Paclitaxel increases axonal localization and vesicular trafficking of Na\textsubscript{V}1.7

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Abstract

The microtubule-stabilizing chemotherapy drug paclitaxel (PTX) causes dose-limiting chemotherapy-induced peripheral neuropathy (CIPN), which is often accompanied by pain. Among the multifaceted effects of PTX is an increased expression of sodium channel Na\textsubscript{V}1.7 in rat and human sensory neurons, enhancing their excitability. However, the mechanisms underlying this increased Na\textsubscript{V}1.7 expression have not been explored, and the effects of PTX treatment on the dynamics of trafficking and localization of Na\textsubscript{V}1.7 channels in sensory axons have not been possible to investigate to date. In this study we used a recently developed live-imaging approach that allows visualization of Na\textsubscript{V}1.7 surface channels and long-distance axonal vesicular transport in sensory neurons to fill this basic knowledge gap. We demonstrate concentration- and time-dependent effects of PTX on vesicular trafficking and membrane localization of Na\textsubscript{V}1.7 in real-time in sensory axons. Low concentrations of PTX increase surface channel expression and vesicular flux (number of vesicles per axon). By contrast, treatment with a higher concentration of PTX decreases vesicular flux. Interestingly, vesicular velocity is increased for both concentrations of PTX. Treatment with PTX increased levels of endogenous Na\textsubscript{V}1.7 mRNA and current density in DRG neurons. However, the current produced by transfection of DRG neurons with Halo-tag Na\textsubscript{V}1.7 was not increased after exposure to PTX. Taken together, this suggests that the increased trafficking and surface localization of Halo-Na\textsubscript{V}1.7 that we observed by live imaging in tranfected DRG neurons after treatment with PTX might be independent of an increased pool of Na\textsubscript{V}1.7 channels. After exposure to inflammatory mediators (IM) to mimic the inflammatory condition seen during chemotherapy, both Na\textsubscript{V}1.7 surface levels and vesicular transport are increased for both low and high concentrations of PTX. Overall, our results show that PTX treatment increases levels of functional endogenous Na\textsubscript{V}1.7 channels in DRG neurons and enhances trafficking and surface distribution of Na\textsubscript{V}1.7 in sensory axons, with outcomes that depend on the presence of an inflammatory milieu, providing a mechanistic explanation for increased excitability of primary afferents and pain in CIPN.

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Introduction

Paclitaxel (PTX) is a chemotherapeutic agent commonly used for the treatment of multiple types of cancers including breast, ovary, and lung. Dose-limiting side effects include chemotherapy-induced peripheral neuropathy (CIPN), which is frequently accompanied by pain and sensory abnormalities which can impact patient quality of life or survival. PTX is a microtubule stabilizer that binds to the inner lumen of the microtubule, promoting microtubule polymerization and stabilizing against depolymerization. This is highly effective in preventing mitosis in proliferating cancer cells; however, it can have off-target effects on non-dividing cells, such as neurons, which lead to CIPN. The mechanisms underlying the development of CIPN and pain have remained elusive and are likely multifaceted. PTX causes multiple changes in neurons including mitochondrial dysfunction, calcium dysregulation, microtubule disruption, and alterations to the expression and function of ion channels. A better understanding of the underlying mechanisms of PTX-induced neuropathy and pain could provide a basis for treatments that mitigate these dose-limiting effects, allowing more effective use of PTX in treating cancers.

Human and rat dorsal root ganglion (DRG) neurons become hyperexcitable after treatment with PTX, due to increased expression of several types of ion channels, including the voltage-gated sodium (Na\textsubscript{v}) channel Na\textsubscript{v}1.7. Na\textsubscript{v}1.7 is an attractive target for understanding PTX induced neuropathy and pain since it is a key driver of pain in humans: gain- or loss-of-function mutations of Na\textsubscript{v}1.7 result in painful neuropathies or insensitivity to pain, respectively. However, studies on the effects of PTX on Na\textsubscript{v}1.7 expression levels and excitability of DRG neurons have been limited to the soma of these neurons and have not addressed underlying mechanisms such as temporal and spatial regulation of channel trafficking or surface distribution in sensory axons. Additionally, the PTX-induced increase in Na\textsubscript{v}1.7 at nerve endings must be reconciled with reported deficits in anterograde axonal trafficking, for example transport of mitochondria.

Due to the role of Na\textsubscript{v}1.7 in human pain, including CIPN, and demonstrated upregulation in response to PTX treatment, there is a need for understanding axonal trafficking of this channel and alterations to its cell surface distribution in response to PTX. Examination of axonal trafficking and membrane localization of Na\textsubscript{v} channels in real-time has previously been hampered by the lack of tools to study dynamics of channel trafficking and membrane localization in live neurons. We recently developed novel fluorescently-tagged Na\textsubscript{v}1.7 constructs to enable visualization of Na\textsubscript{v}1.7...
surface distribution and axonal trafficking in the axons of live cultured sensory neurons with high spatial and temporal resolution. Using these new methods, we demonstrated fast microtubule-based vesicular NaV1.7 transport in sensory axons, which was disrupted by treatment with the microtubule depolymerizing drug nocodazole. Thus, we would expect that PTX-induced changes to microtubules would impact NaV1.7 axonal transport. The new high resolution, real-time imaging techniques are ideally suited for investigation of the dynamic regulation of NaV1.7 channel distribution and trafficking in live sensory neurons in response to treatment with agents such as PTX.

In this study, using a tagged Halo-NaV1.7 construct and live-imaging, we show that treatment with low levels of PTX increases the number of membrane inserted channels at sensory axonal terminals, as well as enhancing neuronal trafficking of vesicles carrying NaV1.7; conversely, higher concentrations of PTX result in axonal degeneration, less channel expression at distal axons, and impaired axonal trafficking. We also show that co-treatment with inflammatory mediators, which mimics the inflammatory milieu in peripheral nerves during chemotherapy, further increases surface expression and axonal transport of NaV1.7.

Materials and methods

DNA constructs

The parent plasmid which encodes human NaV1.7 plasmid that was rendered tetrodotoxin-resistant (TTX-R) by substituting amino acid (a.a.) Tyr362 with serine (Y362S) was previously described. Cloning of the HaloTag in-frame at the N-terminus of the channel to produce Halo-NaV1.7 was previously described and characterized.

