

Sensory and Motor Systems

Pregabalin Silences Oxaliplatin-Activated Sensory Neurons to Relieve Cold Allodynia

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Abstract

Oxaliplatin is a platinum-based chemotherapeutic agent that causes cold and mechanical allodynia in up to 90% of patients. Silent Nav1.8-positive nociceptive cold sensors have been shown to be unmasked by oxaliplatin, and this event has been causally linked to the development of cold allodynia. We examined the effects of pregabalin on oxaliplatin-evoked unmasking of cold sensitive neurons using mice expressing GCaMP-3 in all sensory neurons. Intravenous injection of pregabalin significantly ameliorates cold allodynia, while decreasing the number of cold sensitive neurons by altering their excitability and temperature thresholds. The silenced neurons are predominantly medium/large mechano-cold sensitive neurons, corresponding to the “silent” cold sensors activated during neuropathy. Deletion of $\alpha 2\delta 1$ subunits abolished the effects of pregabalin on both cold allodynia and the silencing of sensory neurons. Thus, these results define a novel, peripheral inhibitory effect of pregabalin on the excitability of “silent” cold-sensing neurons in a model of oxaliplatin-dependent cold allodynia.

Key words: oxaliplatin; pain; pregabalin; sensory neurons; silent nociceptors

Significance Statement

Pregabalin is an analgesic drug in the clinic, that is supposed to act by blocking neurotransmitter release. Here, we show that silent nociceptors that are activated by chemotherapeutic insults like oxaliplatin are silenced by pregabalin, which blocks the associated pain. This mode of action suggests that peripheral acting pregabalin-like drugs could be very useful for pain during chemotherapy, as they would have no CNS side effects, a problem for many patients with pregabalin. This novel effect of pregabalin is mediated by its interaction with the $\alpha 2\delta 1$ calcium channel subunit, but how this works is not yet understood.

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Introduction

Cold allodynia is a debilitating condition where patients experience mild, innocuous cooling as severe pain (Viana, 2018). This ailment is common in many neuropathic pain conditions, from nerve injury to diabetic neuropathy, toxin poisoning and chemotherapy treatment (MacDonald et al., 2020). Anticancer drugs, of which oxaliplatin is an example, are alkylating agents that act through the formation of DNA-platinum adducts to cause DNA damage (Cersosimo, 2005; Carozzi et al., 2015). Patients treated with platinum-based drugs usually develop acute allodynia as well as chronic neuropathic symptoms like numbness, ataxia, loss of reflexes, and of course pain: these side effects are often time and dose related, and in the case of oxaliplatin, they affect almost 90% of patients that undergo chemotherapy (Carozzi et al., 2015; MacDonald et al., 2020; Fumagalli et al., 2021).

The pathophysiological mechanisms of cold allodynia are currently unclear, but a recent article showed a common feature of many neuropathies carrying this condition: a previously unidentified population of large-diameter, silent cold-sensing neurons that become active and contribute to cold allodynia in these states (MacDonald et al., 2021). This shift in neuronal activity was particularly evident in an intraplantar pain model of oxaliplatin-induced neuropathy, one of the conditions that has the highest occurrence of peripheral cold allodynia (Deuis et al., 2013; Safat et al., 2019; MacDonald et al., 2020, 2021). Oxaliplatin is a chemotherapeutic agent associated with acute neurotoxicity that manifests as mechanical and cold allodynia (Y. Yang et al., 2021). Oxaliplatin-dependent neurotoxicity may develop in two ways: an acute and reversible sensory neurotoxicity with cold-triggered paresthesia and dysesthesia of the extremities that occurs within hours from the infusion, and secondly, a chronic sensory neuropathy, again with a stocking-glove distribution, that occurs after accumulation of the drug because of repeated infusions (Cersosimo, 2005; Land et al., 2007; Carozzi et al., 2015).

There is still no clear therapeutic route to alleviate neuropathic pain symptoms and cold allodynia. Few drugs have shown promising results, but gabapentinoids show a promising analgesic function in several neuropathic pain models (Curros-Criado and Herrero, 2007; Han et al., 2007; Gustafsson and Sandin, 2009; Coderre et al., 2010; Mangaiarkkarsi et al., 2015; Chincholkar, 2018). These compounds are thought to act primarily on excitability through their interaction with voltage-gated calcium channels (VGCCs) via the auxiliary subunit $\alpha 2\delta 1$ (Bauer et al., 2009; Chincholkar, 2018), although they can have other molecular targets such as NMDA receptors (Verma et al., 2014; Carozzi et al., 2015; Alles and Smith, 2017; Chincholkar, 2018). Gabapentin, and its successor pregabalin, are thought to act both at spinal and supra-spinal loci, inhibiting ascending nociceptive input from dorsal root ganglion (DRG) neurons and activating descending inhibitor pathways to ameliorate neuropathic pain (Carozzi et al., 2015; Chincholkar, 2018). Its use, alone or in combination with other drugs, has been shown to be effective in numerous preclinical models of neuropathic pain (Verma et al., 2014). This drug has been shown to be particularly effective in the mitigation of cold allodynia, with single dose reversal of cold symptoms occurring after spinal nerve ligation (SNL) and spared-nerve injury (SNI) models as well as an attenuation of allodynia induced by oxaliplatin that showed pregabalin as the most efficacious drug alongside lidocaine and morphine as notable examples (Ling et al., 2008; Gustafsson and Sandin, 2009; Peng et al., 2012; Verma et al., 2014).

To investigate the functional effects of pregabalin on an acute, oxaliplatin-dependent model of cold allodynia, we used behavioral testing as well as *in vivo* calcium imaging to dissect the effect of this compound on DRG neuronal activity. Here, we describe a novel, peripheral action of pregabalin that results in the preferential silencing of the large-diameter, silent cold-sensing neurons involved in

cold allodynia. Furthermore, we show that this effect is dependent on the $\alpha 2\delta 1$ subunit of the VGCCs and is abolished in a global $\alpha 2\delta 1$ transgenic knock-out (KO) mouse line.

