pain perception in control animals was not affected by the TRPA1 antagonist suggesting that the transduction of

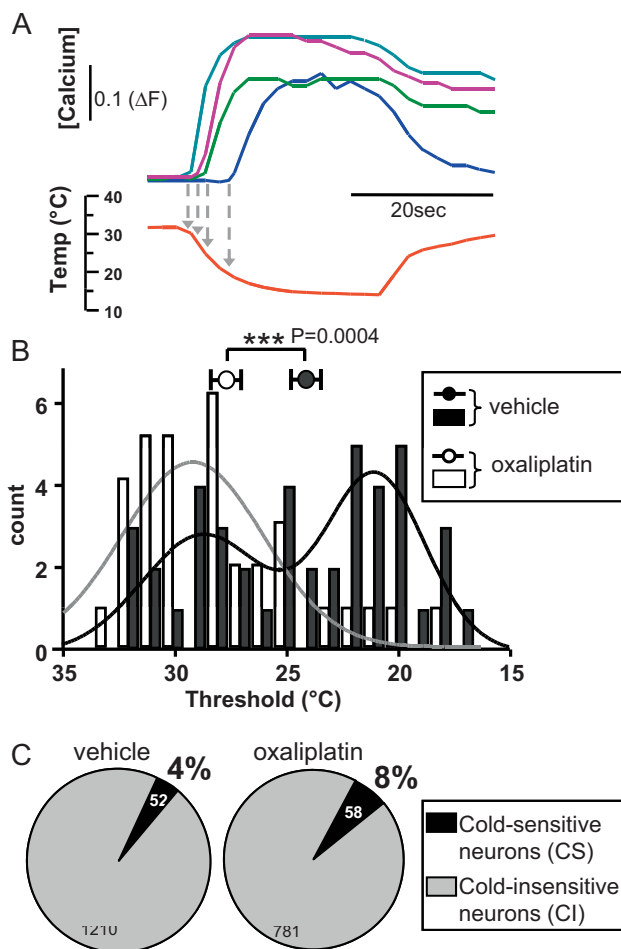


Figure 3. Effect of oxaliplatin treatment on cold-sensing DRG neurons properties measured *in vitro*.

- A. Time course of intracellular calcium elevation in the four cold-sensitive neurons showing the variability in temperature thresholds (represented by grey arrows).
- B. Histogram of frequency distribution of temperature thresholds for cold-sensitive neurons from vehicle- or oxaliplatin-treated mice (dark and open bars, respectively). Distributions of thresholds from vehicle- or oxaliplatin-treated cold-sensitive neurons are fitted, respectively, with a double or a single Gaussian equation. Mean thresholds (\pm SEM) of vehicle (filled circle: $24.2 \pm 0.7^\circ\text{C}$) and oxaliplatin (open circle: $27.7 \pm 0.7^\circ\text{C}$) are displayed behind the histogram ($p = 0.0004$).
- C. Effect of oxaliplatin or vehicle on the percentage of cold-sensitive neurons in the total number of DRG cells analysed.

mechanical stimuli is governed by multiple molecular substrates.

TREK-1 and TRAAK channels are important for oxaliplatin-mediated cold and mechanical hypersensitivity

One of the most marked transcript expression changes observed was a decrease in background potassium channels. Consequently, we asked whether oxaliplatin-induced cold hypersensitivity would still develop in mice invalidated for both TREK1 and TRAAK subunits. As presented in Fig 7A and B, vehicle-treated TREK1-TRAAK KO animals presented a tonic intolerance

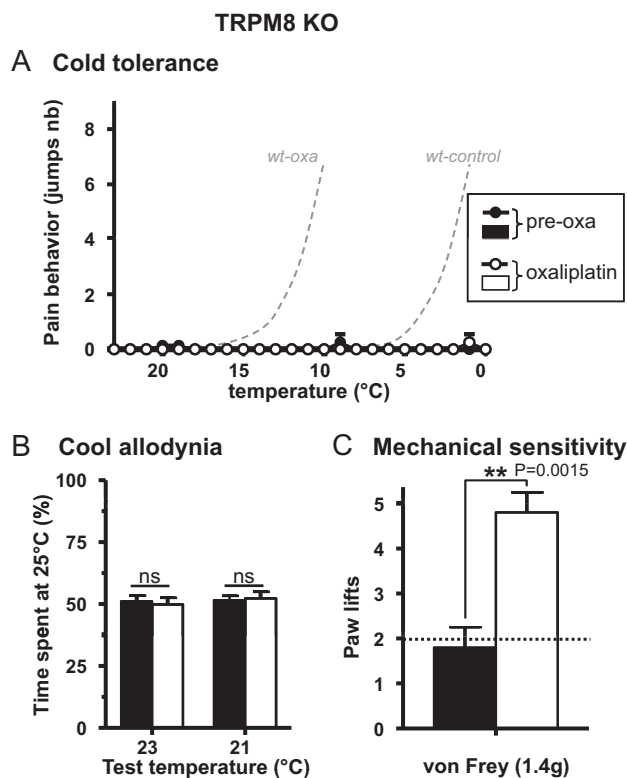


Figure 4. Effect of oxaliplatin (6 mg/kg) on TRPM8 KO mice.