Primary rat DRG neuron culture and transfection

Animal studies followed a protocol approved by the Veterans Administration Connecticut Healthcare System Institutional Animal Care and Use Committee. DRG neurons were isolated from 0 to 5-day-old Sprague–Dawley rat pups and transfected with Halo-NaV1.7 as described previously, and briefly described in Supplemental Information.

Microfluidic chambers
Microfluidic chambers (MFCs) (DOC450, 2-chamber 450 µm groove, Xona Microfluidics, Temecula, California) were placed on 50 mm glass-bottomed dishes (P50G-1.55-30-F, MatTek). In preparation for the assembly of the MFCs, dishes were coated with 0.5 mg/ml poly-L-lysine overnight at 37°C, then the glass surface was washed twice with sterile double distilled water and air dried under the hood. The dishes were then coated with 10 mg/ml laminin for at least two hours at 37°C, excess laminin aspirated, and the dishes dried under the hood before MFCs were adhered. Transfected DRG neuron suspension was applied in the soma chamber and DRG medium supplemented with 50 ng/mg growth factors (NGF, GDNF from PeproTech) and 2x growth factors (100 ng/mg) was added to the axonal chamber. Media was changed to serum-free medium in both chambers after 24 hrs (Neurobasal medium supplemented with 2% B27, 1% Pen/strep, same 1:4 ratio of NGF, GDNF in soma and axonal chambers), and 1 µM Uridine/5-Fluoro-2-deoxyuridine was added to inhibit the growth of fibroblasts and glia. During the 5-7 days before imaging, volumes were kept slightly higher (~50 µl) in the somatic chamber as recommended by the manufacturer to fluidically isolate the chambers and maintain the high concentration of growth factors in the axonal chamber.

Treatments

PTX was prepared in advance as a stock solution and diluted to a final concentration of either 25 nM or 250 nM PTX. Inflammatory mediator cocktail (IM) was prepared in advance as a stock solution and diluted to a final concentration containing (all from Sigma): bradykinin (1 µM), PGE-2 (10 µM), histamine (10 µM), 5-HT (10 µM) and ATP (15 µM). Stock solutions were stored in aliquots and frozen until use. Treatments were added to both the somatic and axonal chambers for 6, 24, or 48 hrs before imaging as indicated. The appropriate volumes of stock solutions (DMSO, PTX, and/or IM) were first mixed with a portion of the media removed from the culture dish or slide before mixing the dilution back into the original well, thus preventing exposure of the cells to high concentrations of the compounds.

Imaging system

Images were acquired using an Andor Dragonfly spinning disk confocal microscope built on a Nikon Eclipse Ti fluorescence microscope. Images were collected using an Andor iXon Ultra 888 EMCCD camera through either a 20x air objective or Plan Apo Lambda 60x (NA1.4) oil objective.
The system includes an Andor Integrated Laser Engine containing 100 mW 405 nm, 150 mW 488 nm, 150 mW 561 nm, and 140 mW 637 nm solid state lasers. The Nikon Perfect-Focus system was used to maintain focus in the z-plane during time-lapse imaging.

**Live-cell imaging and surface labeling**

All experiments were performed at 37°C using a stage incubator (Tokai Hit, Shizuoka, Japan). Neurons were kept in DRG neuronal imaging saline (DRG-NIS) containing (in mM): 136 NaCl, 3 KCl, 1 MgSO4, 2.5 CaCl2, 0.15 NaH2PO4, 0.1 Ascorbic Acid, 20 HEPES, 8 dextrose, pH 7.35 with NaOH (adjusted to 320 mOsmol/L) during incubation and imaging. To surface label Halo-NaV1.7 channels, the culture media in the axonal chamber was removed by washing with NIS, then 100 nM cell-impermeable HaloTag-JF635i ligand (Kind gift from Luke D. Lavis and Jonathan B. Grimm, Janelia Research Campus)\(^{16, 17}\) was added for 20 min. Axons were washed three times with DRG-NIS, then imaged for up to 1 hr after labeling. Analysis of axonal endings (Fig 1) were performed on images acquired using the 20x objective. All other data was acquired using the 60x objective.

**Optical Pulse-chase Axonal Long-distance (OPAL) imaging**

The OPAL imaging technique\(^{12}\) was used to selectively label anterograde vesicles containing Halo-NaV1.7 during long-distance axonal transport, and is described in Supplemental Information.

**Image analysis**

Axon degeneration was measured from three fields of view acquired using the 20x objective from each of three independent cultures. Axon endings were analyzed from compressed confocal z-stacks and designated as either healthy or bulbed as previously described\(^{11,18}\). Healthy axons had similar diameters between the axon shaft and the axon end, or showed branches and extensions. In contrast, bulbed axons were round or oval at the ending with a diameter greater than the axon shaft and did not show any branches or extensions.

To determine the amount of Halo-NaV1.7 on the axonal endings, Fluorescence intensity was measured from z-stacks of surface labeled Halo-NaV1.7 on axon endings in the axonal chamber of the MFCs. A compressed z-stack was created in ImageJ by summing individual z-slices. A region
of interest (ROI) was drawn around the final 60 µm of the axon and the mean fluorescence intensity was background subtracted.

To analyze vesicular flux and velocity, kymographs were created from time-lapse image sequences using ImageJ and KymographClear and analyzed using the automated kymograph analysis software KymoButler, which is based on deep learning. Time-lapse movies were loaded into ImageJ and the KymographClear toolset was used to create kymographs from sections of axons. Axons chosen for analysis were isolated away from other axons and had multiple clearly visible vesicles that moved through the region during the image sequence. Representative axons were chosen from each field of view. Vesicle tracks were automatically detected using KymoButler, which provided velocities for each vesicle track and flux data for each axon. Average velocities included pauses and stops for each vesicle track. Vesicles were classified as stationary if they had an average velocity between -0.1 and 0.1 µm/s, retrograde vesicles had velocities < -0.1 µm/s, and anterograde vesicles had velocities > 0.1 µm/s. Run length was determined as described by Lim et al. by whereby the total length of clearly defined vesicle tracks on kymographs was measured, using only tracks that traveled across the x-axis of the kymograph, and the total number of stops and pauses were measured. A stop or pause was defined as multiple pixels showing a vertical line on the kymographs. Flux was defined by the number of forward moving vesicle tracks that passed the center of the kymograph. Data were imported into Origin for statistical analysis.