Materials and Methods

Experimental model and treatment

Mice

All animal procedures conducted at University College London were approved by University College London ethical review committees and conformed to United Kingdom Home Office regulations (Animals (Scientific Procedures) Act 1986) under Project license P413329A2. For experiments using only wild-type (WT) mice, adult (more than six weeks old) C57BL/6 mice from Charles River were used. Both transgenic mice used in this paper had a C57BL/6 background. *Pirt-GCaMP3* mice were kindly provided by Prof. Xinzhong Dong (John Hopkins University, Baltimore, MD; Kim et al., 2014) and global $\alpha 2\delta 1$ knock-out (KO) mice were generously donated by Prof. Annette Dolphin (University College London, London, United Kingdom; Patel et al., 2013).

For experiments using genetically altered lines, all were performed on adult male and female $\alpha 2\delta 1$ -KO mice and wild-type littermates, obtained by breeding heterozygotes. Mice were housed in groups of no more than five on a 12/12 h light/dark cycle; food and water were available *ad libitum*. Both male and female animals were used for all experiments, in equal numbers where possible. These studies were not however designed to test for sex differences, and sexes were pooled for analysis. The number of animals used to generate each dataset is described in individual figure legends. For genotyping, genomic DNA was isolated from ear tissue followed by PCR. For *Pirt-GCaMP3*, the primers used were the following: forward: TCCCCTCTACTGAGAGCCAG; WT reverse: GGCCCTATCATCCTGAGCAC; GCaMP3 reverse: ATAGCTCTGACTGCGTGACC. For $\alpha 2\delta 1$ -KO, the primers used were the following: primer 01: CATGGGTGGACAAGATGCAGG; primer 02: CTGCACGAGACTA-GTGAGACG; primer 03: CATTCTCAAGACTGTA GGCAGAG.

GCaMP virus injection

Pups from $\alpha 2\delta 1$ breeding heterozygote mice [postnatal day (P)2 to P5] were used for injection of GCaMP-expressing virus. Before the injection, the mother was anesthetized with a low concentration of isoflurane (1%) and pups were anesthetized by hypothermia. Virus pAAV.CAG.GCaMP6s.WPRE.SV40 (plasmid #100844, Addgene) was injected into the plantar area of the hind paw using a 10- μ l Hamilton syringe with a cannula connected to a 30-G needle; 5 μ l of virus (titer = 5×10^{12} GC/ml) was injected into each of the two hindpaws. After injection, the pups were kept on a heating box until their body temperature returned to normal. Then they were put back into the cage before the mother was put back and rubbed against the pups. The mice injected with virus, after weaning, had the genotyping for $\alpha 2\delta 1$ subunit checked by PCR. Both sexes were used for *in vivo* imaging six to eight weeks after injection.

Chemotherapy-induced neuropathy pain model

Chemotherapy-induced neuropathy was studied in mice using the intraplantar oxaliplatin model first described by Deuis and colleagues, because this treatment recapitulates the rapid onset of cold allodynia in human patients infused with the drug (Deuis et al., 2013). Oxaliplatin (Sigma, O9512) was dissolved in 5% glucose dH₂O solution to an equivalent dose of 80 μg in 40 μl since oxaliplatin is unstable in chloride-containing saline solution. Mice were treated by intraplantar injection into the left hindpaw. Behavioral testing or imaging was assessed at least 3 h after injection.

Treatment

After injection of oxaliplatin and the behavioral or calcium imaging baseline measurements (at least 3 h after injection), the mice were treated with either pregabalin (Sigma, Y0001805) at a dose of 2 mg/kg or vehicle (PBS) solutions via intravenous injection. Behavioral testing or calcium imaging were performed up to either 60 or 50 min after injection, respectively.

Behavioral testing

All animal experiments were performed in accordance with Home Office Regulations. The investigator was blind to treatment and/or genotype. Animals were acclimatized to handling and every effort was made to minimize stress during the testing. Both male and female animals were used.

Cold plate

Mice were placed on the Cold Plate apparatus (Ugo Basile). The Cold Plate was maintained at 10°C for 5 min while the animal was free to move around on the plate and the number of nociceptive behaviors (shaking, lifting, licking, guarding, biting) displayed by the injected hindpaw were counted by the observer (Luiz et al., 2019). In the experiments with animals that did not receive any intraplantar treatment, as the nociceptive behaviors are almost absent in the hindpaws, the number of forepaw lifts was assessed by the observer instead.

In vivo calcium imaging

Acquisition

Adult mice expressing GCaMP3 (6–12 weeks, male and female) were anesthetized using ketamine (KETAVET; Zoetis; 100 mg/kg), xylazine (Rompun; Bayer; 15 mg/kg), and acepromazine (Elanco; 2.5 mg/kg). Depth of anesthesia was confirmed by pedal reflex and breathing rate. Animals were maintained at a constant body temperature of 37°C using a heated mat (VetTech). Lateral laminectomy was performed at spinal level L3–5. In brief, the skin was incised longitudinally, and the paravertebral muscles were cut to expose the vertebral column. Transverse and superior articular processes of the vertebrae were removed using OmniDrill 35 (WPI) and microdissection scissors. To obtain a clear image of the sensory neuron cell bodies in the ipsilateral dorsal root ganglion (DRG), the dura mater and the arachnoid membranes were carefully

opened using microdissection forceps. Artificial spinal fluid (values are in mM: 120 NaCl, 3 KCl, 1.1 CaCl₂, 10 glucose, 0.6 NaH₂PO₄, 0.8 MgSO₄, and 18 NaHCO₃, pH 7.4 with NaOH) was constantly perfused over the exposed DRG during the procedure to maintain tissue integrity. The animal was mounted onto a custom-made clamp attached to the vertebral column, rostral to the laminectomy. The trunk of the animal was slightly elevated to minimize interference caused by respiration. The DRG was isolated by coating with silicone elastomer.

