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An HDAC6 inhibitor reverses chemotherapy-induced mechanical hypersensitivity via an IL-10 and macrophage dependent pathway

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) impacts a growing number of cancer survivors and treatment options are limited. HDAC6 inhibitors are attractive candidates because they reverse established CIPN and may enhance anti-tumor effects of chemotherapy. Before considering clinical application of HDAC6 inhibitors, the mechanisms underlying reversal of CIPN need to be identified. We showed previously that deletion of *Hdac6* from sensory neurons did not prevent cisplatin-induced mechanical hypersensitivity, while global deletion of *Hdac6* was protective, indicating involvement of HDAC6 in other cell types. Here we show that local depletion of MRC1 (CD206)-positive macrophages without affecting microglia by intrathecal administration of mannosylated clodronate liposomes reduced the capacity of an HDAC6 inhibitor to reverse cisplatin-induced mechanical hypersensitivity. The HDAC6 inhibitor increased spinal cord *Il10* mRNA and this was M2-macrophage dependent. Intrathecal administration of anti-IL-10 antibody or genetic deletion of *Il10* prevented resolution of mechanical hypersensitivity. Genetic deletion of the IL-10 receptor from Advillin+ neurons prevented resolution of mechanical hypersensitivity in mice treated with the HDAC6 inhibitor. These findings indicate that treatment with an HDAC6 inhibitor increases macrophage derived IL-10 signaling to IL-10 receptors on Advillin+ sensory neurons to resolve mechanical hypersensitivity. Cisplatin decreases mitochondrial function in sensory axons, and HDAC6 inhibition can promote axonal transport of healthy mitochondria. Indeed, the HDAC6 inhibitor normalized cisplatin-induced tibial nerve mitochondrial deficits. However, this was independent of macrophages and IL-10 signaling. In conclusion, our findings indicate that administration of an HDAC6 inhibitor reverses cisplatin-induced mechanical hypersensitivity through two complementary pathways: macrophage HDAC6 inhibition to promote IL-10 production and IL-10 signaling to DRG neurons, and neuronal HDAC6 inhibition to restore axonal mitochondrial health.

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Conflict of interest statement

The HDAC6 inhibitors were provided by Regenacy Pharmaceuticals and this company also funded part of the study.

Keywords

CIPN; HDAC6; IL-10; IL10RA; Macrophage; Pain

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) negatively affects the quality of life of patients treated for cancer and there are no effective interventions to alleviate the pain and other symptoms (Ma et al., 2018; Maihofner et al., 2021). Novel interventions need to be developed to reverse CIPN without interfering with cancer treatment. Recent findings identify histone deacetylase 6 (HDAC6) as a target meeting these requirements (Krukowski et al., 2017; Ma et al., 2019; Van Helleputte et al., 2018). HDAC6 is a class IIb histone deacetylase, and mainly deacetylates non-histone proteins, including tubulin and heat shock proteins (Bali et al., 2005; Hubbert et al., 2002; Parmigiani et al., 2008). Inhibition of HDAC6 has been proposed as a potential treatment for neurodegenerative disorders and also holds promise for the treatment of cancer (Li et al., 2018; Shen and Kozikowski, 2020). Using a model of CIPN induced by cisplatin, we showed that in male mice two weeks of dosing the HDAC6 inhibitor ACY-1083 completely reverses signs of CIPN, including mechanical hypersensitivity, spontaneous pain, and loss of intraepidermal nerve fibers (Krukowski et al., 2017; Ma et al., 2019). Moreover, HDAC6 inhibition reverses paclitaxel-induced peripheral neuropathy and prevents vincristine-induced peripheral neuropathy (Krukowski et al., 2017; Van Helleputte et al., 2018).

Impaired mitochondrial function in peripheral neurons is thought to play a key role in the development of CIPN. We and others have shown that HDAC6 inhibition improves mitochondrial function and transport in peripheral neurons exposed to chemotherapy *in vitro* or *in vivo* (Krukowski et al., 2017; Van Helleputte et al., 2018). Consistently, genetic deletion of *Hdac6* from all cells or from Advillin-positive sensory neurons prevents peripheral nerve mitochondrial damage in mice treated with cisplatin. However, deletion of *Hdac6* from Advillin-positive neurons did not prevent cisplatin-induced mechanical hypersensitivity, while global HDAC6 deletion was protective. These results indicate that HDAC6 inhibition in other cells is required for the reversal of CIPN in response to treatment with an HDAC6 inhibitor.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that can suppress neuropathic pain caused by nerve injury or the chemotherapeutic agent paclitaxel (Ledeboer et al., 2007; Milligan et al., 2005; Wieseler-Frank et al., 2005). We recently showed that endogenous IL-10 is required for the spontaneous resolution of CIPN after a short course of treatment with cisplatin or paclitaxel (Krukowski et al., 2016; Laumet et al., 2020). In vivo and in vitro studies have shown that HDAC6 inhibition can regulate IL-10 production, but the effects are mixed. HDAC6 inhibitors or genetic deletion of *Hdac6* can increase IL-10 production (Wang et al., 2014). However, others reported decrease LPS-induced IL-10 production in vitro in response to HDAC6 inhibition or genetic deletion (Cheng et al., 2014a; Cheng et al., 2014b) and *Hdac6* deletion reduces IL-10 production by macrophages stimulated with polyinosinic-

polycytidylic acid (poly-IC) in vitro (Wang et al., 2020). It is not known whether HDAC6 regulates IL-10 production and signaling in mice treated with chemotherapy.

Here, we determined the contribution of macrophages and IL-10 signaling to the reversal of cisplatin-induced mechanical hypersensitivity in response to treatment with an HDAC6 inhibitor. Using pharmacological, cell depletion, and genetic tools, we show that the reversal of cisplatin-induced mechanical hypersensitivity in response to HDAC6 inhibition is mediated at least in part via effects on non-neuronal cells. Specifically, we show that macrophages and IL-10 signaling to IL-10 receptors on peripheral sensory neurons are required for resolution of cisplatin-induced mechanical hypersensitivity in response to treatment with an HDAC6 inhibitor.

2. Materials and methods

2.1. Animals

Male and female wild type C57BL/6J mice (#000664) and male *Il10*$^{-/-}$ knockout mice (#002251) (Madan et al., 2009) were purchased from Jackson Laboratories (Bar Harbor, ME). Male and female *Avil*^{Cre}*Il10ra*$^{fl/fl}$ mice in which *Il10ra* is deleted from peripheral sensory neurons were obtained by crossing *Il10ra* floxed mice with Advillin-Cre mice (Laumet et al., 2020; Zhou et al., 2010). All mice were genotyped before inclusion in experiments (TransnetYX, Cordova, TN). Mice were used at 8 – 10 weeks of age. Mice were group housed in individually ventilated cages at The University of Texas MD Anderson Cancer Center animal facility (Houston, TX) on a regular 12-hour light/dark cycle with free access to food and water. All experimental procedures were consistent with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the local Institution for Animal Care and Use Committee (IACUC). Mice were randomly assigned to group and all analyses were performed by investigators blinded to group.

2.2. Drug administration

Cisplatin (#NDC 16729-288-11, TEVA Pharmaceuticals, North Wales, PA) was diluted with sterile phosphate buffered saline (PBS, #21-040-CV, Corning, Manassas, VA) to a concentration of 0.23 mg/mL and administered i.p. at a dose of 2.3 mg/kg per day for 5 days followed by 5 days of rest and another 5 days of injections. The HDAC6 inhibitor ACY-1083 (Regenacy Pharmaceuticals, Boston, MA) was prepared in vehicle consisting of 20% 2-hydroxypropyl-B-cyclodextrin (#297565000, ACROS Organics) and 0.5% hydroxypropyl methylcellulose (#Hy124, Spectrum Chemical, Gardena, CA) in sterile water. Mice received daily i.p. injections of ACY-1083 at a dose of 10 mg/kg. The HDAC inhibitor ACY-1215 (Regenacy Pharmaceuticals) was prepared in 10% DMSO, 30% Propylene glycol (#P4347, Sigma-Aldrich) and 60% Polyethylene glycol-300 (#81162, Sigma-Aldrich), and was administered daily via oral gavage at 30 mg/kg (Krukowski et al., 2017).