**Voltage-clamp recordings in DRG neurons**

$\text{Na}_V^{1.7}$ currents were recorded from DRG neurons in voltage-clamp mode using an EPC-10 amplifier and the PatchMaster program (HEKA Elektronik, Holliston MA, USA) at room temperature (22-25°C). Details of the methods are provided in Supplemental Information.

**Real-time RT-PCR**

RNA from DRG neurons in culture was extracted using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. cDNA was generated from 100-200 ng of DRG RNA using Bio-Rad iScript Reverse Transcription Supermix. The following Prime-PCR probe Bio-Rad assays were used: Actb (qRnoCIP0050804), $\text{Na}_V^{1.7}$ (qRnoCIP0023430). Samples of 10 µl PCR reactions were run in triplicate for each sample using the Bio-Rad CFX96 Touch System with the following thermal-cycling procedure; 95°C for 30 sec followed by 40 cycles of 95°C for 15 sec and 60°C for
30 sec. Three independent cultures (biological replicates) were used for each condition. The relative quantity and normalized expression data were processed using Bio-Rad CFX Manager.

**Image analysis and Statistical Analysis**

Images were processed with ImageJ. For figures with multiple panels, matched panels were processed and contrasted identically. Statistical analysis was performed using Origin or Prism and specific tests are identified in the text. Data are presented as mean ± standard error unless otherwise stated, and statistical significance was considered at p < 0.05. All data represent three independent cultures (imaging and RT-PCR, and voltage-clamp recordings of endogenous Na\(_V\)1.7 currents), and four cultures for voltage-clamp recordings of Halo-Na\(_V\)1.7.

**Data and Materials availability**

All information necessary to evaluate the findings of the paper are included in the paper. Additional data can be provided from the authors upon request.
Results

Time and concentration dependent effects of PTX on sensory neuron axons

Effects of PTX treatment have been shown to be time- and concentration-dependent. For example, low concentrations of PTX promote neurite extension in cultured CNS neurons, while higher concentrations prevent neurite extension. In cultured DRG neurons, the degree of axon degradation increases with higher concentrations of PTX or incubation with PTX for longer periods. Therefore, we investigated the effects of multiple concentrations of PTX using a sensory neuronal culture system and optical methods that permit high resolution, real-time imaging of NaV1.7 channels. Importantly, we wanted to find PTX concentrations that do not cause acute axonal degradation so we could study the effects of PTX treatment on NaV1.7 axonal trafficking and surface distribution independent of axonal damage, so as to mimic conditions during early phases of treatment with PTX. Studies investigating the effects of PTX on cultured sensory neurons have used concentrations ranging from 10 nM to more than 2.5 µM. It is difficult to know what concentrations peripheral axons are exposed to in human patients, and further how this translates to cultured neurons with direct exposure to the drug. Human cancer patients have been reported to display peak concentrations reaching greater than 4 µM, but steady state plasma concentrations drop to 21 – 860 nM over the 2-48 hrs following treatment. Based on this information and our desire to investigate neuronal axons prior to axonal degradation, we chose to treat axons with either 25 nM or 250 nM PTX. After treatment with PTX, rats show an accumulation of PTX both within their DRG where the neuronal cell bodies are located, as well as along the nerves. Thus, we chose to incubate both the neuronal cell bodies and axons with PTX to best mimic in vivo conditions.

DRG neurons from neonatal rats were cultured in microfluidic chambers with a microgrooved barrier that separates the soma and axon compartments, enabling better visualization of distal axon endings as described previously. DRG neurons were transfected with a EGFP plasmid to visualize axon morphology. Using spinning disk microscopy, we observed a dose and time-dependent formation of retraction bulbs in response to PTX treatment. Retraction bulbs have been previously described as markers of axonal degeneration, and were defined as axonal ends with round or oval enlargements with diameters greater than the axon. Healthy axon endings displayed visible protrusions including branches or filipodia. We found that treatment with 25 nM PTX
causes minimal formation of retraction bulbs (Fig 1A, orange arrows) over the course of 48 hrs (Fig 1A), suggesting this is a sub-toxic concentration of PTX within this timeframe. By contrast, the frequency of bulbed axonal endings was greater for 250 nM PTX than for 25 nM PTX, showing increased axonal degeneration with increasing time (Fig 1B).

**Low PTX dose increases the amount of Na\textsubscript{V}1.7 at the surface of axonal endings**

Due to its role as a threshold channel that regulates action potential firing in DRG neurons and as the underlying cause in multiple human pain disorders,\textsuperscript{27} an increase in the number of Na\textsubscript{V}1.7 channels at axonal endings of sensory neurons is expected to increase neuronal excitability. DRG neurons expressing Halo-Na\textsubscript{V}1.7 and soluble EGFP were cultured in microfluidic chambers. HaloTag is a self-labeling protein that forms a covalent bond with specific cognate ligands, which can be conjugated to fluorophores or other functional groups. We previously demonstrated that the Halo-Na\textsubscript{V}1.7 channel construct is suitable for live-cell imaging.\textsuperscript{12} The extracellularly tagged channel Halo-Na\textsubscript{V}1.7 allows for visualization of channels inserted within the plasma membrane at axonal endings using cell-impermeable HaloTag fluorescent ligands (Fig 2A). Neurons were treated with either vehicle (0.025% DMSO), 25 nM PTX, or 250 nM PTX for either 6, 24, or 48 hr. Prior to imaging, the axonal chamber was labeled with cell-impermeable HaloTag-JaneliaFluor635i (JF635i) (Fig 2B).

**Figure 2C** shows example axons from each treatment group at 48 hrs, with the pseudo colored images demonstrating surface labeled Na\textsubscript{V}1.7 channels and the EGFP signal showing axon morphology. The surface labeling for the axon treated with 25nM PTX is much brighter than the DMSO control, representing increased numbers of surface Na\textsubscript{V}1.7 channels at the axonal ending. Conversely, the axon treated with 250 nM PTX has less surface labeling and its morphology shows a retraction bulb, as is characteristic of degenerating axons. When looking over time, neurons treated with 25 nM PTX showed an increase in mean fluorescence intensity compared to the DMSO control at all timepoints, with statistically significant increases at 24 and 48 hrs (Fig. 2D) (Table S1). This increase was not observed when neurons were treated with 250 nM PTX, and at 48 hrs axons treated with 250 nM PTX were significantly dimmer than axons treated with 25 nM PTX.