- A. Dynamic cold plate test performed before (filled circles) and 90 h after oxaliplatin injection (open circles, $n = 10$). Nocifensive reactions were measured from 22 to 1°C . Grey dotted lines represent the reactions of vehicle- and oxaliplatin-treated wild type mice.
- B. Thermal place preference before (filled bars) and 90 h after oxaliplatin injection (open bars, $n = 10$). Mice were allowed to choose between adjacent surfaces adjusted to 25°C versus 23°C or 21°C .
- C. Effect of oxaliplatin on mechanical perception on the same TRPM8 KO mice as in (A) and (B) ($n = 10$ per group). Numbers of paw lifts out of 5 mechanical stimulations using a von Frey filament of 1.4 g bending force.

to noxious cold (Fig 7A) and cool allodynia (Fig 7B) similar to that of wild type animals after oxaliplatin treatment (Fig 1B and D). Interestingly, oxaliplatin failed to increase this tonic hypersensitivity to cold in the double KO mice, demonstrating a total loss of oxaliplatin modulation of cold perception in this genotype in agreement with the qPCR results. As previously described, the TREK1-TRAAK KO mice presented a robust mechanical hyperalgesia that could not be further modified by oxaliplatin (Fig 7C).

HCN channel pharmacological inhibition reverses oxaliplatin mediated cool/cold hypersensitivity

Given that the treatment with oxaliplatin resulted in an over-expression of Ih channels, we assessed the effect of the pan HCN inhibitor ivabradine, a recently developed and clinically used compound to treat stable angina pectoris (Berdeaux et al, 2009). This molecule was chosen for its more selective effects compared to other Ih blockers, and, importantly, for its inability

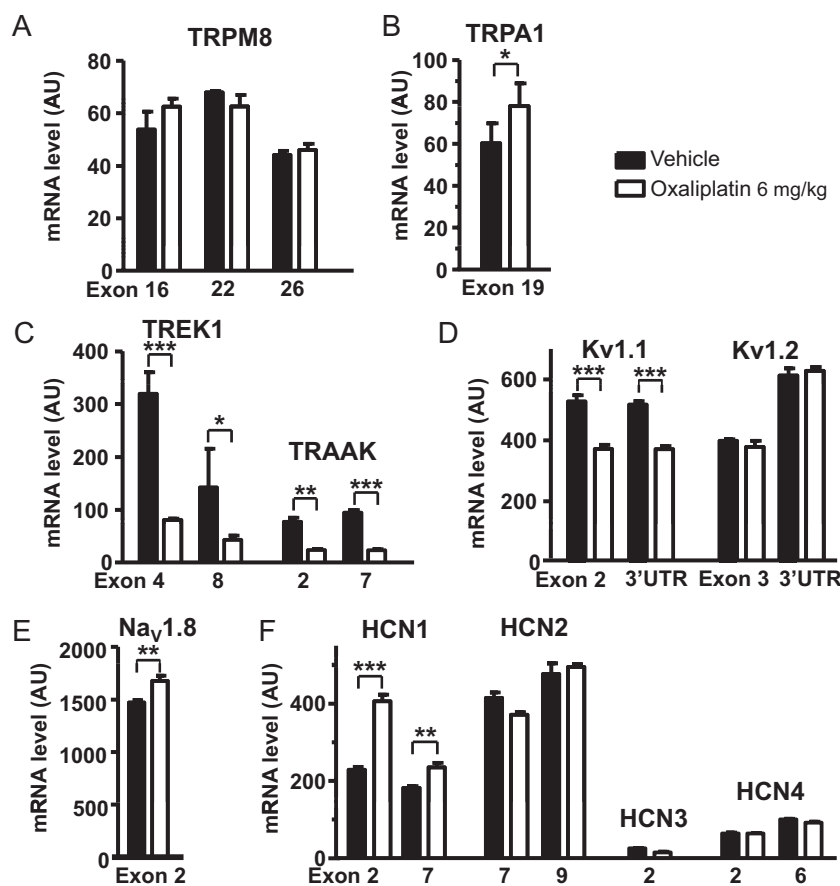


Figure 5. Oxaliplatin (6 mg/kg) effect on the expression profile of a set of ion channels in DRG. The filled and open bars represent the vehicle- and the oxaliplatin-treated samples, respectively. The numbers in the X-axis correspond to the exon number targeted in the given channel transcript analysed.

A,B. Expression of the cold-activated thermo receptors, TRPM8 (A) and TRPA1 (B).

C,D. Expression profile of the potassium channels TREK1 and TRAAK (C), and $K_v1.1$ and $K_v1.2$ (D).

E. Expression of the sodium channel $Na_v1.8$.

F. Expression profile of the HCN1-4 hyperpolarization activated cationic channels.

to cross the blood brain barrier (BBB). Therefore, its exclusive peripheral action would not be complicated by CNS effects. As presented in Fig 8A, oxaliplatin-mediated cold hyperalgesic animals were treated with ivabradine at 3 mg/kg (i.p.) (a clinically relevant dose that keeps the heart rate in the physiological range) or vehicle. Thirty minutes after vehicle or ivabradine injection, the mice were subjected to the cold tolerance paradigm (correct time window for the ivabradine efficacy). Ivabradine clearly reduced the oxaliplatin cold hyperalgesia and normalized the noxious cold perception close to the vehicle-treated thresholds, although return to the initial (pre-treatment) threshold was not completely obtained (Fig 8A). Similarly, ivabradine completely abolished the oxaliplatin-induced cool allodynia (Fig 8B). In vehicle-treated control mice, ivabradine had no statistically significant effect. Importantly, ivabradine did not alter locomotor activity that could have biased result interpretation (Supporting Fig 4). Interestingly, the mechanical hyperalgesia was not corrected by ivabradine (Fig 8C) suggesting that HCN channels are probably more prominent in monomodal nociceptors solely activated by cold. To explore the effect of ivabradine on cold-sensitive nociceptor excitability further, we evaluated the effect of HCN blockade on cold thresholds by measuring fluctuations of intracellular calcium in response to cooling. In cold-sensitive neurons from vehicle-treated mice, ivabradine produced a minimal shift towards colder temperature (Fig 9A). Although

there was a tendency to slightly increase thresholds, this effect was not statistically significant (ctrl: $25.4 \pm 1.3^\circ\text{C}$ versus iva: $23.4 \pm 1.4^\circ\text{C}$, $n = 17$, $p = 0.3230$). In contrast, in nearly all cold-sensitive neurons from oxaliplatin-treated mice (Fig 9B), ivabradine produced an increase in the cold threshold by 5°C towards colder values (ctrl: $27.4 \pm 0.8^\circ\text{C}$ versus iva: $22.9 \pm 0.9^\circ\text{C}$, $n = 19$, $p = 0.0008$). These results indicate that HCN channels are important tuners of cold sensitivity in cold-sensitive DRG nociceptors. Thus, as for TRPA1 and TREK1-TRAAK KO mice, this pharmacological effect nicely corroborates the transcriptome analysis.