Images were acquired using a Leica SP8 confocal microscope. A 10× dry, 0.4-N.A. objective with 2.2 mm working distance was used, with image magnification of 0.75–3× optical zoom. GCaMP3 was excited using a 488 nm laser line (3–10% laser power). GCaMP was detected using a hybrid detector (60% gain). 512 × 512-pixel images were captured at a frame rate of 1.55 Hz, bidirectional scan speed of 800 Hz, and pixel dwell time of 2.44 μs.

Noxious and innocuous stimuli were applied to the left hindpaw, ipsilateral to the exposed DRG. For thermal stimuli, the ventral side of the paw was immersed with ice-water (nominally 0°C), acetone (100%) or water heated to 55°C using a Pasteur pipette. For delivery of precise temperature stimuli, a Peltier-controlled thermode (Medoc) was used. For mechanical stimuli, a pinch with serrated forceps was used. An interval of at least 30 s separated each stimulus application.

Analysis

Image stacks were registered to a reference image, typically, the first frame in the series, using the FIJI plugin TurboReg (accurate rigid body transformation) to correct for XY drift. Stacks that showed excessive Z movement were excluded from analysis. Regions of interest (ROIs) were manually drawn around apparently responding cells using the polygon tool in FIJI. Mean pixel intensity over time for each ROI was extracted and analyzed. The time series of mean pixel intensity for each ROI was smoothed by a five time point rolling average to remove high-frequency noise. Next, we calculated the derivative of the mean pixel intensity. We calculated a mean baseline derivative for the 20 s preceding stimulus application. Neurons were classed as responders if, within 30 s of stimulus application, the maximum derivative was greater than the baseline derivative plus four standard deviations, that is, a Z-score of at least 4. We then calculated the $\Delta F/F_0$ value for each response to obtain a normalized measure of change in fluorescence. Neurons which showed a $\Delta F/F_0 < 0.25$ were then discarded. Each trace was then manually screened as a further precaution against false positives. The remaining neurons that constituted the responding population were then used for statistical analysis.

Quantification and statistical analysis

For *in vivo* imaging experiments, *n* refers to the number of cells responding to any stimulus. For all imaging and data, the number of animals used is indicated in the legend. For behavioral experiments, *n* refers to the number of animals. No power calculations were performed;

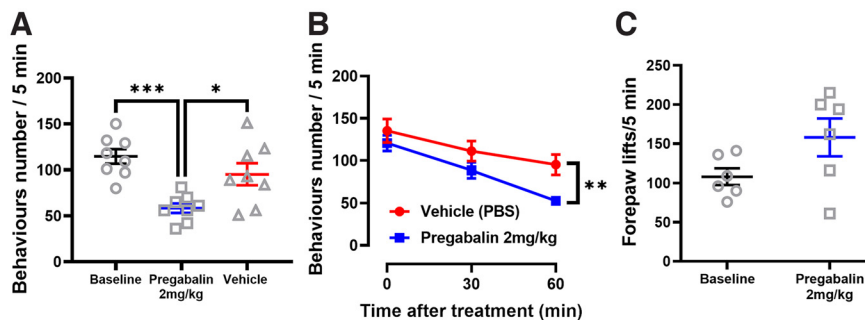


Figure 1. Assessment of the effect of pregabalin on acute oxaliplatin-induced cold allodynia. **A**, Cold-plate assessment at 10°C of mice treated with oxaliplatin and successively either vehicle or 2 mg/kg of pregabalin. Activity was measured as the total number of nociceptive behaviors (hindpaw lifts, shakes, licks) over the test duration. A cutoff time of 300 s was used to limit tissue damage. The baseline data in the graph refers to the mice injected with oxaliplatin. For the baseline versus oxaliplatin-treated animal data please refer to Extended Data Figure 1-1. **B**, Plot showing the decrease of the number of nociceptive behaviors over time following vehicle or pregabalin injection. **C**, Cold-plate assessment at 10°C of mice treated only with pregabalin. Activity was measured as the total number of forepaw lifts over the test duration. A cutoff time of 300 s was used to limit tissue damage. $n=8$ oxaliplatin + pregabalin mice, $n=8$ oxaliplatin + vehicle mice, $n=6$ pregabalin only mice. Statistical analyses in **A** and **B** were performed using one-way and two-way ANOVA tests with multiple comparisons, respectively. Statistical Analysis in **C** was performed using paired t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

however, sample sizes are similar to those used in the field.

Datasets are presented using appropriate summary statistics as indicated in the legend, typically accompanied by raw data points or a representation of the underlying distribution. Behavioral data were generally assumed to be normal, as is typical in the field, and error bars denote mean \pm SEM. When considering *in vivo* imaging experiments, for responding cell population comparisons, percentages were calculated individually from each animal. For polymodality, threshold, and area analyses cells from all animals were pooled. Normality was not assumed when comparing cross-sectional areas or response magnitude of responding cells. Data are summarized using averages and SEMs, as well as cumulative or numerical distribution plots.

Tests of statistical comparison for each dataset are described in detail in figure legends. When comparing two treatment timepoints, paired t test was used. When comparing the distribution of cell cross-sectional areas for two groups, the Wilcoxon test was used. For more than two groups, one-way ANOVA or Kruskal–Wallis test was used with *post hoc* tests corrected for multiple comparisons. When comparing the effect of two factors on multiple groups, a repeated measures two-way ANOVA was used, with *post hoc* tests corrected for multiple comparisons. Curve fitting was performed using linear or nonlinear regression functions.

Statistical tests were all performed using GraphPad Prism 9. An α -value of $p < 0.05$ for significance testing was used. All p -values resulting from planned hypothesis testing are reported.

Data and materials availability

The data that support the findings of this study are openly available at: https://figshare.com/articles/dataset/Pregabalin_silences_oxaliplatin-activated_sensory_neurons_to_relieve_cold_allodynia/20200109.