**Nav1.7 channel transport is altered after treatment with PTX**
Alterations in channel delivery to the plasma membrane at axonal endings could underlie changes in surface expression of Na\textsubscript{v}1.7. In order to test the hypothesis that PTX treatment alters Na\textsubscript{v}1.7 trafficking to distal axons, we used an imaging technique recently developed in our lab, optical pulse-chase axonal long-distance (OPAL) imaging\textsuperscript{12} that enables visualization of vesicular transport of Na\textsubscript{v}1.7 channels with single-molecule sensitivity (Fig 3A). This technique allows imaging of live axonal transport of low abundance proteins that are difficult to image using traditional microscopy methods. For this technique, Halo-Na\textsubscript{v}1.7 expressing neurons cultured in microfluidic chambers are incubated with cell-permeable HaloTag-JaneliaFluor549 only within the somatic chamber. Vesicles carrying Halo-Na\textsubscript{v}1.7 from the somatic chamber into the axonal chamber carry the labeled channels, allowing selective visualization of anterograde Halo-Na\textsubscript{v}1.7 vesicles with minimal background when imaging the axonal chamber. OPAL imaging substantially enhances the signal/noise ratio, which permits observation of vesicles carrying a relatively small number of labeled channels.

*Figure 3B* shows example kymographs depicting time-lapse imaging of axons with Halo-Na\textsubscript{v}1.7 containing vesicles (Movie 1). We observed that cultures incubated with 25 nM PTX had more anterogradely moving vesicles per axon (flux) than control axons at all timepoints (Fig 3C) (Table S2). Conversely, cultures exposed to 250 nM PTX showed similar numbers of vesicles at 6hrs, but had decreased flux at 24 and 48hrs. We next measured the velocity of anterograde vesicles and found that incubation with either 25 nM or 250 nM PTX caused an increase in average anterograde vesicular velocity, even within 6 hrs (Fig 3D) (Table S3). The exception was for 250 nM PTX at 24 hrs, which trended towards faster vesicles compared to controls, but this did not reach statistical significance. Together, these data suggest that the number of vesicles traveling along each axon is sensitive to PTX concentrations, while vesicular speed is much less concentration dependent. Although OPAL imaging enhances visualization of anterograde vesicles, a small number of stationary and retrograde-moving vesicles can be visualized. The distribution of vesicle velocities for all tracks, including stationary and retrograde tracks are shown in *Figure S1* and the percentage of vesicles for each direction is shown in *Figure S2*. To further investigate the vesicle behaviors that contributed to differences in vesicle velocities, we determined the average distance each particle traveled before pausing or stopping (run length) under each condition. We used the method of Lim et al.,\textsuperscript{21} whereby we summed the total distance traveled for all clearly visible vesicles that traveled the full distance (x-axis) of each kymograph and then divided this distance by the number
of stops and pauses observed. We found that the run length trended longer for all the conditions with PTX treatment compared to control conditions. (In order of control, 25 nM PTX, 250 nM PTX (in µM): 18.41, 27.17, 32.18 (6 hrs), 20.84, 24.35, 23.67 (24 hrs), 16.40, 29.81, 25.14 (48 hrs) (Table S4).

Next, we assessed the effects of PTX treatment on the expression levels of endogenous NaV1.7 channels as well as the current density in DRG neurons transfected with the Halo-NaV1.7 channels. Exposure of DRG neurons to 25 nM PTX for 24 hours resulted in a 1.43-fold increase in endogenous NaV1.7 mRNA as determined by real-time PCR, normalizing the expression levels of NaV1.7 by that of actin (DMSO: 1.03 ± 0.05 a.u.; PTX 1.43 ± 0.08 a.u., p=0.0002, Student t-test). In parallel experiments using voltage-clamp recordings we found that the ProTx-II-sensitive NaV1.7 current density from PTX-treated DRG neurons was significantly larger than that from DMSO-treated neurons (Fig. 4A); (DMSO: 126.33 ± 12.06 pA/pF, n = 7; PTX: 237.26 ± 31.36 pA/pF, n = 8, Student’s t-test p = 0.008). By contrast, Halo-NaV1.7 current levels (TTX-R and ProTx-II-sensitive) in transfected DRG neurons were unaltered by PTX treatment (Figure 4B); (DMSO: 197.18 ± 60.00 pA/pF, n = 7; PTX: 200.13 ± 27.75 pA/pF, n = 9, Student’s t-test p = 0.96).

**Inflammatory mediators further increase surface labeling and vesicular trafficking of NaV1.7 at axonal endings**

Chemotherapy using PTX can also produce nerve inflammation.28, 29 Our results thus far showing increased surface labeling and enhanced vesicular trafficking in response to exposure to PTX are similar to previous results 12 showing that treatment with inflammatory mediators (IM) increases the presence of NaV1.7 channels in the plasma membrane of axonal endings and increases vesicle flux and velocity, thus we investigated the effects of both PTX and an inflammatory milieu on NaV1.7 trafficking in sensory axons. Since differential surface levels and vesicular trafficking were seen for 25nM vs 250nM PTX at 24 hrs, we investigated the influence of co-treatment with IM on NaV1.7 trafficking at this timepoint.

Neurons were co-treated with or without IM and either DMSO, 25 nM PTX, or 250 nM PTX for 24 hrs and Halo-NaV1.7 was labeled with cell-impermeable JF635i-HaloTag ligand as before. **Figure 5A** shows example axon endings from cultures exposed to vehicle or 25 nM PTX, with or
without co-treatment with IM. An increase in mean fluorescence intensity of Na\textsubscript{v}1.7 surface labeling in axons treated with IM was seen for all conditions (DMSO, 25 nM PTX, 250 nM PTX) compared to axons not receiving IM (Fig 5B) (Table S5).