2.3. Intrathecal injections

Intrathecal injections were performed under 1.5% isoflurane anesthesia as described previously using a sudden tail flick response as confirmation of correct insertion of the needle (Krukowski et al., 2016). Goat-anti-IL-10 IgG (#I5145, Sigma-Aldrich) or normal goat IgG (IgG) (#I5256, Sigma-Aldrich) were administered intrathecally at 10 μg/mouse (5 μL) together with each ACY-1083 administration. For macrophage depletion, the mannosylated macrophage depletion kit (#CLD-8914, Encapsula Nano Sciences) was used. Mice were injected i.t. with 10 μL control empty mannosylated liposomes (m-Encapsome, m-Lip-E), or clodronate-containing mannosylated liposomes (m-Clodrosome, m-Lip-C) (Niehaus et al., 2021).

2.4. Von Frey test

Mechanical sensitivity was measured using the von Frey up and down method with von Frey hairs (0.02, 0.07, 0.16, 0.4, 0.6, 1.0, and 1.4 g) (Stoelting, Wood Dale, Illinois, USA) as described previously (Krukowski et al., 2017; Ma et al., 2019).

2.5 Immunofluorescence analysis

Mice were transcardially perfused with prechilled ice cold PBS followed by freshly made 4% paraformaldehyde (PFA) in PBS. Lumbar spinal cords with meninges were dissected and postfixed in 4% PFA for at least 2 hours. After cryoprotected in 30% sucrose, the tissues were embedded and frozen in Tissue-Tek O.C.T. Compound (#4583, Sakura, Torrance, CA) and cut at 20 μM using Leica CM3050S cryostat. Sections were washed with PBS and blocked with 10% normal donkey serum (NDS) or normal goat serum (NGS) (2% BSA + 10% NDS/NGS + 0.1% Saponin in PBS) for 90 minutes at room temperature. Sections were then incubated with primary antibody in antibody buffer (2% BSA + 2% NDS/NGS + 0.1% Saponin in PBS) overnight at 4° C, washed three times in PBS with 0.1% Tween (PBS-T) and incubated with secondary antibodies for 2 hours at room temperature. After washing with PBS-T, DAPI (1:5000 in PBS, 5 min) was used to stain nuclei. Antibodies used were as follows: primary goat polyclonal anti-MRC1/CD206 (#AF2535, R&D Systems, 1:200); primary rabbit polyclonal anti-IBA1 (#019-19741, WAKO, 1:500); secondary goat anti-rabbit IgG (Alexa Fluor 488, #A-11034, Invitrogen, 1:500); secondary donkey anti-goat IgG (Alexa Fluor 488, #A-11055, 1:500). Sections were imaged using a Nikon A1R Confocal Microscope and the intensity of regions of interest was analyzed using ImageJ plugins RGB Measure. Data were normalized to the DAPI signal in that region.

2.6. mRNA expression analysis

Total RNA was extracted from lumbar spinal cords (L4–L6) using TRIzol (#15596018, Invitrogen, Carlsbad, CA) and converted to cDNA with high-capacity cDNA reverse transcription kit (#4368813, Applied Biosystems, Foster City, CA). Gene expression level was measured with the following PrimeTime probes: *Il10* (#Mm.PT.58.13531087, exon 3–5), *Il6* (#Mm.PT.58.10005566, exon 4–5), *Tnf* (#Mm.PT.58.12575861, exon 2–4), and normalized to *Gapdh* (#Mm.PT.39a.1, exon 2–3) (Integrated DNA Technologies, Coralville, IA). Quantitative amplification was performed using the PrimeTime™ Gene Expression

Master Mix (#1055772, Integrated DNA Technologies) with a running program (95°C 3 min and 40 cycles of 95°C for 5 s and 60°C for 30 s).

2.7. Mitochondrial bioenergetics analysis

Mitochondrial bioenergetics was measured with the XF24 Flux Analyzer (Agilent Technologies Inc, Santa Clara, CA) as previously described (Krukowski et al., 2017; Ma et al., 2019). Briefly, tibial nerves were freshly isolated and put into the islet capture XF24 microplate (Seahorse Bioscience, North Billerica, MA) in Seahorse XF base media (#102353-100, Agilent technologies) supplemented with 5 mM glucose (#G7021, Sigma-Aldrich), 0.5 mM sodium pyruvate (#25-000-CI, Corning, Manassas, VA), and 1 mM glutamine (#G8540, Sigma-Aldrich). Oligomycin A (#75351, Sigma-Aldrich, final concentration is 12 μM), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; #C2920, Sigma-Aldrich , final concentration is 20 μM), and rotenone (#R8875, Sigma-Aldrich, final concentration is 20 μM) and antimycin A (#A8674, Sigma-Aldrich, final concentration is 20 μM) were used to determine mitochondrial respiratory properties. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were normalized to protein contents and were used as indicators for the statuses of mitochondrial oxidative phosphorylation and glycolysis, respectively.

2.8. Statistics

Data are expressed as mean ± SEM. Minimum group sizes were based on power analysis of results of previous experiments. Statistical analysis of the differences between groups was performed using two-way ANOVA (GraphPad8, San Diego, CA) or one-way ANOVA as appropriate; $P<0.05$ was considered statistically significant. All raw data will be made available upon request.

3. Results

3.1. Treatment with an HDAC6 inhibitor reverses cisplatin-induced mechanical hypersensitivity

Our previous studies in male mice showed that two weeks of treatment with an HDAC6 inhibitor after completion of chemotherapy reverses cisplatin-induced mechanical hypersensitivity (Krukowski et al., 2017). Here, we first determined whether HDAC6 inhibitors also reverse cisplatin-induced mechanical allodynia in females. Female mice were treated with 2 cycles of cisplatin for a cumulative dose of 23 mg/kg (Figure 1A) to induce mechanical hypersensitivity (Figure 1B). Subsequently, mice were treated with three doses of the HDAC6 inhibitors ACY-1083 (Figure 1B) or ACY-1215 (Ricolinostat; Figure 1C) starting 3 days after completion of cisplatin. Both HDAC6 inhibitors reversed cisplatin-induced mechanical hypersensitivity in females (Figure 1B–C), which is consistent with our earlier results in males (Krukowski et al., 2017).

3.2. Macrophages are required for resolution of mechanical hypersensitivity by HDAC6 inhibition

Our previous studies showed that deletion of *Hdac6* from Advillin+ sensory neurons did not prevent cisplatin-induced mechanical hypersensitivity (Ma et al., 2019).These

findings indicate the involvement of another cell type in the beneficial effects of HDAC6 inhibitors in models of CIPN. MRC1 (CD206) positive macrophages producing IL-10 are a potential candidate (Laumet et al., 2020; Niehaus et al., 2021). To test this hypothesis, we treated mice with cisplatin to induce mechanical hypersensitivity and intrathecally administered mannosylated clodronate liposomes (Figure 2A). This intervention has been shown to locally reduce the number MRC1 (CD206) positive spinal cord border-associated macrophages, a specific subset of macrophages lining the meninges (Niehaus et al., 2021). The data in Figure 2B and 2C confirm that intrathecal administration of mannosylated clodronate liposomes depletes MRC1+ macrophages surrounding the spinal cord. Intrathecal injection of mannosylated clodronate liposomes did not affect the number of IBA1+ cells in the spinal cord (Figure 2D and 2E). These findings are in line with a previous report showing that this intervention does not affect spinal cord microglia (Polfliet et al., 2001). Intrathecal administration of mannosylated clodronate liposomes significantly reduced the capacity of the HDAC6 inhibitor to reverse mechanical hypersensitivity in both male and female mice (Figure 2F and 2G). We did not detect any effect of the mannosylated clodronate liposomes on mechanical hypersensitivity in mice treated with cisplatin only (Figure 2F and 2G). These results indicate that MRC1 (CD206+) spinal cord macrophages are required for reversal of cisplatin-induced mechanical hypersensitivity in response to the HDAC6 inhibitor.

3.3. Treatment with an HDAC6 inhibitor upregulates IL-10 production in the spinal cord

Previous studies have shown that border associated macrophages suppress pain via production of IL-10 (Niehaus et al., 2021). Moreover, IL-10 is required for the spontaneous resolution of CIPN in mice treated with a short course of cisplatin or paclitaxel (Krukowski et al., 2016; Laumet et al., 2020). To assess the involvement of IL-10 in the MRC1+ macrophage-dependent reversal of CIPN in response to HDAC6 inhibition mice were treated with 2 cycles of cisplatin followed by 1 dose of liposome and 3 doses of ACY-1083 (Figure 2A). Quantitative real-time PCR analysis demonstrated that administration of ACY-1083 significantly increased *Il10* mRNA levels in the spinal cord of cisplatin-treated mice (Figure 3A). Depletion of spinal cord MRC1 (CD206) positive macrophages by intrathecal administration of mannosylated clodronate liposomes prevented the increase in spinal cord *Il10* mRNA in response to HDAC6 inhibitor treatment (Figure 3A). Expression of the pro-inflammatory cytokines *Tnf* and *Il6* in the spinal cord was not affected by cisplatin or the HDAC6 inhibitor (Figure 3B–C). In the DRG, we did not detect *Il10* mRNA. These data indicate that IL-10 produced by spinal cord MRC1-positive macrophages may contribute to the reversal of mechanical hypersensitivity after treatment with an HDAC6 inhibitor.