Results

Pregabalin ameliorates oxaliplatin-dependent cold allodynia by suppressing silent cold-sensing neurons

Pregabalin alleviates cold allodynia in oxaliplatin-dependent neuropathy. Cold allodynia following oxaliplatin treatment is a side effect that occurs in humans within a few hours from the injections. The model used in this paper, developed to isolate the activity of oxaliplatin on peripheral sensory neurons, consists of an intraplantar injection of 80 μ g (in 40 μ l) of oxaliplatin that elicits strong nociceptive responses, that comprise licking, flinching, and shaking of the injected paw as soon as 3–4 h from the injection, assessed using a cold plate assay (Deuis et al., 2013; MacDonald et al., 2021). We observed the same increase in nociceptive responses on oxaliplatin injection when performing a cold plate assay over 5 min at 10°C (Extended Data Fig. 1-1). When the mice were treated with 2 mg/kg of pregabalin via intravenous injection, the number of nociceptive behaviors decreased significantly from 115 (± 7.9) to 58 (± 5.1). Control mice injected with vehicle showed a slight decrease in the total number of behaviors with high variability and no significant difference from the untreated ones (95 ± 11.9 ; Fig. 1A). Nociceptive responses to cold temperature were measured 30 and 60 min after intraplantar injection, and a significant difference between the mice treated with pregabalin and those treated with vehicle is evident only after 60 min (Fig. 1B). Next, we assessed the specificity of the effect of pregabalin on cold allodynia by treating mice with 2 mg/kg pregabalin without the prior oxaliplatin injection. In this case, no difference was observed in the behavior of the mice maintained 5 min on a 10°C cold plate (from 108 ± 10.6 to 158 ± 24.2 ; Fig. 1C).

Pregabalin silences cold-sensing cells by decreasing their excitability. Next, to dissect the functional effect of pregabalin on cold allodynia, we used *in vivo* calcium imaging to investigate whether and how pregabalin

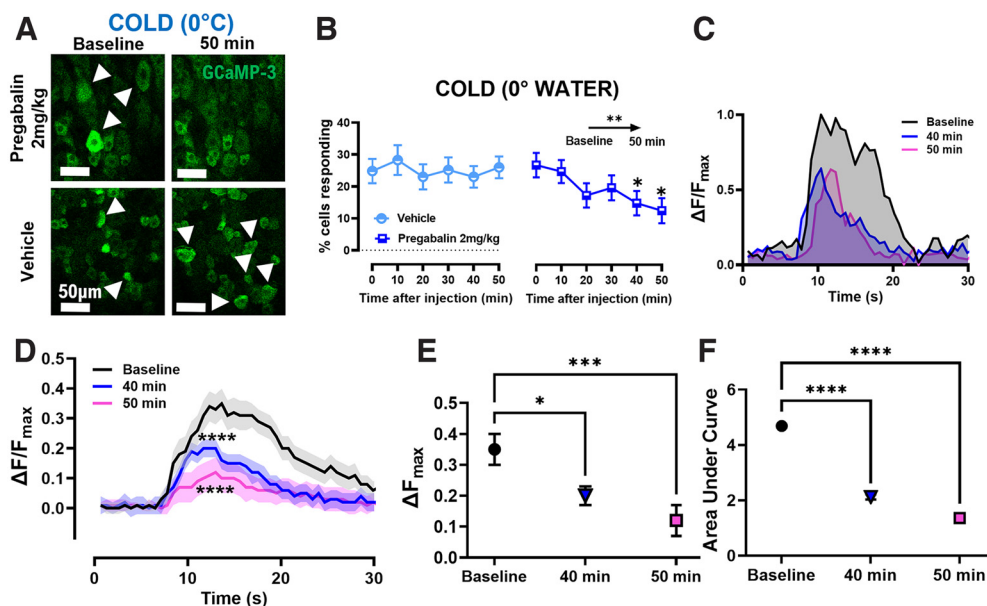


Figure 2. Pregabalin treatment decreases both the number of neurons responding to cold stimuli and the intensity of their responses. **A**, Example images showing the reduction in the population of DRG neurons responding to ice-water 50 min after treatment with 2 mg/kg of pregabalin. **B**, Graph showing the decrease of the percentages of cell responding to ice-water stimulus. **C**, Example traces showing the decrease in the intensity of the calcium signals over time from pregabalin injection. **D**, Plot showing the mean response amplitude before (black trace) and 40 min (blue) and 50 min (pink) after pregabalin injection. $n = 45$ cells for all time points. **E**, Graphs showing the ΔF_{\max} changes before and 40 and 50 min after pregabalin injection. **F**, Graphs showing the area under curve changes before and 40 and 50 min after pregabalin injection. $n = 6$ pregabalin-treated mice, $n = 5$ vehicle-treated mice. Statistical analysis in **B** was performed using repeated measures ANOVA test with multiple comparisons. Statistical analysis in **D** was performed using multiple Wilcoxon tests. Statistical analyses in **E** and **F** were performed using one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. For the data regarding acetone, hot water, and mechanical stimulations please refer to Extended Data Figures 2-1 and 2-2.

treatment affects the responses of sensory neurons to various mechanical and thermal stimuli, with particular attention to any changes in their responses to cold temperatures. It is known that the acute oxaliplatin-dependent neuropathy model used in this study causes a change in the peripheral representation of cold, with the appearance of a population of large, normally silent, cold responding cells (MacDonald et al., 2021). Using laser-scanning confocal imaging, we performed one-photon calcium imaging on the L4 DRG of oxaliplatin-treated mice to image the calcium signals of the neurons' somata. Our experiments show a similar result, with a population of cells responding to ice-water and acetone stimuli of 27% and 17%, respectively (Fig. 2A,B; Extended Data Fig. 2-1). Interestingly, after intravenous injection of 2 mg/kg of pregabalin, we observed a decrease of the percentage of cells responding to either ice-water or acetone that becomes significant as early as 40 min after injection and decreases to 13% and 8% 50 min after injection (Fig. 2B; Extended Data Fig. 2-1B). Moreover, following pregabalin treatment, we observed a decrease in the intensity of the calcium signals evoked in cold-responding cells: both the peak fluorescence intensity (ΔF_{\max}) and the area under the curve decreased significantly after 40–50 min from the injection of pregabalin (Fig. 2C–F).