We previously demonstrated that in addition to increased surface expression, treatment with inflammatory mediators enhances vesicular trafficking.\textsuperscript{12} Thus, we used OPAL imaging to investigate axonal transport for cultures treated with both PTX and IM. Fig 6A shows example kymographs representing vesicular trafficking of axons from cultures treated with 25 nM PTX, with or without IM for 24 hrs (Movie 2). The number of vesicles per axon (flux) and vesicle velocities were measured. Compared to axons not treated with IM, all conditions showed an increase in vesicular flux when treated with IM (Fig 6B) (Table S6). Vesicle velocities were also increased for all conditions (Fig 6C) (Table S7).
Discussion

PTX-related neuropathies most often and most severely affect the extremities which are innervated by DRG neurons with the longest axons. This has led to a focus on the effects of PTX on axonal transport which may be most apparent in the longest axons. Studies have shown that PTX impairs axonal trafficking,\textsuperscript{10} although it is unclear whether this is a cause or a result of axonal degradation.\textsuperscript{11} This reported loss of anterograde trafficking\textsuperscript{10} needed to be reconciled with the upregulation of proexcitatory ion channels including Na\textsubscript{v}1.7 at axonal endings in animals treated with PTX. Using high-resolution live imaging in sensory axons, we found that the effects of PTX are time- and concentration-dependent. At low concentrations (25 nM PTX), we found increased levels of Na\textsubscript{v}1.7 at the surface in distal axons at 24 and 48 hrs, which is accompanied by an enhancement of vesicular trafficking with increases in both the flux and velocity of Na\textsubscript{v}1.7-containing vesicles. Using a higher concentration (250 nM PTX) we do not observe a change in surface expression relative to the DMSO control, but see a decrease in the number of vesicles per axon. Treatment of DRG neurons with PTX increased endogenous Na\textsubscript{v}1.7 mRNA levels and current density. However, the current produced by transfection of DRG neurons with Halo-Na\textsubscript{v}1.7 was not increased after exposure to PTX. We also found that mimicking PTX-induced tissue inflammation\textsuperscript{28, 29} in our culture with an IM cocktail augments the effects of PTX on surface levels and vesicular trafficking of Na\textsubscript{v}1.7.

To investigate the dynamic regulation of channel trafficking in sensory axons treated with PTX, we used full-length Na\textsubscript{v}1.7 constructs and live-cell imaging to assess the effect of this treatment on surface expression and vesicular trafficking of Na\textsubscript{v}1.7. High-resolution imaging of channel trafficking after exposure to PTX requires study of cells in culture, because it is currently not technically feasible to obtain sufficient signal-to-noise ratio for live imaging of channels in \textit{in vivo} preparations. Neuronal cell cultures for the study of CIPN have been widely used and allow a highly controlled environment whereby specific effects can be studied quickly and efficiently.\textsuperscript{30} Our studies used \textit{in vitro} assessment of DRG neurons from young animals, which were required for transfecting and imaging full-length Na\textsubscript{v} channels and maintaining these transfected cultures for several days. Ideally, future studies can employ the use of human induced pluripotent stem cells (iPSCs) to provide a model that can better simulate human disease.
In vitro studies of PTX have used a wide variety of concentrations, ranging from 10 nM to more than 2.5 µM. Human cancer patients have peak concentrations reaching greater than 4 µM, but steady-state plasma concentrations drop to 21–860 nM over the 2-48 hrs following treatment, suggesting that sub-micromolar concentrations may be best to model CIPN. Furthermore, neurons studied in a dish have more immediate exposure to PTX than axons embedded in tissues, suggesting lower concentrations of PTX may be most applicable for in vitro studies. It is also important to note that different compartments of DRG neurons can be exposed to different concentrations of PTX. A study of rats treated with PTX over 6 days showed higher concentrations within the DRG, compared to the dorsal and ventral roots and the sciatic nerve. Thus, studies of multiple concentrations of PTX as done here might mimic temporal and spatial effects of the drug on sensory neurons.

In several cell types, lower concentrations of PTX can promote the formation of growth cones and regeneration of axons, while higher concentrations of PTX can be damaging. This is consistent with our observation of apparently normal axonal morphology in cultures treated with 25 nM PTX, while axons with end bulbs are evident in cultures treated with 250 nM PTX, indicative of axonal degeneration. Furthermore, axonal trafficking of Na\textsubscript{v}1.7 is promoted by exposure to 25 nM PTX, while it is inhibited by the higher concentration of 250 nM. The underlying mechanisms for the observed differential effects on Na\textsubscript{v}1.7 trafficking remain speculative at this time. Low levels of PTX cause modest microtubule stabilization, while higher concentrations of PTX increase the number of microtubules and cause microtubule bundling. One hypothesis is that different concentrations of PTX interact with microtubule-based kinesin motors differently; PTX binds to the inner lumen of microtubules where it would not sterically interfere with kinesin transport. Indeed, purified microtubules are commonly stabilized using PTX for in vitro assays of motor proteins. However, PTX can affect the posttranslational modifications of tubulin, or the compliment of microtubule associated proteins (MAPs). These modifications can interfere with the affinity of kinesin motors for microtubules, or their ability to navigate the microtubules. Thus, it is possible that low concentrations of PTX, through microtubule stabilization and modification, enable some kinesin-based axonal transport while higher concentrations can disrupt it.
Most studies have reported impaired, rather than enhanced axonal transport after PTX treatment. However, many used micromolar concentrations of PTX where signs of axonal degeneration were also observed.\textsuperscript{10} Using isolated squid axoplasm, a decrease in transport velocity was observed for kinesin-1, but not dynein, after 50 min of incubation with 10 µM PTX.\textsuperscript{37} Another study with cultured mouse DRG axons showed decreased anterograde velocities of mitochondria after exposure to 25 nM PTX for one day, or 2.5 µM PTX for 6-9 hrs.\textsuperscript{11} Our observations of increased vesicle velocities after incubation with similar PTX concentrations suggest that transport of organelles and transport of vesicles may be differentially altered in response to PTX treatment. This could be due to the involvement of molecular motors that have different sensitivities to alterations in microtubule stability or posttranslational modifications. The observed changes in mitochondrial transport\textsuperscript{11} treated with a wide range of concentrations of PTX (25 nM to 2.5 µM) did not correlate well with the axonal degeneration. Our data show that Na\textsubscript{V}1.7 is transported to distal axons even in the context of axonal degeneration (presence of bulbed axonal ends), especially in the presence of inflammatory mediators, the clinically-relevant condition in chemotherapy.

The use of the OPAL imaging technique to observe Na\textsubscript{V}1.7-containing vesicles with single-molecule resolution\textsuperscript{12} enabled the first real-time, live-cell visualization of altered ion channel trafficking after exposure to PTX. This technique capitalizes on the labeling of channels in the somatic chamber and imaging the transported channels in the axonal chamber, which preferentially captures anterograde moving vesicles. We observed enhanced anterograde trafficking under conditions that also demonstrated increased surface levels of Na\textsubscript{V}1.7 at axonal endings which suggested that this increased trafficking is linked to the increased surface expression. However, these experiments do not rule out the contributions of decreased retrograde trafficking or altered surface stability of membrane-inserted Na\textsubscript{V}1.7 at axonal endings. Alternate labeling and imaging paradigms will need to be used to address retrograde trafficking and protein turnover at axonal endings.