3.4. The reversal of cisplatin-induced mechanical hypersensitivity in response to an HDAC6 inhibitor is IL-10 dependent

To determine whether IL-10 signaling contributes to the reversal of mechanical hypersensitivity in response to treatment with the HDAC6 inhibitor in cisplatin-treated mice, we intrathecally administered anti-IL-10 neutralizing antibody together with each dose of ACY-1083. Notably, co-administration of anti-IL-10 antibody prevented the reversal of mechanical hypersensitivity in response to the HDAC6 inhibitor in both male and female mice (Figure 4A). In contrast, intraplantar injection of the same amount of IL-10 antibody

did not interfere with the resolution of cisplatin-induced mechanical hypersensitivity in mice treated with cisplatin followed by the HDAC6 inhibitor (Figure 4B).

To confirm that IL-10 is required for the reversal of cisplatin-induced mechanical hypersensitivity in response to the HDAC6 inhibitor, we used *Il10*$^{-/-}$ mice (Figure 4C–D). Following cisplatin treatment, *Il10*$^{-/-}$ mice developed mechanical hypersensitivity to a similar extent as WT mice (Figure 4D). As expected, the HDAC6 inhibitor failed to reverse mechanical hypersensitivity in *Il10*$^{-/-}$ mice, while it normalized mechanical sensitivity in WT mice (Figure 4C–D). Collectively, our data indicate that IL-10 signaling at the level of the spinal cord or DRG contributes to reversal of mechanical hypersensitivity in response to treatment with an HDAC6 inhibitor.

The IL-10 receptor is expressed by multiple cell types, including sensory neurons, glia, and infiltrating immune cells (Laumet et al., 2020). We hypothesized that IL-10 receptors on sensory neurons contribute to the resolution of cisplatin-induced mechanical hypersensitivity after treatment with an HDAC6 inhibitor. To test this hypothesis, we used mice with conditional deletion of the IL-10 receptor (*Il10ra*) in DRG neurons by crossing *Il10ra*-floxed mice with Advillin-Cre mice (*Avil*^{Cre}*Il10ra*$^{fl/fl}$). We confirmed previously that these mice show a decrease in IL10R1 protein on DRG neurons while IL10R1 expression on satellite glia is not affected (Laumet et al., 2020). In control mice (without Cre), cisplatin-induced mechanical hypersensitivity was reversed by 3 doses of ACY-1083 (Figure 5). Deletion of *Il10ra* from Advillin+ neurons did not affect the development or maximal severity of cisplatin-induced mechanical hypersensitivity (Figure 5). However, in *Avil*^{Cre}*Il10ra*$^{fl/fl}$ mice, ACY-1083 failed to reverse mechanical hypersensitivity caused by cisplatin treatment (Figure 5). Taken together, these results indicate a critical role of IL-10 signaling to IL-10 receptors on Advillin+ sensory neurons in the resolution of cisplatin-induced mechanical hypersensitivity in response to treatment with an HDAC6 inhibitor.

3.5. Treatment with an HDAC6 inhibitor reverses cisplatin-induced mitochondrial deficits in tibial nerves independently of MRC1+ macrophages or IL-10

Cisplatin treatment induces deficits in mitochondrial bioenergetics in dorsal root ganglia and in peripheral axons as measured in the tibial nerve (Krukowski et al., 2017; Ma et al., 2019; Maj et al., 2017). Reversal of mechanical hypersensitivity in response to treatment with the HDAC6 inhibitor ACY-1083 was associated with normalization of tibial nerve mitochondrial bioenergetics, while normalization of DRG mitochondrial bioenergetics is delayed (Krukowski et al., 2017). To determine whether the early effect of the HDAC6 inhibitor on mitochondrial bioenergetics in the tibial nerve is macrophage dependent, we tested tibial nerve mitochondrial function in mice that had received an intrathecal injection of mannosylated clodronate liposomes prior to treatment with the HDAC6 inhibitor (Figure 2A). Cisplatin-treated mice showed a significant decrease in both oxygen consumption rate (OCR) as well as in extracellular acidification rate (ECAR), which serve as indicators of mitochondrial oxidative phosphorylation and glycolysis, respectively (Figure 6). Three doses of ACY-1083 normalized mitochondrial bioenergetics in the tibial nerves (Figure 6). The results in figure 6 show that macrophage depletion did not interfere with the capacity of the HDAC6 inhibitor to normalize tibial nerve mitochondrial function (Figure 6A–B).

To determine the contribution of IL-10 to restoration of tibial nerve mitochondrial health, we treated mice with cisplatin followed by 3 doses of ACY-1083 together with anti-IL-10 neutralizing antibody or control IgG and analyzed mitochondrial function in the tibial nerves. OCR and ECAR were significantly decreased by cisplatin which was normalized by 3 doses of ACY-1083 treatment (Figure 7A–B). Notably, the HDAC6 inhibitor also normalized mitochondrial bioenergetics in tibial nerve when IL-10 signaling was blocked with anti-IL-10 antibody (Figure 7), even though this intervention prevented resolution of mechanical hypersensitivity. Collectively, these data indicate that normalization of tibial nerve mitochondrial function by an HDAC6 inhibitor is independent of macrophages in spinal cord/DRG and IL-10 signaling and is not sufficient to reverse mechanical hypersensitivity.

4. Discussion

HDAC6 inhibitors are promising candidates for the treatment of chemotherapy-induced peripheral neuropathy. HDAC6 inhibition reverses established CIPN induced by cisplatin (Krukowski et al., 2017; Ma et al., 2019) or paclitaxel (Krukowski et al., 2017). HDAC6 inhibition also prevents CIPN induced by vincristine without interfering with its anti-tumor effects of chemotherapy (Van Helleputte et al., 2018). Here, we demonstrate that reversal of mechanical hypersensitivity in response to treatment with an HDAC6 inhibitor depends on MRC1 (CD206) -positive spinal macrophages and IL-10 signaling to IL-10 receptors on peripheral sensory neurons. We show that administration of an HDAC6 inhibitor after cisplatin treatment increased the expression of spinal cord *Il10* mRNA and that local depletion of MRC1 (CD206) positive macrophages without affecting microglia, global genetic deletion of *Il10*, intrathecal administration of IL-10 antibody, or genetic deletion of the IL-10 receptor (*Il10ra*) from peripheral sensory neurons abolished the beneficial effects of the HDAC6 inhibitor on cisplatin-induced mechanical hypersensitivity. Importantly, the intervention used to deplete MRC1 positive macrophages did not affect spinal cord microglia. We also show that although IL-10 is required for the reversal of mechanical hypersensitivity induced by cisplatin, it is, however, not required for the normalization of mitochondrial function in the peripheral nerve of mice treated with cisplatin. Together, our current and previous findings indicate that HDAC6 inhibition reverses mechanical hypersensitivity by activating a required spinal cord MRC1-positive macrophage and IL-10 dependent pain resolution pathway in addition to restoring axonal mitochondrial health (Figure 8). We recently demonstrated that spontaneous resolution of CIPN also depends on endogenous IL-10 signaling indicating that treatment with an HDAC6 inhibitor does not just provide symptom relief in CIPN, but acts by accelerating resolution (Laumet et al., 2020). This notion is supported by our previous study showing that reversal of CIPN is maintained for at least 30 days in mice treated with only 14 daily doses of an HDAC6 inhibitor (Krukowski et al., 2017).