DISCUSSION

Chemotherapy-induced peripheral neuropathy is a common, often severe and dose limiting toxic side effect of cancer treatment (Wolf et al, 2008). Despite its clinical relevance, several important issues are still to be addressed for a less empirical therapeutic management of these pain symptoms. These include a better understanding of the underlying mechanisms of these neuropathies. Among the currently used chemotherapy treatments, the third generation platinum compound oxaliplatin is unique in producing early onset neuropathic pain signs associated specifically to exacerbated cold perception in almost all patients (Attal et al, 2009).

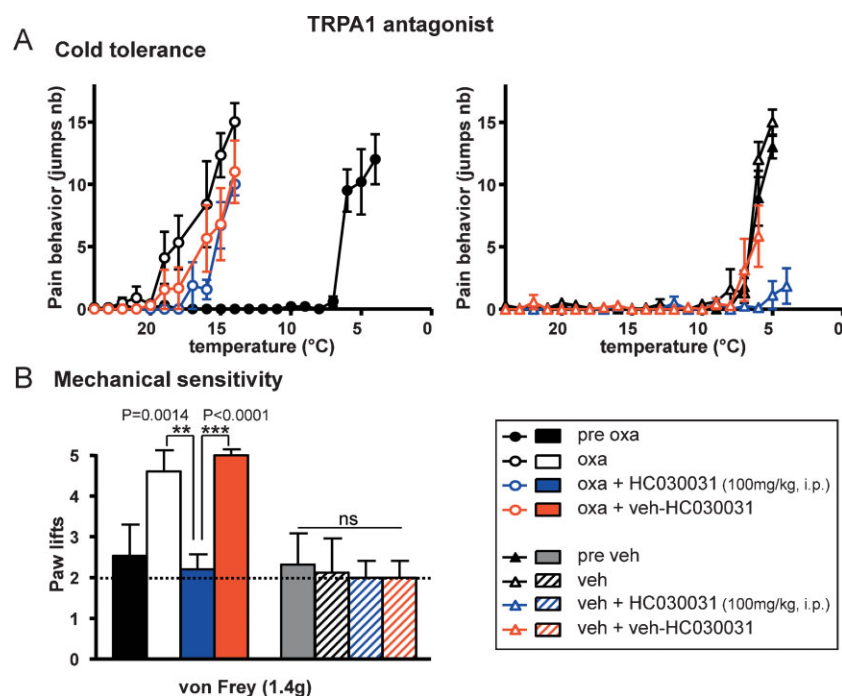


Figure 6. The TRPA1 channel blocker HC030031 does not affect oxaliplatin mediated cold hypersensitivity but reverses mechanical hyperalgesia. Filled black circles and bars represent the basal values before oxaliplatin injection, while the open circles and bars corresponds to the oxaliplatin (6 mg/kg) treated animals at 90 h ($n = 20$) prior to treatments with HC03031 (100 mg/kg i.p.) or vehicle. The red circles/bars and the blue circles/bars represent, respectively, the oxaliplatin-vehicle and the oxaliplatin-HC030031 groups ($n = 10$ per group). Filled black triangle and grey bars represent the basal values before vehicle injection, while the black open triangle and hatched bars corresponds to the vehicle treated animals at 90 h ($n = 20$) prior to treatments with HC03031 (100 mg/kg i.p.) or its vehicle. The red triangle/hatched bars and the blue triangles/hatched bars represent, respectively, the vehicle-vehicle and the vehicle-HC030031 groups ($n = 10$ per group).

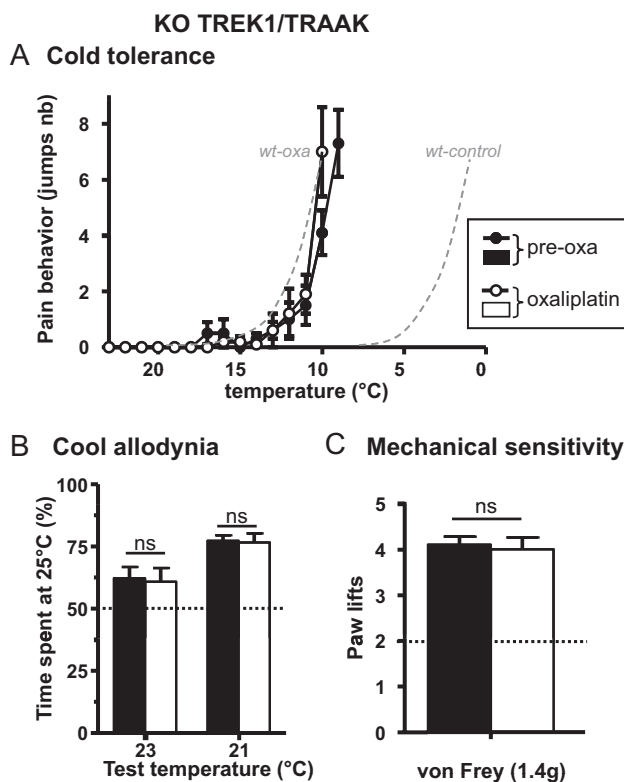
- A.** Lack of effect of TRPA1 channel blockade with acute HC030031 treatment on oxaliplatin cold hyperalgesia (left panel). The same treatment reduces normal cold tolerance in control mice (right panel).
- B.** Reversal of oxaliplatin-mediated mechanical hyperalgesia by HC030031 in similar experimental conditions as in (A) ($n = 20$ or 10 per group). Numbers of paw lifts out of five mechanical stimulations using a von Frey filament of 1.4 g bending force.