Our results suggest that DRG neurons regulate Nav1.7 expression on multiple levels in response to PTX. Treatment with 25 nM PTX increased mRNA levels and current density of endogenous Nav1.7 in DRG neurons, which is consistent with a previous report\textsuperscript{6}. Our data, together with the increased protein levels of Nav1.7 shown in Li et al., suggest that increased Nav1.7 protein after
PTX treatment contributes to increased somatic surface levels of Na\textsubscript{V}1.7. In contrast to the endogenous Na\textsubscript{V}1.7 current, PTX treatment did not increase the current density produced by transfecting DRG neurons with the Halo-Na\textsubscript{V}1.7 channels. Although the reasons for this difference in the response of endogenous and Halo-Na\textsubscript{V}1.7 channels are not well understood, we interpret our data to suggest that the increased axonal vesicular trafficking and surface channels which we observed by live imaging for Halo-Na\textsubscript{V}1.7 might be independent of increased overall protein levels of the channel. Further experiments will be needed to fully determine the contributions of transcription, translation, trafficking, and protein turnover of Na\textsubscript{V}1.7 to the PTX-induced pathology \textit{in vivo}.

Paclitaxel induced peripheral neuropathy has been shown to involve the activity of several additional ion channels including T-type voltage-gated calcium channels and transient receptor potential channels TRPV1, TRPA1, and TRPV4\textsuperscript{38-40}. It is probable that these and other ion channels, including other Na\textsubscript{V}1 isoforms, would also show similar enhanced anterograde trafficking and increased surface levels at axon endings. We previously demonstrated that Na\textsubscript{V}1.7 is transported in Rab6A-containing vesicles\textsuperscript{12} and future studies will determine whether other Na\textsubscript{V}1 isoforms or other ion channels would be transported in overlapping or distinct vesicle populations and whether they would be differentially regulated by PTX.

Paclitaxel has been shown to trigger inflammation in patients receiving PTX\textsuperscript{28, 29, 41-44}. To make the culture system more clinically-relevant, we mimicked the inflammatory milieu in the peripheral nerves in patients receiving PTX by treating the cultures with an inflammatory mediator cocktail\textsuperscript{15}. Thus, while cultured neurons have limitations because this model does not recapitulate the complex cellular interactions of the in vivo environment, our studies nevertheless provide an unprecedented view of the dynamic regulation of channel trafficking and surface expression of Na\textsubscript{V}1.7 under conditions that recapitulate multiple aspects of CIPN. We demonstrate here that IM can cause an increase in surface levels of Na\textsubscript{V}1.7 and vesicular trafficking above and beyond the modifications observed for PTX treatment alone. The experiments presented here are not able to determine whether the increased trafficking and surface expression seen for PTX and IM are due to overlapping pathways. However, these data provide insight into how axons respond to the combination of PTX and inflammation, which occurs in human patients. Additionally, this suggests that some of the off-target effects of PTX chemotherapy treatment may not be due directly
to modifications of microtubules, but additionally are the effect of the accompanying inflammatory response which may exacerbate the direct effects of the chemotherapeutic agents on neurons.

There is a pressing need for a better understanding of CIPN associated with agents such as PTX. We have shown in this study that vesicular flux and velocity in distal axons are significantly increased upon treatment with 25 nM PTX. This data may reflect an early effect of the treatment when the cumulative dose is still relatively low. The accumulation of the Na$_V$1.7 channels at the surface of distal axons during the early phase of treatment may contribute to the spontaneous activity of nociceptors that has been previously reported. Even in the event of distal axon degeneration, the increased surface levels of Na$_V$1.7 channels may continue to support ectopic firing, leading to pain and paresthesias that are associated with PTX treatment, analogous to the proposed role of this channel in spontaneous firing within painful neuromas. These data suggest that when determining dosing regimens for chemotherapy treatments, it is important to identify both peak plasma levels, as well as steady state levels of PTX. Our data also suggest that both the concentrations of PTX as well as the inflammatory response need to be addressed when considering treatments for CIPN. Together with the elevated levels of Na$_V$1.7 transcripts and protein that have been demonstrated in human and rodent DRG soma treated with PTX shown here and in the literature, our data demonstrate a previously undescribed phenomenon of enhanced trafficking of Na$_V$1.7 to distal axons driven by both the direct effects of PTX and the chemotherapy-induced inflammatory response.

**Contributions**

E.J.A., S.D.D.-H., S.G.W. and G.P.H. designed the experiments. E.J.A., M.A., and F.B.D-H performed experiments and analyzed data. S.L. and P. Z. provided critical technical support. E.J.A., M. A., S.D.D.-H., and S.G.W. wrote the manuscript. S.D.D.-H, S.G.W, E.J.A., M. A., and G.P.H. revised and edited the manuscript.

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**Competing interests**

The authors report no competing interests.
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Figure legends

Figure 1. Formation of retractions bubs in cultured DRG neurons treated with PTX is concentration and time dependent. DRG neurons expressing soluble EGFP to visualize axonal morphology were cultured in microfluidic chambers and treated with either DMSO (Cntrl), 25nM PTX, or 250nM PTX. A) Example axon endings from the axonal chamber. Control axon endings generally had defined morphologies with protrusions or branches. Cultures treated with PTX showed some axons with bulbed endings (arrowheads), consistent with axonal degeneration. B) Percentage of healthy versus bulbed axonal endings. Bulbed endings were seen more frequently with higher concentrations of PTX, as well as increasing over time of incubation. Number of axons for each condition indicated in white, from 3 independent cultures.