Recent studies showed the sex-independent capacity of HDAC6 inhibitors to reverse mechanical hypersensitivity in the spared nerve injury model of neuropathic pain (Sakloth et al., 2020), a migraine model (Bertels et al., 2021), and in the CFA model of inflammatory pain (Sakloth et al., 2020). We previously showed that HDAC6 inhibition during chemotherapy prevents CIPN in both sexes and that inhibiting HDAC6 reverses

established CIPN in males (Krukowski et al., 2017; Ma et al., 2019). Here, we show that administration of an HDAC6 inhibitor after completion of cisplatin treatment also reverses mechanical hypersensitivity in female mice, thereby further validating the sex-independent pain reversing effects of HDAC6 inhibitors. Moreover, we show that reversal of mechanical hypersensitivity in response to an HDAC6 inhibitor is dependent on MRC1 (CD206)-positive macrophage and IL-10 in both sexes.

Our current findings indicate that the HDAC6 inhibitor increases spinal cord IL-10 expression in mice treated with cisplatin. The cytosolic histone deacetylase HDAC6 has been implicated as a regulator of inflammatory and immune responses in other studies as well (de Zoeten et al., 2011; Youn et al., 2017; Zhang et al., 2008). Relevant for our current study, HDAC6 has been implicated in the regulation of the anti-inflammatory cytokine IL-10 (de Zoeten et al., 2011; Di Liddo et al., 2016; Oh et al., 2017; Wang et al., 2014), which can provide enduring pain relief in various pain models (Eijkelkamp et al., 2016; Krukowski et al., 2016; Vanderwall et al., 2018; Wahlman et al., 2018). The upregulation of IL-10 by the HDAC6 inhibitor in our model of CIPN is in line with previous studies showing increased IL-10 production in response to HDAC6 inhibition; in *in vitro* cultures of T-regulatory cells (de Zoeten et al., 2011); in explants and surrounding tissues from mice transplanted with silicone subcutaneously (Di Liddo et al., 2016); in mice bone marrow-derived primary macrophages treated with lipopolysaccharide (LPS) *in vitro*; and in serum isolated from *Hdac6* knockout mice challenged with LPS in vivo (Wang et al., 2014). However, some reports have shown that genetic deletion of *Hdac6* downregulated IL-10 *in vitro* in LPS-treated peritoneal elicited macrophages and bone marrow-derived dendritic cells (Cheng et al., 2014a; Cheng et al., 2014b). The apparently contradictory results on regulation of IL-10 in response to HDAC6 inhibition may result from comparing in vitro and in vivo studies, the differences in the inducing stimulus, the local microenvironment, as well as the cell type producing IL-10. Mechanistically, cell or activation state dependent difference in the HDAC6 interacting partners or effectors may also contribute to the differential effects of HDAC6 inhibitors. For example, the suppressive effects of HDAC6 inhibition on IL-10 have been ascribed to interference with STAT3 or HDAC11 (Cheng et al., 2014a; Cheng et al., 2014b), while the activating effects are thought to be mediated by changes in acetylation of cytosolic proteins, such as heat shock protein 90 and α-tubulin (de Zoeten et al., 2011; Wang et al., 2014). Future studies should aim at identifying the mechanisms whereby the HDAC6 inhibitor increases IL-10 in our CIPN model.

Based on the contribution of MRC1-postive spinal cord macrophages to resolution of cisplatin-induced mechanical hypersensitivity in response to HDAC6 inhibitor treatment we propose that macrophages are the major source of the IL-10 needed for resolution. Support for a model in which spinal cord macrophages produce IL-10 to suppress pain comes from a recent report using a peripheral nerve injury model. This study showed that spinal cord border associated MRC1+ macrophages suppress pain hypersensitivity after mild nerve injury in an IL-10-dependent manner. It also showed that increasing spinal cord macrophage IL-10 production by overexpression of CD163 reduced pain in a model of severe injury (Niehaus et al., 2021). Moreover, we previously identified a role of IL-10 producing macrophages in the spontaneous resolution of inflammatory pain (Willemen et al., 2014). However, we cannot exclude that other cell types, e.g. T lymphocytes contribute

to producing the IL-10 needed for the reversal of hypersensitivity in response to HDAC6 inhibitor treatment (Durante et al., 2021).

Notably, increasing evidence, including the findings provided here, points to a critical role of IL-10 signaling to IL10RA on sensory neurons in resolution of mechanical hypersensitivity in models of neuropathic and inflammatory pain (Laumet et al., 2020; Prado et al., 2021; Shen et al., 2013). Although initially characterized as an immune system receptor, IL10RA is enriched in DRG neurons (Laumet et al., 2020; Prado et al., 2021; Shen et al., 2013). Electrophysiology recording data showed that in vitro application of IL-10 can silence the spontaneous activity in DRG neurons isolated from paclitaxel-treated rats (Krukowski et al., 2016) and cisplatin-treated mice (Laumet et al., 2020). Moreover, intrathecal injection of IL-10 reverses the increased excitability of DRG neurons in rats with nerve injury (Shen et al., 2013). Multiple mechanisms have been proposed for the suppression of neuronal excitability by IL-10, including down-regulation of voltage gated sodium channels and changes in phosphorylation of NMDA receptors leading to altered calcium signaling (Durante et al., 2021; Shen et al., 2013; Turovskaya et al., 2012). The current data using *Il10*$^{-/-}$ knockout mice and mice with *Il10ra* deletion in sensory neurons indicate that the HDAC6 inhibitor reverses mechanical hypersensitivity in mice with CIPN via promoting IL-10 signaling to sensory neurons. However, it is likely IL-10 independent effects of the HDAC6 inhibitor contribute to reversal of CIPN. Indeed, we show that although IL-10 signaling is required for the recovery from cisplatin induced hypersensitivity, IL-10 is not essential for the restoration of mitochondrial function in tibial nerves of cisplatin-treated mice.

Mitochondrial toxicity and the resulting mitochondrial dysfunction is considered a key contributor to the pathogenesis of CIPN (Bennett et al., 2014). We have shown previously that cisplatin induces mitochondrial bioenergetic deficits in peripheral sensory neurons isolated from dorsal root ganglia as well as in the tibial nerves, which comprise the distal axons of the sensory neurons (Krukowski et al., 2017; Maj et al., 2017). Reversal of CIPN in response to HDAC6 inhibition was associated with normalization of tibial nerve mitochondrial function (Krukowski et al., 2017; Ma et al., 2019) that is likely mediated by changes in α-tubulin acetylation (Rossaert and Van Den Bosch, 2020) and/or Miro 1 activity (Kalinski et al., 2019). These studies led to the conclusion that normalization of axonal mitochondrial health was responsible for the reversal of mechanical hypersensitivity (Krukowski et al., 2017; Ma et al., 2019; Van Helleputte et al., 2018). However, in a subsequent study, we discovered that deleting *Hdac6* from peripheral sensory neurons in mice prevents axonal mitochondrial deficits and the loss of intraepidermal nerve fibers induced by cisplatin, but did not prevent mechanical hypersensitivity (Ma et al., 2019). In the current study we show that the normalization of tibial nerve mitochondrial function in response to the HDAC6 inhibitor is not dependent on IL-10 signaling while resolution of mechanical hypersensitivity is IL-10 dependent. Together these findings indicate that there is no causal relation between prevention or reversing axonal mitochondrial damage and prevention or reversal of mechanical hypersensitivity. However, it may well be possible that the IL-10-independent normalization of axonal mitochondrial health is required for reversal of the chemotherapy-induced loss of intraepidermal nerve fibers in response to HDAC6 inhibition (Krukowski et al., 2017). Notably, we showed earlier that although HDAC6

inhibition after completion of cisplatin treatment normalizes axonal mitochondrial health, mitochondrial function in the cell bodies in the DRG is still impaired (Krukowski et al., 2017). It is possible that the persistent mitochondrial abnormalities in the cell bodies of DRG neurons are contributing to the persistence of mechanical hypersensitivity even when axonal mitochondrial health is normalized after treatment with an HDAC6 inhibitor.

In conclusion, our current study further characterizes the mechanism underlying the reversal of signs of CIPN in response to treatment with HDAC6 inhibitors and identifies MRC1 (CD206) positive macrophages and increased IL-10 signaling to IL-10 receptors on peripheral sensory neurons as an indispensable contributor for reversal of cisplatin-induced mechanical hypersensitivity in response to HDAC6 inhibitors.