Although antineoplastic action of platinum compounds is believed to be a consequence of DNA alkylation, the rapid and specific cold hyperalgesic and allodynic effects of oxaliplatin suggest a unique pathophysiological mechanism. These clinical characteristics of oxaliplatin-mediated sensory troubles can be duplicated in rodents (Authier et al, 2009; Joseph et al, 2008; Joseph & Levine, 2009; Ling et al, 2007b; Ling et al, 2008), offering the opportunity to use a preclinical neuropathic pain model, which is highly relevant to the clinical situation, to basic research.

The early onset of oxaliplatin-mediated sensory troubles that precedes the structural alteration of the peripheral nerve integrity suggests a consequence on nerve excitability. In line with this hypothesis, a direct effect on sodium and potassium channels has been described (Grolleau et al, 2001; Kagiava et al, 2008). However, these immediate actions do not correlate well with the neuropathy that develops within a time scale of hours and persists for days. Corroborating the beneficial effects of antioxidant treatments in patients, the role of oxidative stress in the oxaliplatin painful effects has been demonstrated in rats

Figure 7. Effect of oxaliplatin (6 mg/kg) on TREK1-TRAAK KO mice.

- A.** Dynamic cold plate test performed before (filled circles, $n = 10$) and 90 h after oxaliplatin injection (open circles, $n = 10$). Nocifensive reactions were measured from 22 to 1°C.
- B.** Thermal place preference before (filled bars) and 90 h after oxaliplatin injection (open bars, $n = 10$). Mice were allowed to choose between adjacent surfaces adjusted to 25°C versus 23°C or 21°C.
- C.** Effect of oxaliplatin on mechanical perception on the same TREK1-TRAAK KO mice as in (A) and (B) ($n = 10$ per group). Numbers of paw lifts out of five mechanical stimulations using a von Frey filament of 1.4 g bending force.



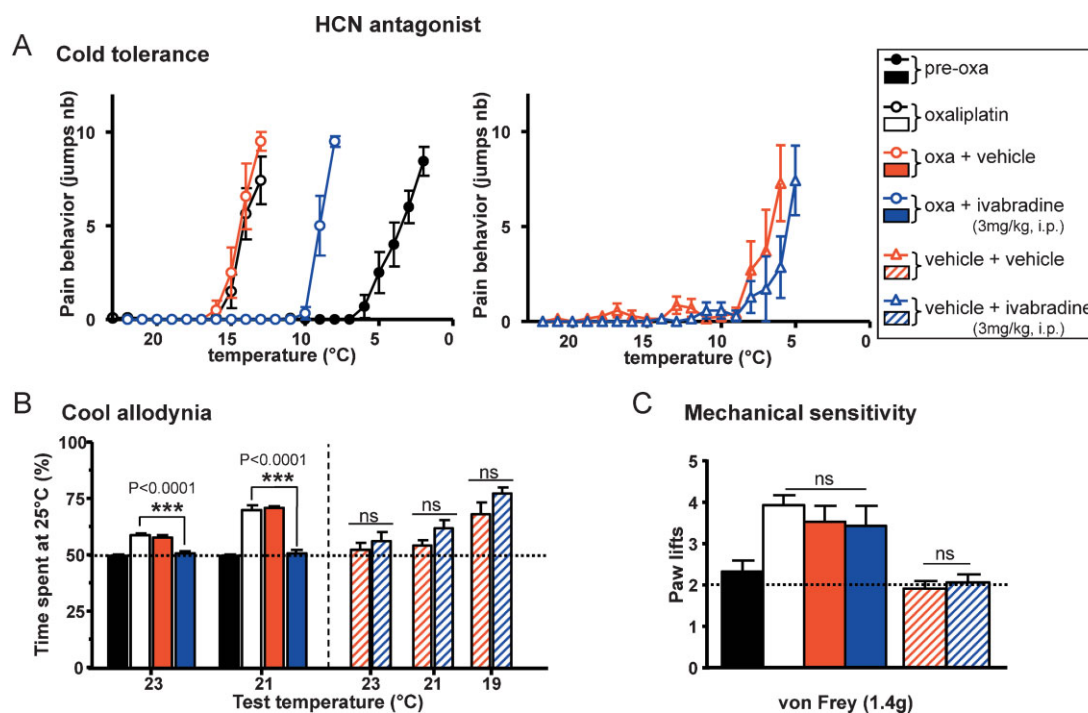


Figure 8. Reversal of oxaliplatin-mediated cold hypersensitivity by the HCN channel blocker ivabradine. Filled black circles and bars represent the basal values before oxaliplatin injection, while the black open circles and bars corresponds to the oxaliplatin (6 mg/kg) treated animals at 90 h ($n = 16$) prior to vehicle or ivabradine treatment (3 mg/kg i.p.). The red circles/bars and the blue circles/bars represent, respectively, the oxaliplatin-vehicle and the oxaliplatin-ivabradine groups ($n = 8$ per group). The red triangle/hatched bars and the blue triangles/hatched bars represent, respectively, the vehicle-vehicle and the vehicle-ivabradine groups ($n = 8$ per group).