This effect of pregabalin is specific to cold sensory stimuli, the ones most significantly impacted by oxaliplatin: we

observed no significant change in the number of cells responding to either mechanical or heat stimulation at any point during the treatment with pregabalin (Extended Data Fig. 2-2).

The oxaliplatin treatment is known not to affect the temperature threshold or excitability of cold sensing neurons, while unmasking silent cold nociceptors (MacDonald et al., 2021). We investigated any possible change in the thermal activation thresholds of cold responding cells after treatment with pregabalin by delivering transient 4°C temperature drops, from 30°C to 2°C, through a Peltier-controlled thermode (Fig. 3A). While at baseline the cell recruitment rises in a linear fashion with temperature decrease, after treatment with pregabalin the linear correlation skews toward lower temperatures, this is a significant change and an indicator that pregabalin increases the activation threshold of cold responding cells (Fig. 3B; Extended Data Fig. 3-1).

Pregabalin treatment suppresses predominantly “silent” cold sensors. Acute oxaliplatin injection indeed causes the recruitment of cells that do not respond to cold stimuli before treatment. It also increases receptor polymodality, with a marked increase in neurons responding to both mechanical and cold stimuli: the population of silent mechano-sensors primarily respond to high-threshold mechanical stimuli (MacDonald et al., 2021). We observed similar percentages in our model, and furthermore the injection of pregabalin decreased

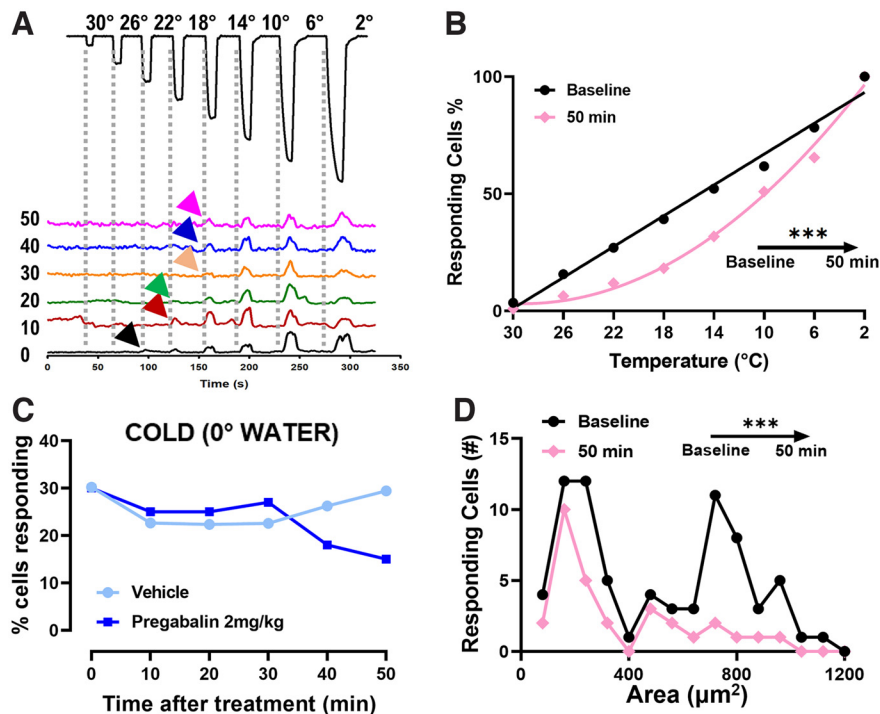


Figure 3. Pregabalin treatment significantly reduces the excitability of cold responding cells and targets preferentially medium-large mechano-cold sensors. **A**, Example traces of a representative cold-sensing cell in response to a series of 4°C temperature drops from a holding temperature of 32°C. The traces represent the change in the threshold of this neuron over time (0–50 min) from pregabalin injection. Arrowheads mark the first response to temperature drops in every timepoint. **B**, Graph showing the change of the relationship between the number of cold-responding neurons and the temperature drop over time from pregabalin injection. While the relationship at baseline can be fit with a linear equation ($y = -3.292x + 99.84$; $r^2 = 0.9880$; $n = 115$), the one at 50-min fits a quadratic equation ($y = 110.8 - 7.381x + 0.1265x^2$; $r^2 = 0.9927$; $n = 109$). The slopes at baseline and 50 min after treatment are significantly different ($***p < 0.001$), as analyzed by Wilcoxon test. For a complete visualization of the data regarding the cold threshold changes in all time points please refer to Extended Data Figure 3-1. **C**, Graphs showing the change in the number of total mechano-responding neurons that respond also to ice-water stimulus. Mice treated with vehicle do not exhibit the decrease over time from treatment as the mice treated with pregabalin do. For the graphs showing the mechano-acetone and mechano-heat responding cells trends please refer to Extended Data Figure 3-2. **D**, Numeric plots of the distribution of cold-responding cell areas in oxaliplatin-treated mice before (black trace) and after 50 min (pink trace) from pregabalin injection. The difference between the distributions at different timepoints after treatment is statistically significant ($***p = 0.0001$), as analyzed by Wilcoxon test. For a complete visualization of the numeric distributions of cold responding cells areas in all time points please refer to Extended Data Figure 3-3. $n = 7$ mice for temperature threshold experiments; $n = 6$ pregabalin-treated mice, $n = 5$ vehicle-treated mice.

the population of cells responding to both mechanical and ice-water and acetone from 30% to 15% and from 20% to 6%, respectively (Fig. 3C; Extended Data Fig. 3-2A). This effect is specific to mechano-cold responding cells, as the percentage of mechano-heat polymodal neurons did not vary significantly after treatment in either condition (Extended Data Fig. 3-2B). When analyzing the area of cold-responding neurons we find the same two different populations found in MacDonald et al. (2021). Pregabalin treatment significantly affects the peripheral representation of cold allodynia: the analysis of the cross-sectional area of cold-responding cells shows a significant difference between the area of responding neurons after 50 min from pregabalin injection. Indeed, the treatment, although not completely, preferentially silences larger cold-responding cells, those “silent” cold sensors that are unmasked in the oxaliplatin-dependent neuropathy (Fig. 3D; Extended Data Fig. 3-3).