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- An HDAC6 inhibitor reverses cisplatin-induced mechanical hypersensitivity in both sexes
- MRC1 (CD206)-positive spinal cord macrophages are required for reversal of cisplatin-induced mechanical hypersensitivity by the HDAC6 inhibitor
- The HDAC6 inhibitor increases IL-10 in spinal cord of cisplatin-treated mice and this requires MRC-positive macrophages
- IL-10 signaling to IL-10 receptors on Advillin-positive sensory neurons is required for reversal of cisplatin induced mechanical hypersensitivity by the HDAC6 inhibitor
- Reversal of cisplatin-induced mitochondrial deficits in the tibial nerves by the HDAC6 inhibitor is independent of macrophages and IL-10

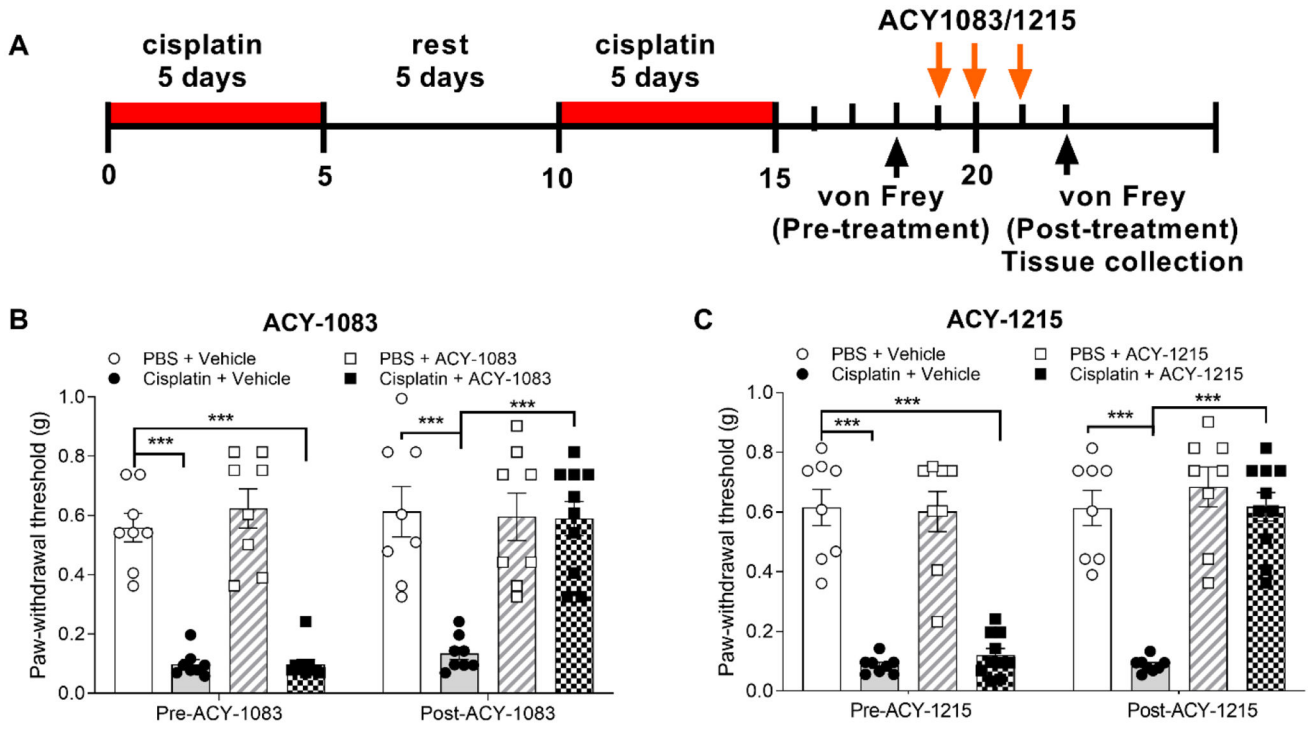

Figure 1.

The HDAC6 inhibitors ACY-1083 and ACY-1215 reverse cisplatin-induced mechanical hypersensitivity in female mice. A. Experimental timeline to test the effect of the HDAC6 inhibitors ACY-1083 and ACY-1215 on mechanical hypersensitivity induced with cisplatin in female mice. Mice were treated with cisplatin (2.3 mg/kg/day, i.p. 5 days on, 5 day rest, 5 days on) followed by three daily doses of ACY-1083 (10 mg/kg, i.p) or ACY-1215 (30 mg/kg, orally) starting 3 days after completion of cisplatin treatment. Black arrows indicate timepoints for the von Frey testing to assess mechanical sensitivity and for tissue collection (the last timepoint). B. The HDAC6 inhibitor ACY-1083 reverses cisplatin-induced hypersensitivity in female mice. Mechanical hypersensitivity of the hind paws was monitored using the von Frey test. *** $P<0.001$, two-way ANOVA with Dunnett test, n=8–10 mice per group. C. The HDAC6 inhibitor ACY-1215 reverses cisplatin induced hypersensitivity in female mice. Mechanical hypersensitivity of the hind paws was monitored using the von Frey test. *** $P<0.001$, two-way ANOVA with Dunnett test, n=8–10 mice per group.

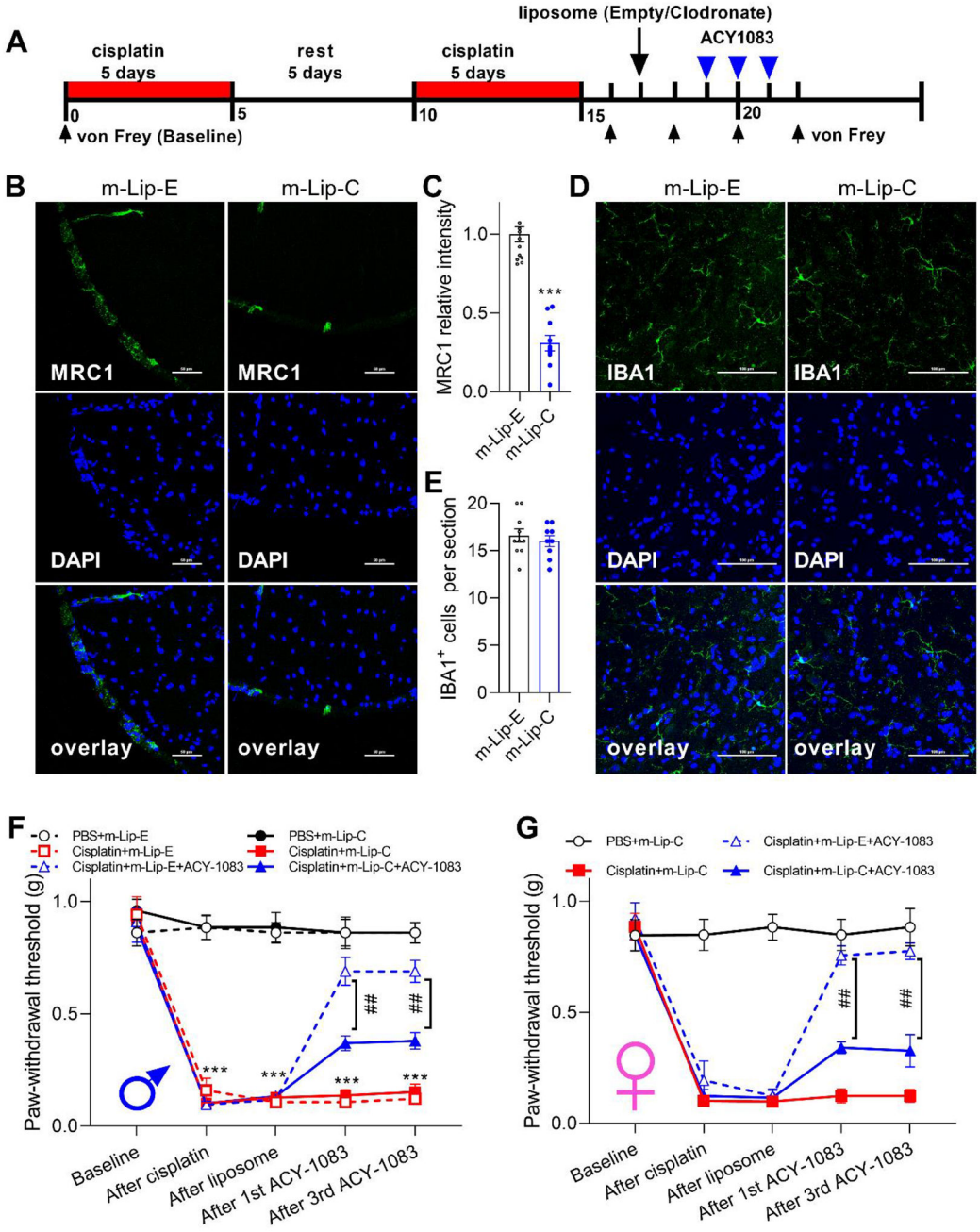

Figure 2.