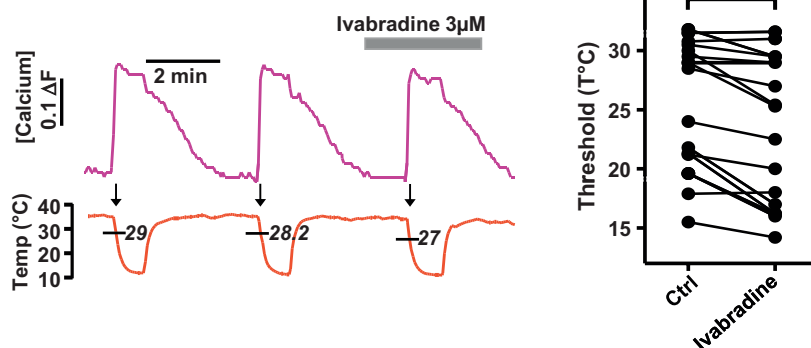
- A.** Effect of HCN channel blockade with acute ivabradine treatment on oxaliplatin-induced cold hyperalgesia measured on the dynamic cold plate (left panel). The same treatment minimally affects normal cold tolerance (right panel).
- B.** Acute ivabradine treatment reverses cool allodynia measured in the thermal place preference test for two temperature choices (25 versus 23 or 21°C) whilst it does not affect place preference in control animal (25 versus 23, 21 or 19°C) ($n = 8$ per group).
- C.** Lack of effect of ivabradine on oxaliplatin-mediated mechanical hyperalgesia or on acute mechanical perception in similar experimental conditions as in (A) and (B) ($n = 8$ per group). Numbers of paw lifts out of five mechanical stimulations using a von Frey filament of 1.4 g bending force.

(Joseph et al, 2008; Joseph & Levine, 2009). Nonetheless, since the molecular understanding of cold perception by the peripheral nerves has increased recently with the use of mice deficient for specific ion channels underlying cold excitability, we evaluated the neurotoxic effects of oxaliplatin in mice. Our results clearly demonstrate that single injection of oxaliplatin induces a dose-dependent development of neuropathic signs with the characteristic hallmark of enhanced cold perception. This analysis demonstrates the hypersensitivity to noxious cold as described in rats (Joseph et al, 2008; Joseph & Levine, 2009; Ling et al, 2007b), as well as allodynia to innocuous cool. The behavioural paradigms used here such as the dynamic cold plate and the thermal place preference test on freely moving animals implemented the knowledge on the effects of oxaliplatin by providing robust and clear quantification of the hypersensitivity to cold that has not been previously reported. Along with this aversion to cold, we demonstrated that oxaliplatin induces a dose-dependent mechanical allodynia and hyperalgesia. At the cellular level, our data show that cold-sensitive DRG neurons have a broad range of activation thresholds as previously shown for cold-sensitive trigeminal

nociceptors (Madrid et al, 2009). Oxaliplatin narrows this distribution towards an homogeneous population of low threshold cold-sensitive neurons activated by moderate cooling.

In view of the role of the thermoreceptor TRPM8 to sense environmental innocuous and noxious cold (Bautista et al, 2007; Colburn et al, 2007; Dhaka et al, 2007), we examined a possible role for this channel in oxaliplatin-mediated cold hypersensitivity. Consistent with a preponderant role of TRPM8-expressing nociceptors, depletion of TRPM8 suppressed cool allodynia. Conversely, mechanical hypersensitivity was still present in the TRPM8 KO genotype, which is congruent with the specific role of TRPM8 on cold sensing. Considering that a fraction of cold-sensing afferent fibres are polymodal and also activated by mechanical stimuli (Abrahamsen et al, 2008; Zimmermann et al, 2007, 2009), these data suggest that, despite the loss of the cold transducer in these sensory endings, oxaliplatin affects the general excitability of these neurons rather than a unique action on TRPM8 channels. Moreover, we have shown that *in vitro*, an absence of direct modulation of recombinant TRPM8 by oxaliplatin or its metabolites. Additionally, the time course to reach the cold hypersensitivity acme (dozens of hours) suggests

A CS neurons / vehicle mice



B CS neurons / Oxaliplatin mice

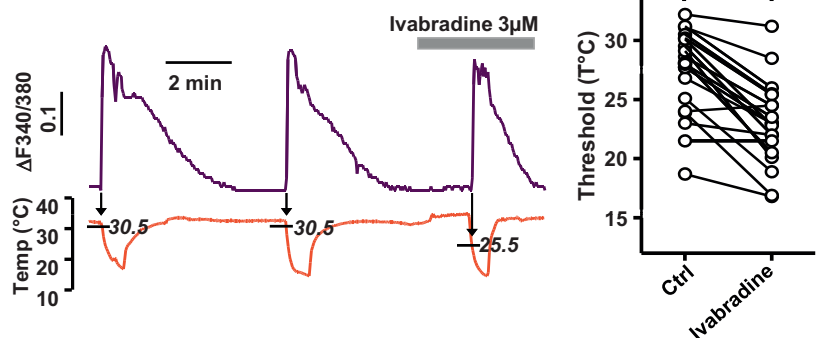


Figure 9. Effect of ivabradine on cold-sensitive DRG neurons thresholds.

A. Time course of intracellular calcium elevation in a cold-sensitive neuron from a vehicle-treated animal showing that cooling elicits elevations of intracellular calcium with reproducible thresholds that are not affected by ivabradine treatment. The histogram on the right shows the thresholds before and during ivabradine perfusion for all the cells tested with no statistical differences ($n = 18$).