Taken together, these results highlight the presence of a new, peripheral effect of pregabalin that inhibits the activity of de-novo cold sensing neurons by decreasing their excitability and thus ameliorates cold allodynia in a model of acute oxaliplatin-dependent neuropathy.

The peripheral effect of pregabalin is dependent on the voltage-gated calcium channel subunit $\alpha 2\delta 1$

Next, we investigated the mechanism of action of pregabalin on the silencing of the unmasked cold sensors by studying the role of its best-known target: $\alpha 2\delta 1$. We performed the same cold plate assay described before on WT and global $\alpha 2\delta 1$ -KO mice: we treated all mice with oxaliplatin and 2 mg/kg of pregabalin, and we observed a significant difference between the two groups. Moreover, when the results were compared with the previous ones, there is an overlap between the vehicle-treated group and the $\alpha 2\delta 1$ -KO mice, suggesting an almost complete loss of the effect of pregabalin (Fig. 4A).

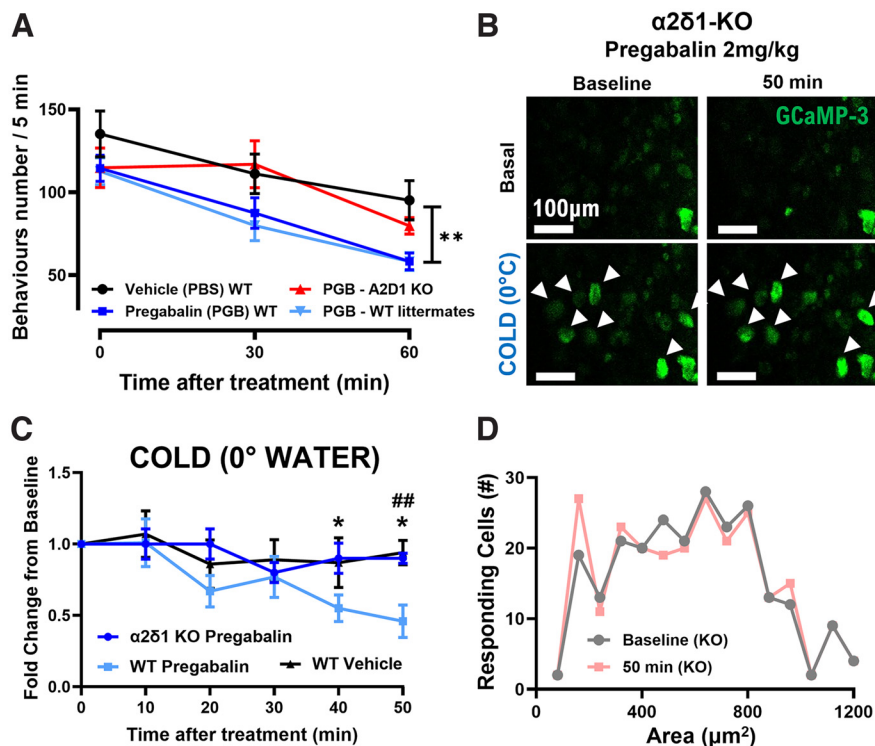


Figure 4. Knock-out of the $\alpha 2\delta 1$ subunit of the VGCCs is sufficient to abolish the effects of pregabalin both on cold allodynia and on silencing of cold responding DRG neurons. **A**, Plot showing the decrease of the number of nociceptive behaviors over time from vehicle ($n=8$; black trace) or pregabalin injection in WT [$n=8$ (blue trace) + 10 (light blue trace)] and $\alpha 2\delta 1$ -KO mice ($n=10$; red trace) when exposed to a 10°C cold plate for 5 min (** $p < 0.01$). **B**, Example images showing that the reduction of the population of DRG neurons responding to ice-water 50 min after treatment with pregabalin is absent in $\alpha 2\delta 1$ -KO mice. **C**, Graph showing the disappearance of the decrease of the percentages of cell responding to ice-water stimulus in $\alpha 2\delta 1$ -KO mice. Change is quantified as fold-change to baseline. **D**, Numeric plots of the distribution of cold responding cell areas in oxaliplatin-treated $\alpha 2\delta 1$ -KO mice before (gray trace) and 50 min after pregabalin injection (light pink trace). $n=7$ $\alpha 2\delta 1$ -KO mice, $n=6$ pregabalin-treated mice, $n=5$ vehicle-treated mice for imaging data. Statistical analyses in **C** were performed using repeated measures ANOVA test with multiple comparisons. * $p < 0.05$ (WT vehicle vs WT pregabalin treated); ## $p < 0.01$ ($\alpha 2\delta 1$ -KO vs WT pregabalin treated). For the data regarding acetone, hot water, and mechanical responses, please refer to Extended Data Figure 4-1. For the data regarding the area distribution of cold responding cells after pregabalin treatment in $\alpha 2\delta 1$ -KO animals, please refer to Extended Data Figure 4-2.