Macrophages are required for the reversal of cisplatin-induced mechanical hypersensitivity in response to an HDAC6 inhibitor. A. Experimental schedule for treatment with cisplatin, mannosylated clodronate liposomes (m-Lip-C) or mannosylated empty liposomes (m-Lip-E), and the HDAC6 inhibitor ACY-1083 as well as von Frey assay to test the involvement of MRC1 (CD206) positive macrophages in the reversal of cisplatin-induced mechanical hypersensitivity by an HDAC6 inhibitor. Liposomes were administered intrathecally (10 μl/mouse) one day after completion of cisplatin treatment; ACY-1083 (10 mg/kg/day, i.p. for 3 days) was administered starting 1 day after liposome treatment. Tissues were

collected 1 day after the last dose of ACY-1083. B. Confocal images of MRC1 (CD206) positive macrophages in spinal cord sections from mice treated with mannosylated empty liposome (m-Lip-E) or mannosylated clodronate liposomes (m-Lip-C). Scale bar = 50 μm. C. Quantification of macrophages (MRC1) after treatment with m-Lip-E or m-Lip-C in spinal cord. *** $P<0.001$, two-tailed unpaired t-test, n=10–13 sections from 4 mice per group. D. Confocal images of IBA1 positive microglia in spinal cord sections from mice treated with m-Lip-E or m-Lip-C. Scale bar = 100 μm. E. Quantification of the number of microglia (IBA1) after treatment with m-Lip-E or m-Lip-C in spinal cord. N=9–10 sections from 4 mice per group. F/G. Mechanical sensitivity of the hind paws was monitored using the von Frey test at indicated timepoint in males (F) and females (G). *** $P<0.001$ compared to PBS+m-Lip-C; ## $P<0.01$, Cisplatin+m-Lip-E+ACY-1083 versus Cisplatin+Lip-C+ACY-1083; two-way ANOVA with Dunnett test, n=6 male mice per group (F) and n=4 female mice per group (G).

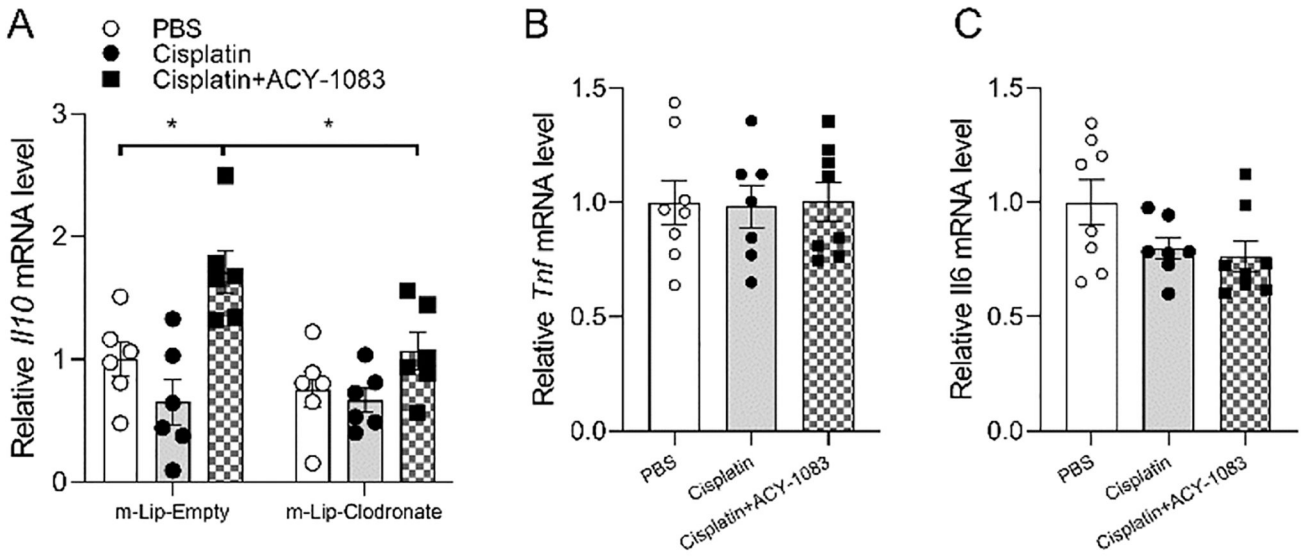

Figure 3.

The HDAC6 inhibitor ACY-1083 increases the expression of *Il10* in spinal cord of cisplatin treated mice and this requires MRC1 (CD206) positive macrophages. A. Quantitative real-time PCR analyzed gene expression levels of *Il10* in spinal cord from mice treated with 2 rounds of cisplatin followed by liposomes and then 3 doses of ACY-1083 (Treatment schedule in Figure 2A). * $P<0.05$, one-way ANOVA with Dunnett test, n=6 male mice per group. B-C. The expression of *Tnf* and *Il6* mRNA in spinal cord of mice treated with 2 rounds of cisplatin followed by 3 doses of ACY-1083 (Treatment schedule in Figure 1A). N=7–8 male mice per group.

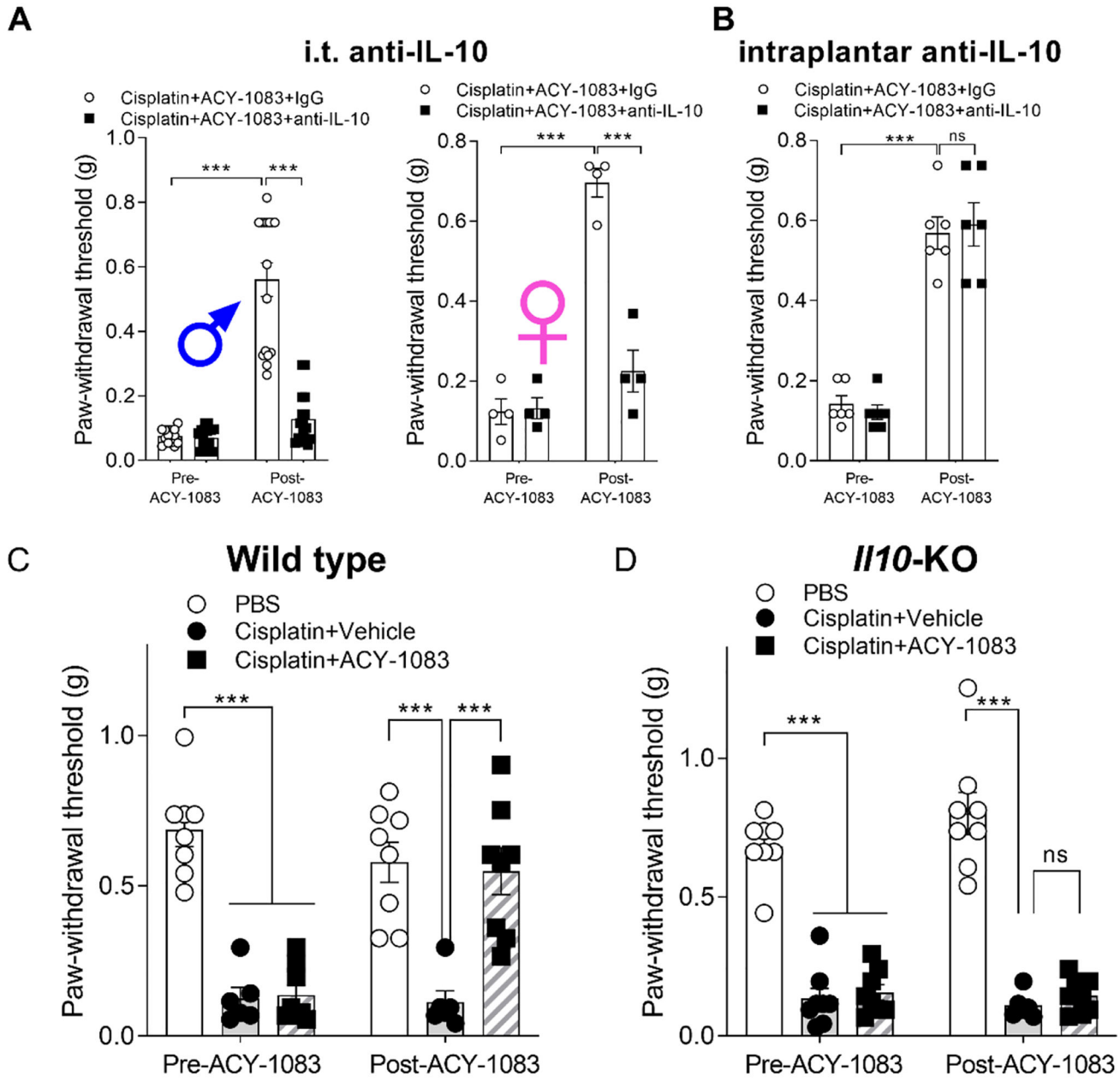

Figure 4.