B. Same representation as in (A) for cold-sensitive DRG neurons from oxaliplatin-treated mice. The time course shows that ivabradine alters the cold threshold. The histogram displaying all cold-sensitive neurons tested reveals a significant effect of ivabradine ($p = 0.0008$, $n = 18$).

a change in expression of regulators of membrane excitability after oxaliplatin treatment including ion channels involved downstream from TRPM8. The notion that cold detection in cold nociceptors is driven by the coordinated action of a set of ionic channels has been clearly demonstrated previously (Madrid et al, 2009; Momin et al, 2008; Viana et al, 2002). Furthermore, the capacity of oxaliplatin to alter gene expression is documented (Martinez-Cardus et al, 2009; Meynard et al, 2007), and transcriptional changes are critical to most neuropathies (Persson et al, 2009) with a contribution of epigenetic regulations (Uchida et al, 2010), supporting that these effects arise in nociceptors upon oxaliplatin treatment. The transcriptional analysis performed confirmed this notion. The lumbar DRG contain the cell bodies of cold-sensing neurons innervating the hindpaws concerned by the behavioural exploration performed. In contrast with a recent report (Ta et al, 2009), we did not observe any difference in TRPM8 expression in our conditions despite the use of several sets of primers. We confirmed the original observations (Peier et al, 2002) that the amplified transcripts were more abundant in trigeminal ganglion compared to DRG (not shown). We observed that TRPA1 expression is slightly increased within the DRG but since this channel is more implicated in cold perception in vagal or trigeminal neurons (Fajardo et al, 2008; Karashima et al, 2009) as well as in the detection of irritant chemicals (Bautista et al, 2006; Macpherson et al, 2007; Talavera et al, 2009), its implication in the oxaliplatin neuropathy seems less probable. Nevertheless, the contribution of TRPA1 to noxious cold pain is still a matter of debate, however, its role in inflammatory or

neuropathic pain of traumatic etiology has been recently demonstrated (del Camino et al, 2010). In addition, the implication of TRPA1 in mechanical hyperalgesia has also been documented (Eid et al, 2008). Our results, obtained using the TRPA1 antagonist, clearly corroborate its role in mechanosensation. With respect to the oxaliplatin-induced cold hypersensitivity, TRPA1 does not seem to play a major role, confirming the essential and major contribution of TRPM8 expressing fibres in this phenomenon. However, results obtained in the cold tolerance test in naïve animals did reveal a protective effect of the TRPA1 antagonist. Thus, at very cold temperatures, TRPA1 might play a role in cold sensing, although the effect is clearly less dramatic than the TRPM8 KO phenotype using the same test. Therefore, the main picture emerging from these results is a clear participation of TRPA1 in the mechanical hyperalgesia aspect of oxaliplatin-induced neuropathy, suggesting its implication in excitatory mechanotransduction complexes whose molecular entities are still being uncovered (Coste et al, 2010).

Particular subtypes of potassium channels have been shown to actively control the membrane potential of cold-sensing neurons and consequently regulate cold perception (Madrid et al, 2009; Noel et al, 2009). The repression of the TREK1 and TRAAK channels by oxaliplatin treatment is in line with the marked cold hypersensitivity of TREK1-TRAAK KO mice (Noel et al, 2009). In agreement, we show that oxaliplatin-induced cold allodynia is similar to that of TREK1-TRAAK KO animals and that oxaliplatin does not further enhance this cold allodynia. These findings fully agree with functional exploration of isolated

DRG neurons from these KO mice showing that cold and menthol sensitivity is largely increased in calcium imaging experiments suggesting a large overlap in expression of TREK1/TRAAK with TRPM8 (Noel et al, 2009). Furthermore, we confirmed that the loss of these background cold and mechanosensitive potassium conductances (Maingret et al, 2000) leads to a mechanical hypersensitivity (Alloui et al, 2006; Noel et al, 2009) comparable with that observed in wild type animals with oxaliplatin treatment. This mechanical hypersensitivity is not modified by oxaliplatin. TREK1/TRAAK channels are broadly expressed in primary afferents, including heat-sensing nociceptors. Decrease of their expression would predict a hypersensitivity to heat as reported for the double KO (Alloui et al, 2006; Noel et al, 2009). However, we found that oxaliplatin does not modify mice reactions to noxious heat. This indicates a probable pronounced tropism of oxaliplatin on cold and mechanically activated subtypes of sensory neurons with a minimal effect on heat-sensitive fibres. Also consistent with previous observations on the role of I_{K_D} potassium currents in cold sensitive nociceptors (Madrid et al, 2009), K_{V1} , one of the major subunits coding for these currents, is down-regulated by oxaliplatin treatment.

Pro-excitatory channels have also been implicated in cold perception. The $Na_{V1.8}$ sodium channels have been shown to be essential to the excitability of cold sensing terminal nerve

endings (Zimmermann et al, 2007). We found an up-regulation of this subunit that could participate in the effects of oxaliplatin. Finally, we assessed whether Ih channels play a role in the effects of oxaliplatin treatment. Ih channels encoded by the HCN subunits have been linked to cold perception (Momin et al, 2008; Orio et al, 2009). Evaluation of the expression of all the members of this channel family revealed that HCN1 and 2 are predominant in sensory ganglia, and that oxaliplatin up regulates HCN1. This increase in HCN1 is consistent with data on neuropathic pain of traumatic etiology (Chaplan et al, 2003) and inflammatory cold pain (Momin et al, 2008). As for TREK1 and TRAAK, large HCN1 like Ih currents were found to have a nearly total overlap expression with cold or menthol activated currents from isolated sensory neurons in rat (Kondrats'kyi et al, 2008) and mice (Madrid et al, 2009; Orio et al, 2009). Furthermore, *in vivo* microneurography recordings of single cold-sensing C-fibres in rats suggested the importance of HCN channels in their firing (George et al, 2007). In addition, cold-sensing fibres have been described to prominently elicit rhythmic firing (Orio et al, 2009), including in humans with persisting ongoing activity following cold exposure (Serra et al, 2009), which is compatible with HCN channel activity, also known as the 'pacemaker channels'. Indeed, HCN channels also shape the excitability of heart pacemaker cells and a pan HCN inhibitor, ivabradine, has been marketed to treat angina pectoris