Figure 2. Increased membrane levels of Na\(_{\text{V}}\)1.7 after 25nM PTX treatment. DRG neurons expressing Halo-Na\(_{\text{V}}\)1.7 and soluble EGFP were cultured in microfluidic chambers. A) Schematic of Halo-Na\(_{\text{V}}\)1.7. HaloTag was fused to an extra transmembrane segment such that channels inserted in the plasma membrane can be labeled using cell-impermeable fluorescently-conjugated HaloTag ligands. B) Schematic of neurons cultured in microfluidic chambers where the cell bodies are plated within the somatic chamber and the axons extend through the microbarrier. For surface labeling of axonal endings (shown as red axons), cell-impermeable HaloTag ligand was added to the axonal chamber for 20 min, then excess label washed off. C) Example axons demonstrating relative labeling of surface Halo-Na\(_{\text{V}}\)1.7 by Halo-JF635i under different experimental conditions (left) and the EGFP shows axon morphology (right). Fluorescence intensity of the JF635i is shown by a graded scale with the greatest fluorescence intensity displayed as white and the lowest fluorescence intensity displayed as dark blue. D) Quantification of Halo-Na\(_{\text{V}}\)1.7 surface levels at axonal endings. Box plots of the mean fluorescence intensities of Halo-Na\(_{\text{V}}\)1.7 in axons treated with 25nM PTX are significantly greater than DMSO controls at 24 and 48 hrs. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75\(^{\text{th}}\) and 25\(^{\text{th}}\) percentiles, respectively. The whiskers extend to the maximum and minimum values. Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 31, 31, 19 (6 hrs); 21, 19, 13.
Figure 3. Vesicular trafficking of Halo-Na\(_v\)1.7 containing vesicles is altered after PTX treatment. A) Schematic depicting the OPAL imaging technique used to visualize vesicular trafficking. DRG neurons expressing Halo-Na\(_v\)1.7 were cultured in MFCs for 5-7 days. Cell-permeable JF635i-HaloTag ligand was added to the soma chamber for 15 min, then anterogradely moving vesicles in the axonal chamber were imaged using spinning disk confocal microscopy. B) Example kymographs of line scans along axons from cultures treated for 48hrs. Vesicle movement is displayed as position (x-axis) over time (y-axis). C) Quantification of vesicular flux (number of vesicles passing along each axon per minute). Number of axons analyzed (in order of control, 25 nM PTX, 250 nM PTX): 39, 34, 28 (6 hrs), 24, 22, 16 (24 hrs), 39, 30, 7 (48 hrs). D) Quantification of anterograde vesicular velocity. Vesicle were considered anterograde if they had a velocity >0.1 \(\mu\)m/s. Number of vesicles analyzed (in order of control, 25 nM PTX, 250 nM PTX): 627, 566, 516 (6 hrs), 306, 250, 121 (24 hrs), 438, 502, 68 (48 hrs). The horizontal line indicates the median, while the top and the bottom of the box indicate the 75\(^{th}\) and 25\(^{th}\) percentiles, respectively. The whiskers extend to the maximum and minimum values. * p < 0.05, ** p < 0.01. *** p < 0.001; one-way ANOVA with Bonferroni correction.

Figure 4. Currents produced by endogenous Na\(_v\)1.7 but not Halo-Na\(_v\)1.7 channels increases following treatment with PTX. (A) Family of somatic endogenous Na\(_v\)1.7 current traces evoked by 40 ms depolarizing voltage steps from -80 mV to +10 mV in 5 mV increments from a holding potential of -100 mV. Representative smoothed (10 points averaged) ProTx-II sensitive Na\(_v\)1.7 traces from rat DRG neurons treated with DMSO control (black) and 25 nM PTX (red) neurons are displayed. Traces illustrate the current evoked during the test-pulse and omit the response from the pre-pulse stimulus to inactivate axonal Na\(_v\) currents. (B) Comparison of peak endogenous Na\(_v\)1.7 current between control (black) and PTX treated conditions (red). (C) Comparison of peak Halo-Na\(_v\)1.7 current (TTX-R and ProTX-II sensitive) between control (black) and PTX treated conditions (red). Na\(_v\)1.7 current density was measured by normalizing maximal peak currents with

(24 hrs), 25, 31, 18 (48 hrs). * p < 0.05, ** p < 0.01. *** p < 0.001; one-way ANOVA with Bonferroni correction.
cell capacitance. Scatter plots showing current density of endogenous Na\textsubscript{v}1.7 current (B) and Halo-Na\textsubscript{v}1.7 (C); mean and SEM are indicated.

**Figure 5. Membrane levels of Halo-Na\textsubscript{v}1.7 at axonal endings treated with PTX are increased with treatment with IM.** DRG neurons were transfected with Halo-Na\textsubscript{v}1.7 and cultured in MFCs and treated with DMSO, 25nM PTX, or 250nM PTX alone, or co-treated with IM for 24hrs. Channels in the plasma membrane were labeled using cell-impermeable JF635i-HaloTag ligand. 

A) Example axons showing surface labeling and axon morphology at axonal endings. Fluorescence intensity of the JF635i is shown by a graded scale with the greatest fluorescence intensity displayed as white and the lowest fluorescence intensity displayed as dark blue. 

B) Quantification of the mean fluorescent signal of the distal 60 µm at axonal endings. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75\textsuperscript{th} and 25\textsuperscript{th} percentiles, respectively. The whiskers extend to the maximum and minimum values.

Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 21, 19, 13 (no IM); 25, 26, 16 (+IM). * p < 0.05, ** p < 0.01. *** p < 0.001; Students t-test or Mann-Whiney U-test.

**Figure 6. The velocity and flux of Halo-Na\textsubscript{v}1.7 vesicles treated with PTX are increased with treatment by IM.** DRG neurons were transfected with Halo-Nav1.7 and cultured in MFCs and treated with DMSO, 25nM PTX, or 250nM PTX alone, or co-treated with IM for 24hrs. OPAL imaging was used to visualize axonal transport of Halo-Nav1.7 containing vesicles. 

A) Example kymographs of axons treated for 24hrs with 25nM PTX, either with or without addition of IM. 

B) Quantification of anterograde vesicular flux. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75\textsuperscript{th} and 25\textsuperscript{th} percentiles, respectively. The whiskers extend to the maximum and minimum values. Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 24, 23, 16 (no IM); 24, 21, 10 (+IM). 