Reversal of cisplatin-induced mechanical hypersensitivity in response to an HDAC6 inhibitor is IL-10 dependent. A. von Frey data present the mechanical sensitivity in cisplatin treated male and female mice before or after administrating 3 daily doses of ACY-1083 together with anti-IL-10 or IgG (10 μg/mouse, i.t injection). *** $P<0.001$, two-way ANOVA with Sidak test, n=16 male mice for each group and 4 females per group. B. von Frey data present the mechanical sensitivity in cisplatin treated mice before or after administering 3 doses of ACY-1083 together with anti-IL-10 or IgG (10 μg/mouse, intraplantar injection). *** $P<0.001$, two-way ANOVA with Sidak test, n=6 male mice for each group. C-D. von Frey data present the mechanical sensitivity in cisplatin treated wild type (C) and *Il10*$^{-/-}$ knock out (*Il10-KO*) mice (D) before and after ACY-1083 administration following the

treatment schedule in Figure 1A. *** $P<0.001$; ns, not significant; two-way ANOVA with Dunnett test, n=6–8 male mice per group.

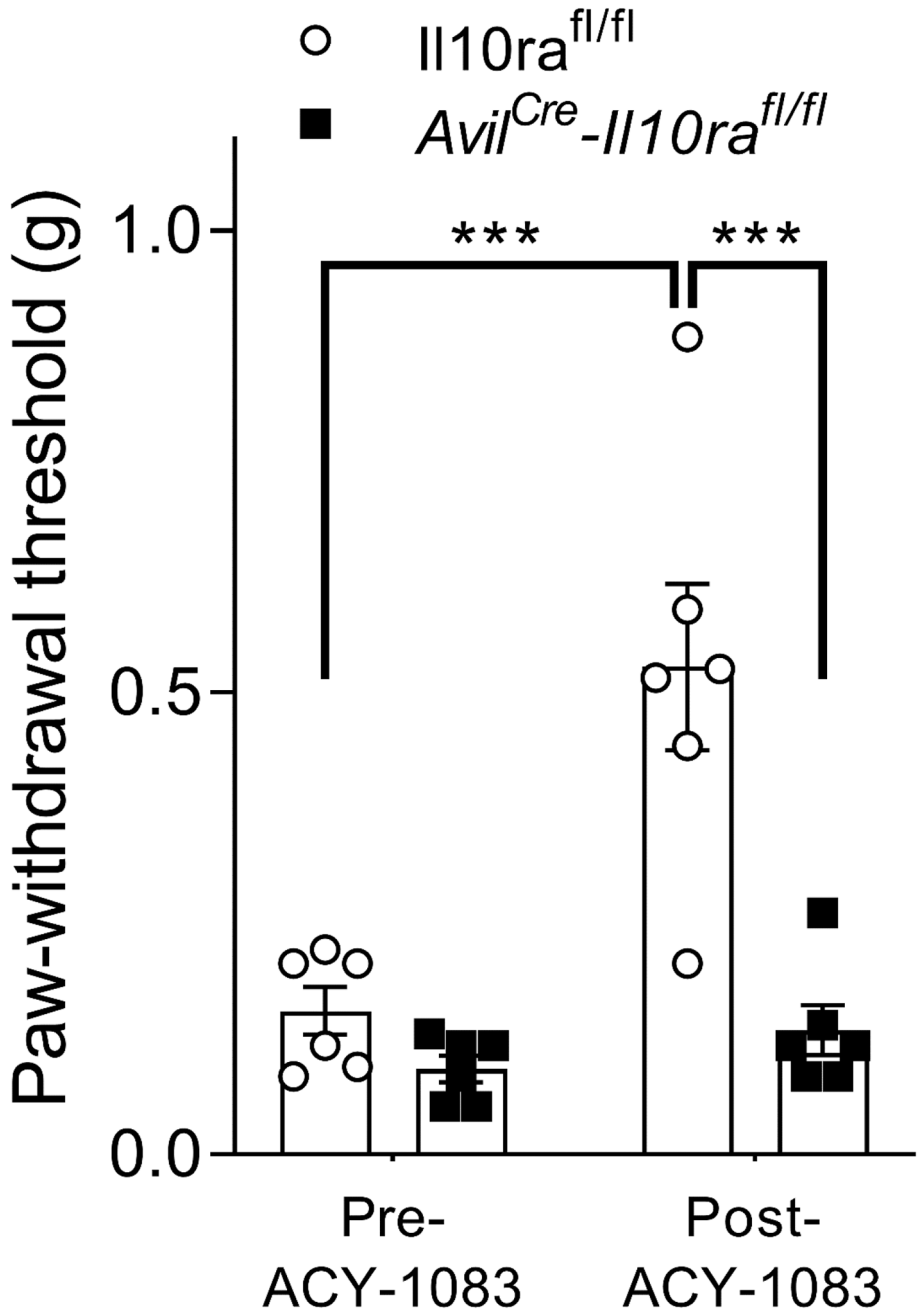

Figure 5.

IL-10 receptors on Advillin-positive sensory neurons mediate the effect of an HDAC6 inhibitor on cisplatin-induced pain hypersensitivity. von Frey data present mechanical sensitivity in cisplatin-treated control (*Il10ra*$^{fl/fl}$) and IL-10 receptor knock out (*Avil*^{Cre}-*Il10ra*$^{fl/fl}$) mice before and after ACY-1083 administration. *** P<0.001, two-way ANOVA with Sidak test, n=6 mice (3 male and 3 female) for each group.

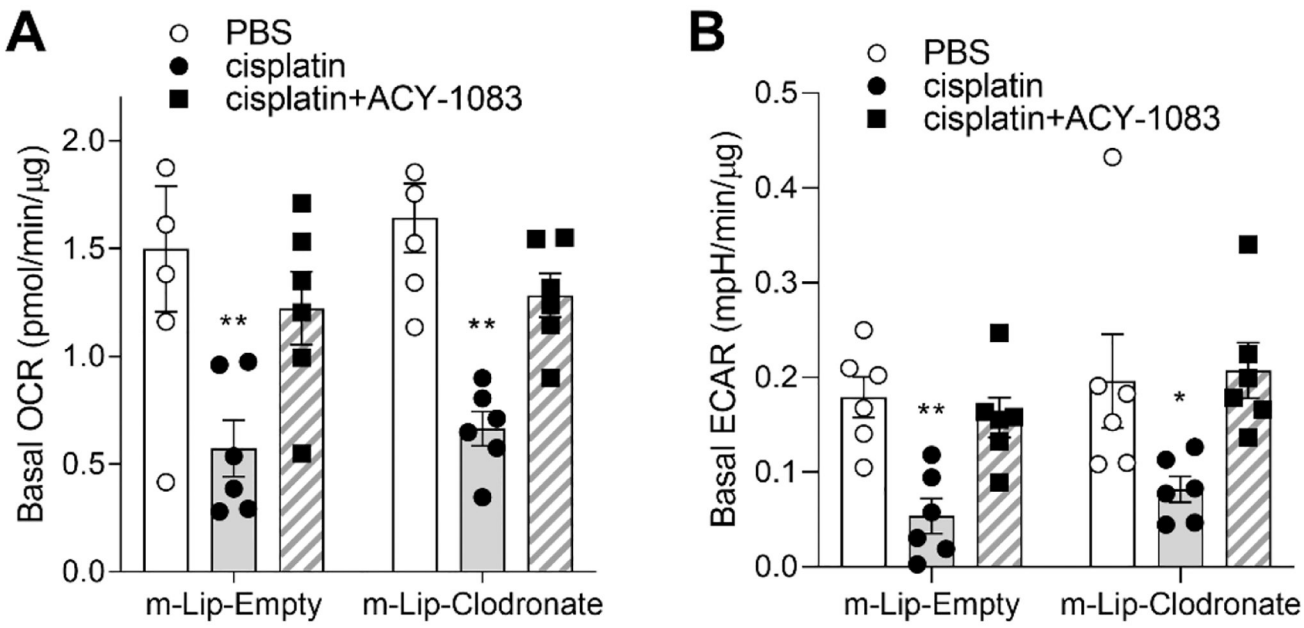

Figure 6.