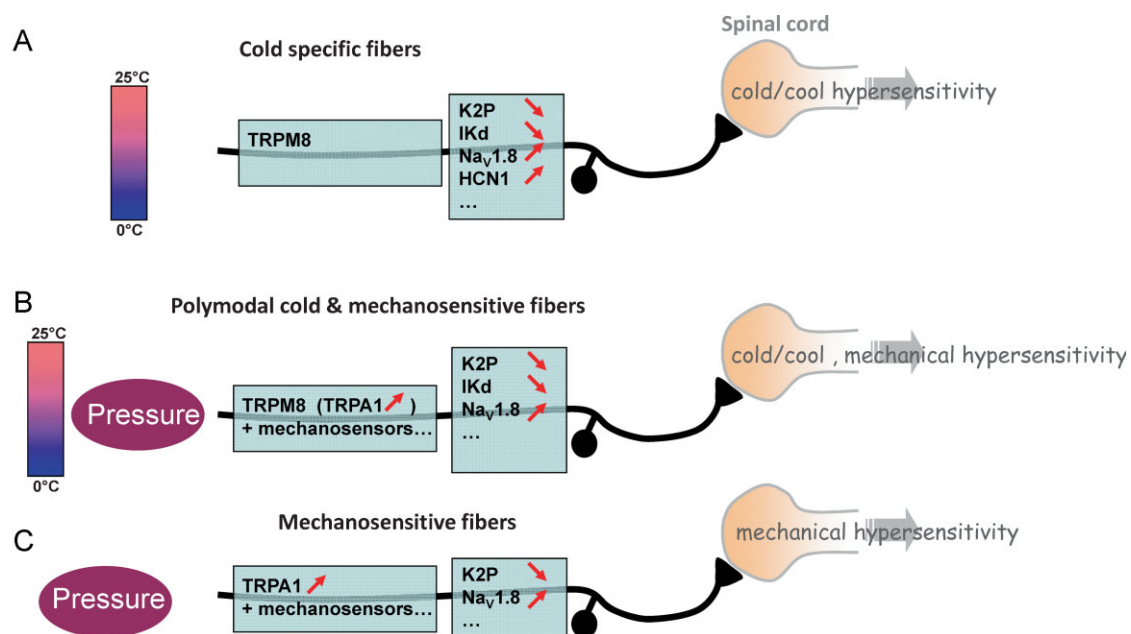


Figure 10. Schematic representation of oxaliplatin-mediated changes in cold and mechanically sensitive primary afferent fibres (adapted from (Madrid et al, 2009)).

- Monomodal cold-specific fibres use TRPM8 as the main detector of innocuous cool and noxious cold stimuli. Oxaliplatin modifies their excitability by decreasing inhibitory potassium channels and increasing excitatory channels with a prominent effect on HCN1.
- Polymodal cold and mechanosensitive fibres affected by oxaliplatin also use TRPM8 as cold detector in addition to yet to be identified excitatory mechanosensors. Distinct from cold specific fibres, HCN channels are not present in these neurons reflecting the lack of ivabradine effect in mechanical pain and the incomplete reversal of cold tolerance.
- Mechanosensitive fibres with up-regulated TRPA1 and down-regulated K2P in their mechanosensory machinery convey oxaliplatin-mediated mechanical hypersensitivity.

The paper explained

PROBLEM:

Oxaliplatin is a first line chemotherapy treatment for several cancers including colorectal cancer, but in nearly all patients it induces a hypersensitivity to cool and cold as a side effect. This highly prevalent neuropathic pain among oxaliplatin-treated patients reduces their quality of life and can lead to cessation of the chemotherapy. Preventive clinical management of this neuropathy is not yet available. To gain insight into the pathological mechanisms underlying sensitization of cold-sensitive sensory neurons by oxaliplatin, we developed a mouse model of oxaliplatin-induced cold hypersensitivity in mice. We used several mouse strains that do not express specific genes coding for ion channels known to be involved in cold detection to ascertain their role in oxaliplatin-mediated neuropathy.

RESULTS:

Hypersensitivity to cold develops in mice much like in patients as shown with new and original approaches of behavioural exploration of cold perception. In sensory neurons, oxaliplatin modulates the expression of a set of ion channels known to be

important for cold perception. The implications of the altered expression of these distinct ion channels (e.g. TRPA1, TREK1, TRAAK, HCN1) on the oxaliplatin-mediated neuropathy has been demonstrated using behavioural studies on KO mice and by using selective antagonists. Furthermore, at the cellular level, the oxaliplatin-mediated alteration of cold sensitivity has been demonstrated *in vitro*.

IMPACT:

Of particular translational pharmacological interest, we used ivabradine, a recently introduced clinically used antagonist of one of the ion channels (HCN1), which we identified to be transitionally upregulated by oxaliplatin in cold-sensitive primary afferent neurons. Ivabradine, which has been developed to treat stable angina pectoris, is able to selectively and strongly attenuate the cold sensitization effects of oxaliplatin in mice. Therefore, as a drug already used in the clinic, it could rapidly become a new potential preventive analgesic treatment in patients undergoing oxaliplatin chemotherapy.

and myocardial ischemia (Berdeaux et al, 2009). Moreover, ivabradine does not penetrate the CNS but can access the cold-sensing afferent fibres as well as the DRG that sits outside the BBB (Arvidsson et al, 1973). The use of a clinically relevant dose of ivabradine strongly and selectively attenuated the oxaliplatin-induced cold hyperalgesia. Additionally, this behavioural effect is corroborated by the demonstration that HCN blockade on cold-sensing neurons *in vitro* is able to increase the threshold of cold detection, thereby directly lowering the excitability of this subclass of nociceptors.