We then proceeded with *in vivo* imaging experiments on $\alpha 2\delta 1$ -KO mice. Similar to the results of the behavioral tests, the decrease of the percentage of cold-sensing neurons is completely abolished in the $\alpha 2\delta 1$ -KO mice, both in the case of ice-water and acetone stimuli (Fig. 4B; Extended Data Fig. 4-1A,B). This effect of $\alpha 2\delta 1$ is specific again to cold stimuli, as the percentages of heat and mechanical-responding neurons did not vary significantly between conditions (Extended Data Fig. 4-1A,C,D). Furthermore, the cross-sectional area of the cold-responding neurons is similar to that of WT animals and did not vary on treatment with pregabalin (Fig. 4D; Extended Data Fig. 4-2), consistent with the behavioral results and suggesting a strong dependence of the effect of pregabalin on its main molecular target $\alpha 2\delta 1$.

Discussion

Pregabalin exerts a novel, peripheral effect on cold allodynia induced by oxaliplatin in mice

We have identified a novel effect of pregabalin on “silent” cold sensing neurons that acquire *de novo* sensitivity

to cold after cold allodynia induction by acute injection of oxaliplatin. Pregabalin, in this model, acts peripherally on this subpopulation of peptidergic A-fiber nociceptors by decreasing their excitability and thus mitigating the increased responses to cold stimuli that is thought to be a common pathophysiology underlying cold allodynia in various models of neuropathic pain (MacDonald et al., 2021).

Oxaliplatin mediates a *de novo* sensitization of previously silent nociceptors to cold stimuli: pregabalin reverses it

Chemotherapy-induced peripheral neuropathy (CIPN) is a side effect of oxaliplatin treatment, as it is for many anti-cancer therapies.

Many animal models of oxaliplatin-dependent neuropathy have been developed that replicate the acute and chronic symptoms experienced by human patients (Ling et al., 2008; Deuis et al., 2013; Yamamoto et al., 2016). We chose a model of acute neuropathy to focus the attention on the early changes in cold perception and

understand the molecular basis of cold allodynia. Indeed, there is strong evidence of changes in the coding of the responses to cold stimuli in the DRG: in the healthy state, only a limited population of small-diameter neurons responds to a cooling stimulus in a very specific fashion. After treatment with oxaliplatin, cold stimuli elicit the response of a population of previously silent neurons responding to both cold and noxious mechanical stimuli. The investigation of their molecular identity suggests they could be classified as A-fiber peptidergic nociceptors that express the sodium channel Nav1.8, whose key role in the sensation of prolonged, extreme cold in normal conditions has been previously shown (Luiz et al., 2019; MacDonald et al., 2021).

Pregabalin is a well-known anti-epileptic and analgesic drug that has been one of the staples in the treatment of neuropathic pain for years. Pregabalin was effective in ameliorating CIPN in numerous clinical trials of cancer-related neuropathic pain and allodynia after treatments with vincristine, paclitaxel, and drug cocktails comprising oxaliplatin (Mishra et al., 2012; Atreya, 2016; Avan et al., 2018; Sugimoto et al., 2021). However, data regarding its effectiveness in the treatment of cold allodynia are more controversial: earlier clinical trials and case studies found clear effectiveness of pregabalin to treat oxaliplatin-dependent neuropathy, whereas a more recent double-blinded trial found no significant difference from placebo on oxaliplatin-dependent CIPN, and ASCO guidelines do not fully recommend the use of gabapentinoids to treat CIPN (Saif et al., 2010; de Andrade et al., 2017; Loprinzi et al., 2020).

Nevertheless, this article confirms previous data showing the effectiveness of pregabalin in treating oxaliplatin-dependent symptoms and highlights its potential role as a functional counter to the increased excitability caused by the chemotherapeutic treatment (Ling et al., 2008; Deus et al., 2013; Starobova and Vetter, 2017). The injection of 2 mg/kg of pregabalin ameliorated cold allodynia symptoms with a significant decrease in the number of nociceptive responses of mice exposed to a 10°C cold plate assay. Furthermore, the treatment caused a significant decrease of the number of neurons responding to different cold stimuli within the DRG to almost normal levels, with a time frame that corresponds to the effect seen on the behavioral tests and is in concert with other experiments done in similar conditions (Ling et al., 2008; Deus et al., 2014).

Furthermore, there seems to be a direct effect on cellular excitability: the temperature threshold of cells responding to cold, that is not affected by oxaliplatin, shifts significantly toward lower temperatures after treatment with pregabalin, suggesting that pregabalin may silence cold-sensing neurons by increasing their response threshold.

Finally, the investigation on the changes in polymodality and cross-sectional area of neurons responding to cold before and after pregabalin injection strongly indicates that, albeit not being a direct counter to the alterations elicited by oxaliplatin, pregabalin indeed inhibits preferentially the mechano-cold “silent” sensors that are the functional result of its pathologic action.

Taken together, these data suggest that pregabalin acts directly on DRG cells by reverting the functional representation of cold stimuli back to almost normal levels, and this in turn ameliorates the symptoms of cold allodynia.

Pregabalin efficacy is dependent on the $\alpha 2\delta 1$ subunit of calcium channels

The main analgesic effect of pregabalin is assumed to be because of its action on the VGCCs through its direct binding with the $\alpha 2\delta 1$ subunit. This subunit is expressed in primary afferent fibers and is fundamental for a correct release of neurotransmitters and neuropeptides at the presynaptic site in the dorsal horn (DH) of the spinal cord (Hoppa et al., 2012; Dolphin, 2016; Alles and Smith, 2017). This protein has been extensively studied for its impact in neuropathic pain; it has been demonstrated to be upregulated in various models of neuropathic pain, and its upregulation alone is sufficient to drive allodynia, even in absence of a neuropathic injury (Li et al., 2006; Alles and Smith, 2017; Chincholkar, 2018). Furthermore, it has been observed that knock-down of the $\alpha 2\delta 1$ subunit has an impact on acute sensation: $\alpha 2\delta 1$ -KO mice in fact have impaired mechanical and cold sensitivity, as well as showing a marked delay in the onset of mechanical hypersensitivity in a neuropathic model of partial sciatic nerve ligation (PSNL). Moreover, in the same model, the absence of $\alpha 2\delta 1$ abolishes the anti-allodynic effect of a normally effective dose of pregabalin (Patel et al., 2013).