Reversal of cisplatin-induced mitochondrial deficits in the tibial nerves in response to an HDAC6 inhibitor is not macrophage dependent. Mice were treated as in Figure 2. The oxygen consumption rate (OCR, A) and extracellular acidification rate (ECAR, B) of mitochondria in tibial nerve were analyzed using Seahorse assay. * $P<0.05$, ** $P<0.01$, repeated-measures two-way ANOVA with Sidak's test, n=6 male mice per group.

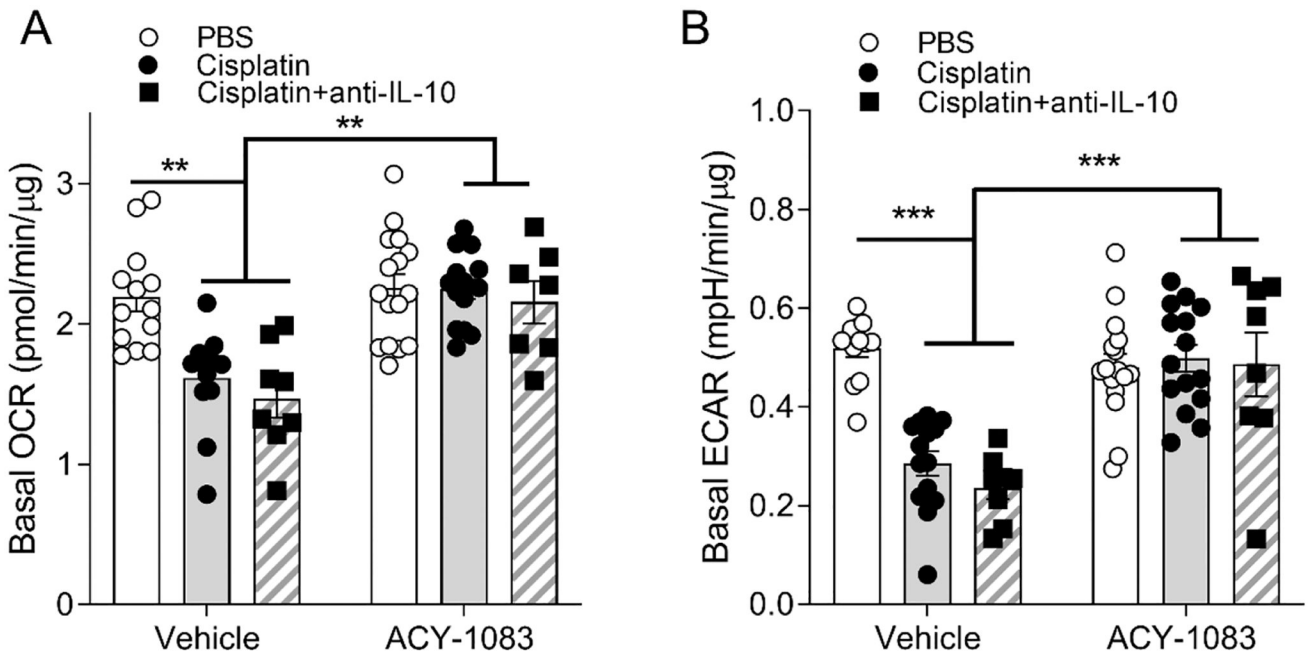

Figure 7.

Reversal of cisplatin-induced mitochondrial deficits in the tibial nerves in response to an HDAC6 inhibitor is not IL-10 dependent. The oxygen consumption rate (OCR, A) and extracellular acidification rate (ECAR, B) of mitochondria in tibial nerve were analyzed using seahorse assay. Tibial nerves were collected after 3 doses of ACY-1083 or Vehicle together with anti-IL-10 or IgG control (intrathecal injection, 10 µg/mouse). ** $P<0.01$, *** $P<0.001$, two-way ANOVA with Sidak's post-test, n=7–16 male mice per group.

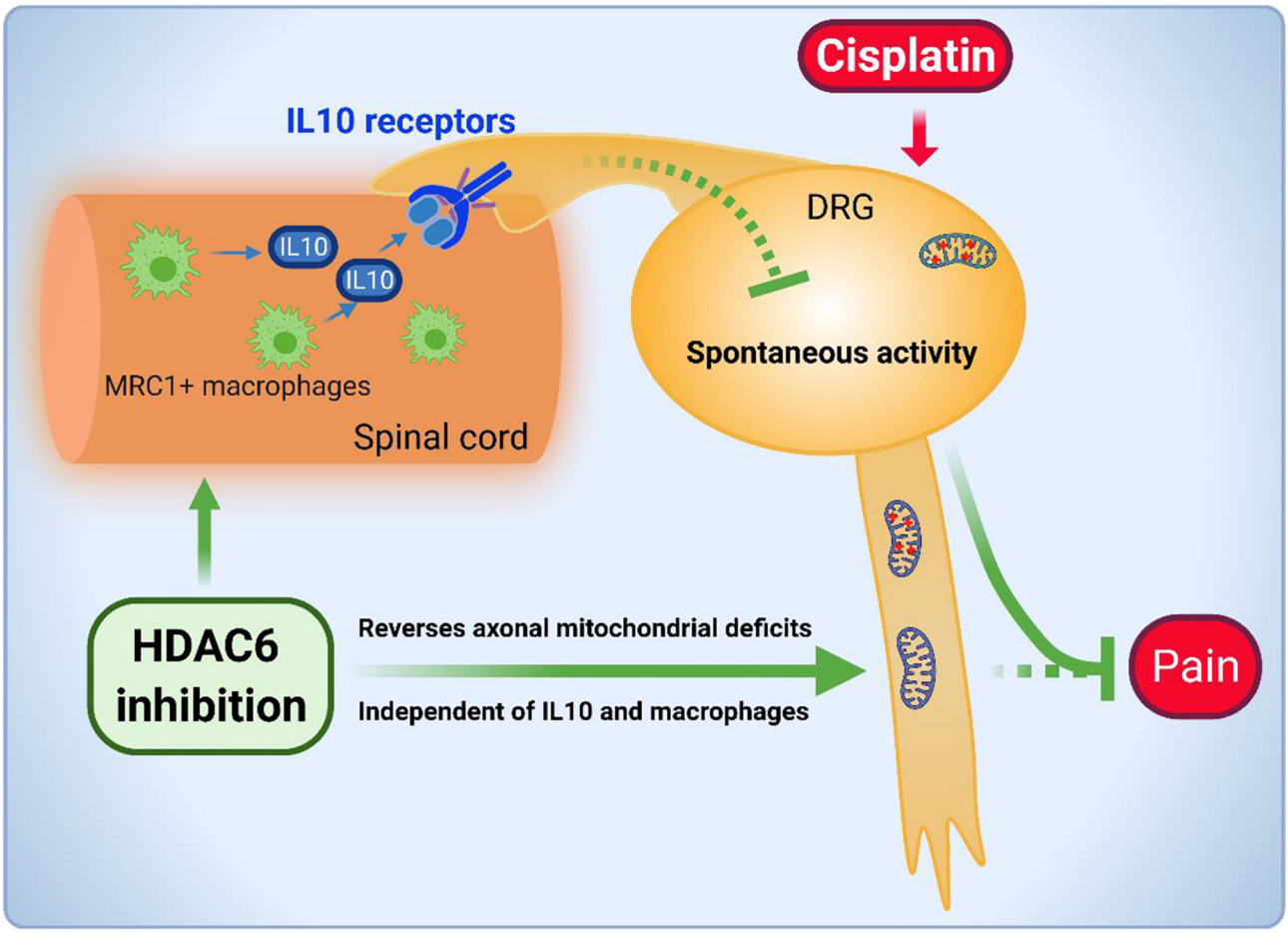

Figure 8.

Proposed model: Treatment with an HDAC6 inhibitor reverses mechanical hypersensitivity and tibial mitochondrial deficits via a concerted action on two different cell types. In sensory neurons, the HDAC6 inhibitor normalizes axonal mitochondrial function, but this is not sufficient to reverses mechanical hypersensitivity. In addition, the HDAC6 inhibitor increases IL-10 signaling, which requires macrophages, to IL-10 receptors on DRG neurons leading to suppression of spontaneous activity (Laumet et al., 2020) and reversal of hypersensitivity.