Collectively, our results demonstrate that oxaliplatin induces peripheral neuropathy in mice with a clear exacerbation of cold detection and development of mechanical hyperalgesia. Cold-sensitive sensory fibres expressing TRPM8 and mechano-sensitive fibres expressing TRPA1 are potentially affected by this toxic chemotherapy side effect. We found that within these neurons, oxaliplatin alters ion channel gene expression in agreement with transcriptional effects reported on cancer cell lines. The potassium channels TREK1, TRAAK, and, to a lesser extent, $K_V1.1$ are repressed while TRPA1, $Na_V1.8$, and HCN1 channels are transcriptionally up-regulated in these particular subclasses of sensory fibres as illustrated in Fig 10. The translational consequences of these findings for patients would be that pharmacological activators of the repressed potassium channels or antagonists of the up-regulated channels are potential tailored preventive treatments of the painful side effects of oxaliplatin. The availability of such molecules like ivabradine currently used in clinic could be of interest, especially as effective drugs for prevention are few and do not exist for curative care (Wolf et al, 2008). Further development of even more specific ligands for the identified channels is

pivotal in future treatment of chemotherapy-induced neuropathies.

MATERIALS AND METHODS

Treatments

Single i.p. injections of oxaliplatin (Sanofi Aventis, Montpellier France) were performed at three doses (1, 3 and 6 mg/kg) in male C57BL6J mice (20–25 g). Ivabradine (3 mg/kg) (Servier, Courbevoie France) and HC030031 (100 mg/kg) was injected i.p. Vehicle solutions were injected in the control groups.

Behaviour

Pain scores were determined with strict adherence to ethical guidelines (Zimmermann, 1983) (Supporting information). Threshold reflex responses to noxious cold or innocuous cool temperatures were assessed using tail-immersion in a water bath set at 10 or 21°C, respectively (Allchorne et al, 2005). Noxious cold tolerance was assessed using a dynamic cold plate (Bioseb, France) (Yalcin et al, 2009). Animals were placed on the test arena with the floor temperature progressively cooled from 30 to 1°C at a rate of $-1^\circ\text{C}/\text{min}$. This procedure allows the paw surfaces to be cooled at the same rate as the floor arena. Nocifensive behaviours (jumps) were noted as function of cooling. Cool allodynia was assessed with a thermal place preference choice test (Bioseb). Animals were placed in an arena containing identical adjacent platforms, one set to 25°C and the other adjusted to various temperatures. Mice were free to explore the arena and the time spent on each surface was recorded over a 3 min period. The percentage of time spent on the 25°C side was scored. Mechanical allodynia and hyperalgesia were assessed using

the von Frey hair filaments of three different bending forces (0.07, 0.6 and 1.4 g). For each filament, five stimuli were applied with an interval of 3–5 s.

Ca²⁺ imaging

Lumbar DRGs were prepared from vehicle or oxaliplatin (6 mg/kg) treated mice 90 h post injection as previously described. Neurons were seeded on laminin coated glass bottom chambers (fluorodish WPI) and cultivated for 12–18 h at 37°C in B27 supplemented Neurobasal A medium (Invitrogen, France) with 100 ng/ml NGF 7S (Sigma–Aldrich, France). Prior to recording, cells were incubated with 5 μM fura-2AM in Tyrode's solution for 1 h at 37°C. Fluorescence measurements were made with an inverted microscope (Olympus IX70) equipped with a coolsnap HQ camera (Roper Scientific, France). Fura-2 was excited at 340 and 380 nm and ratios of emitted fluorescence at 510 nm were acquired simultaneously with bath temperature using Metafluor software (Universal Imaging). Temperature was controlled with a gravity driven perfusion (1–2 ml/min) cooled with a peltier device mounted in series with a resistive heater (CellMicroControls). Perfusion was first cooled at 12°C then heated at 37°C before application onto the chamber. Temperature was monitored with a thermistor probe located near the perfusion outlet always at the same place. Rapid cooling from 37°C to less than 15°C, achieved by switching off the heating, took typically less than 40sec. Threshold temperature of the cold evoked response on intracellular calcium was determined on individual cells.

Molecular biology

RNA extraction, reverse transcription and quantitative PCR were performed as previously reported ((Moore-Morris et al, 2009), supplement). The expression levels of 11 genes encoding ion channels known to regulate cold perception in sensory neurons were selected. Data were analysed using the threshold cycle (Ct) relative quantification method. Results are expressed as the percentage relative to the geometric average of the expression levels of the two selected housekeeping genes.

Statistical analysis

Treatments were randomized within each cage. Behavioural data were analysed using ANOVA followed by a *post hoc* Tukey's *t*-test. QPCR data were analysed with student's *t*-test. Data were expressed as mean ± S.E.M., and the levels of significance were set at **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

Author contributions

JD, VP, AP, AF performed acquisition of data; BL technical, concept and design; VM technical; BC technical, acquisition of data; JB, CC interpretation of data; JN interpretation of data, critical revision of the manuscript; ML interpretation of data, critical revision of the manuscript, material support; AE concept and design, critical revision of the manuscript, study supervision, obtained funding; NA concept and design, critical revision of the manuscript, study supervision; EB concept and design, drafting of the manuscript, study supervision, obtained funding.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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