We do not observe any effect of the $\alpha 2\delta 1$ KO on the basal cold responses after oxaliplatin treatment, but we do show that the rapid effect of pregabalin on both the behavioral and functional representation of cold allodynia that were observed are almost completely abolished in the $\alpha 2\delta 1$ -KO mice. Indeed, the same treatment that elicited a significant reduction both in the number of nociceptive behaviors and of cold-responding cells did not have any effect in the $\alpha 2\delta 1$ -KO mice, further confirming the dependence of pregabalin on the $\alpha 2\delta 1$ molecule for its early action in this CIPN pain model.

Site of action of pregabalin: central, peripheral, or both?

Although gabapentinoids have been widely used in curing patients with neuropathic symptoms, their mechanism of action is not fully understood. They are thought to act mainly by interfering with trafficking of CaV2.2 VGCCs to the presynaptic site of the dorsal laminae in the spinal cord, as well as blocking their function through the $\alpha 2\delta 1$ subunit, decreasing in turn the release of neurotransmitters and neuropeptides from DRG presynaptic sites and overall neuronal excitability in the DH (Verma et al., 2014; Chincholkar, 2018). However, decreased expression of VGCCs in the terminal may not be important, as their blockade by Mn^{2+} has a negligible effect on neurotransmitter release (Biggs et al., 2014; Alles and Smith, 2017). Other putative mechanisms of action include an inhibition of descending serotonergic facilitatory and

an excitation of descending noradrenergic inhibitory pathways, as well as an effect on synaptogenesis by blocking the interactions of thrombospondin to $\alpha 2\delta 1$ (Suzuki et al., 2005; Eroglu et al., 2009; Suto et al., 2014). Another recently discovered effect of gabapentinoids is the decrease of trafficking of NMDA glutamatergic receptors to presynaptic sites in the DH through a direct interaction of the C terminus of $\alpha 2\delta 1$ with these receptors (Chen et al., 2018). Other recently discovered binding partners of the $\alpha 2\delta 1$ subunits are neurexin-1 α , whose weak interaction with $\alpha 2\delta 1$ increases presynaptic release, and calcium-activated potassium (BK) channels, which competes with $\alpha 2\delta 1$ for the binding to calcium channels and reduces overall calcium currents (Zhang et al., 2018; Alles et al., 2020; Taylor and Harris, 2020). These recent finds have highlighted how the signaling cascade downstream $\alpha 2\delta 1$, and subsequently the range of effects of pregabalin, may be much more complex than previously thought. Indeed, taken together, these findings show how pregabalin, thanks to its interaction with the $\alpha 2\delta 1$ subunit, has many different targets and sites of action throughout the nervous system, corroborated also by the fact that an increase of $\alpha 2\delta 1$ expression has been observed in multiple sites within the peripheral and central nervous system (Luo et al., 2001; Cole et al., 2005; Bauer et al., 2009; Yamamoto et al., 2016).

The principal site of action of pregabalin has been thought to be central, mainly at the spinal cord level but without ruling out the possibility of supra-spinal actions (Bauer et al., 2009; Verma et al., 2014; Chincholkar, 2018). Indeed, electrophysiological studies on intact and spinalized rat showed that the inhibitory effect observed with pregabalin treatment on C-fiber mediated nociceptive activity is abolished in spinalized animals, suggesting a strong involvement of supra-spinal centres (You et al., 2009). Nevertheless, our data strongly suggest a direct effect on the excitability of a specific subpopulation of peripheral DRG neurons that acquire cold sensitivity after oxaliplatin-dependent neuropathic injury. Albeit we cannot infer a direct causal effect between the antiallodynic effect of pregabalin and the decrease in the population of cold-sensing cells at the DRG level, we suggest a strong correlation between the behavioral data and the functional changes in the peripheral representation of noxious cold stimuli. Indeed, it has been observed that gabapentinoids have a direct effect on the activity of specific DRG neuronal populations: gabapentin inhibits persistent sodium currents in medium sized neurons, and has an inhibitory effect exerted preferentially on small IB4-negative and medium sized neurons (R.H. Yang et al., 2009; Biggs et al., 2014). The subpopulation of neurons preferentially hit by pregabalin, as shown in this paper, seems to be the silent cold sensors described previously (MacDonald et al., 2021); they are medium sized neurons, positive for Nav1.8 and CGRP. These results are further supported by microarray data on DRG of Nav1.8-DTA mice that show a strong reduction in the transcripts for CGRP- α and CGRP- β , as well as in CaV2.2 and $\alpha 2\delta 1$ (Abrahamsen et al., 2008). Furthermore, it has been

shown that gabapentinoids influence neurons of the substantia gelatinosa in the DH, with an overall decrease in excitability that cannot be explained just by a decrease of calcium currents, since, as was said previously, their blockade by Mn^{2+} does not affect overall neurotransmitter release in the DH (Biggs et al., 2014). On the other hand, IB4-negative neurons and A δ fibers from medium-sized neurons project primarily to excitatory DH neurons (Lu and Perl, 2005; Braz et al., 2014); these cells may be preferentially targeted by gabapentinoids, but we have also shown that the central action of pregabalin may in part depend on its selective inhibition of a specific subpopulation of neurons in the DRG.

These findings thus support a peripheral locus of action of gabapentin in diminishing the cold allodynia evoked by chemotherapeutic insults. The long-term nature of changes associated with neuronal damage and the clinical utility of peripheral application of gabapentin have still to be assessed. The precise mechanism of $\alpha 2\delta 1$ -dependent analgesia evoked by gabapentinoids is also an important topic of future research. Nevertheless, the selective action of gabapentin on unmasked silent nociceptors highlights the role of silent nociceptors in cold allodynia and defines them as a potentially significant clinical target.

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