C) Quantification of vesicular velocity. Number of vesicles analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 306, 386, 121 (no IM); 567, 506, 167 (+IM). * p < 0.05, ** p < 0.01. *** p < 0.001; Students t-test or Mann-Whiney U-test.
Figure 1. Formation of retractions bubs in cultured DRG neurons treated with PTX is concentration and time dependent. DRG neurons expressing soluble EGFP to visualize axonal morphology were cultured in microfluidic chambers and treated with either DMSO (Cntrl), 25nM PTX, or 250nM PTX. A) Example axon endings from the axonal chamber. Control axon endings generally had defined morphologies with protrusions or branches. Cultures treated with PTX showed some axons with bulbed endings (arrowheads), consistent with axonal degeneration. B) Percentage of healthy versus bulbed axonal endings. Bulbed endings were seen more frequently with higher concentrations of PTX, as well as increasing over time of incubation. Number of axons for each condition indicated in white, from 3 independent cultures.
Figure 2. Increased membrane levels of NaV1.7 after 25nM PTX treatment. DRG neurons expressing Halo-Nav1.7 and soluble EGFP were cultured in microfluidic chambers. A) Schematic of Halo-Nav1.7. HaloTag was fused to an extra transmembrane segment such that channels inserted in the plasma membrane can be labeled using cell-impermeable fluorescently-conjugated HaloTag ligands. B) Schematic of neurons cultured in microfluidic chambers where the cell bodies are plated within the somatic chamber and the axons extend through the microbarrier. For surface labeling of axonal endings (shown as red axons), cell-impermeable HaloTag ligand was added to the axonal chamber for 20 min, then excess label washed off. C) Example axons demonstrating relative labeling of surface Halo-Nav1.7 by Halo-JF635i under different experimental conditions (left) and the EGFP shows axon morphology (right). Fluorescence intensity of the JF635i is shown by a graded scale with the greatest fluorescence intensity displayed as white and the lowest fluorescence intensity displayed as dark blue. D) Quantification of Halo-Nav1.7 surface levels at axonal endings. Box plots of the mean fluorescence intensities of Halo-Nav1.7 in axons treated with 25nM PTX are significantly greater than DMSO controls at 24 and 48 hrs. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75th and 25th percentiles, respectively. The whiskers extend to the maximum and...
minimum values. Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 31, 31, 19 (6 hrs); 21, 19, 13 (24 hrs), 25, 31, 18 (48 hrs). * p < 0.05, ** p < 0.01. *** p < 0.001; one-way ANOVA with Bonferroni correction.
Figure 3. Vesicular trafficking of Halo-NaV1.7 containing vesicles is altered after PTX treatment. A) Schematic depicting the OPAL imaging technique used to visualize vesicular trafficking. DRG neurons expressing Halo-NaV1.7 were cultured in MFCs for 5-7 days. Cell-permeable JF635i-HaloTag ligand was added to the soma chamber for 15 min, then anterogradely moving vesicles in the axonal chamber were imaged using spinning disk confocal microscopy. B) Example kymographs of line scans along axons from cultures treated for 48hrs. Vesicle movement is displayed as position (x-axis) over time (y-axis). C) Quantification of vesicular flux (number of vesicles passing along each axon per minute). Number of axons analyzed (in order of control, 25 nM PTX, 250 nM PTX): 39, 34, 28 (6 hrs), 24, 22, 16 (24 hrs), 39, 30, 7 (48 hrs). D) Quantification of anterograde vesicular velocity. Vesicles were considered anterograde if they had a velocity >0.1 µm/s. Number of vesicles analyzed (in order of control, 25 nM PTX, 250 nM PTX): 627, 566, 516 (6 hrs), 306, 250, 121 (24 hrs), 438, 502, 68 (48 hrs). The horizontal line indicates the median, while the top and the bottom of the box indicate the 75th and 25th percentiles, respectively. The whiskers extend to the maximum and minimum values. * p < 0.05, ** p < 0.01, *** p < 0.001; one-way ANOVA with Bonferroni correction.
Figure 4. Currents produced by endogenous NaV1.7 but not Halo-NaV1.7 channels increases following treatment with PTX. (A) Family of somatic endogenous NaV1.7 current traces evoked by 40 ms depolarizing voltage steps from -80 mV to +10 mV in 5 mV increments from a holding potential of -100 mV. Representative smoothed (10 points averaged) ProTx-II sensitive NaV1.7 traces from rat DRG neurons treated with DMSO control (black) and 25 nM PTX (red) neurons are displayed. Traces illustrate the current evoked during the test-pulse and omit the response from the pre-pulse stimulus to inactivate axonal NaV currents. (B) Comparison of peak endogenous NaV1.7 current between control (black) and PTX treated conditions (red). (C) Comparison of peak Halo-NaV1.7 current (TTX-R and ProTX-II sensitive) between control (black) and PTX treated conditions (red). NaV1.7 current density was measured by normalizing maximal peak currents with cell capacitance. Scatter plots showing current density of endogenous NaV1.7 current (B) and Halo-NaV1.7 (C); mean and SEM are indicated.
Figure 5. Membrane levels of Halo-NaV1.7 at axonal endings treated with PTX are increased with treatment with IM. DRG neurons were transfected with Halo-NaV1.7 and cultured in MFCs and treated with DMSO, 25nM PTX, or 250nM PTX alone, or co-treated with IM for 24hrs. Channels in the plasma membrane were labeled using cell-impermeable JF635i-HaloTag ligand. A) Example axons showing surface labeling and axon morphology at axonal endings. Fluorescence intensity of the JF635i is shown by a graded scale with the greatest fluorescence intensity displayed as white and the lowest fluorescence intensity displayed as dark blue. B) Quantification of the mean fluorescent signal of the distal 60 µm at axonal endings. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75th and 25th percentiles, respectively. The whiskers extend to the maximum and minimum values. Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 21, 19, 13 (no IM); 25, 26, 16 (+IM). * p < 0.05, ** p < 0.01. *** p < 0.001; Students t-test or Mann-Whiney U-test.

76x103mm (300 x 300 DPI)
Figure 6. The velocity and flux of Halo-NaV1.7 vesicles treated with PTX are increased with treatment by IM. DRG neurons were transfected with Halo-NaV1.7 and cultured in MFCs and treated with DMSO, 25nM PTX, or 250nM PTX alone, or co-treated with IM for 24hrs. OPAL imaging was used to visualize axonal transport of Halo-NaV1.7 containing vesicles. A) Example kymographs of axons treated for 24hrs with 25nM PTX, either with or without addition of IM. B) Quantification of anterograde vesicular flux. The horizontal line indicates the median, while the top and the bottom of the box indicate the 75th and 25th percentiles, respectively. The whiskers extend to the maximum and minimum values. Number of axons analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 24, 23, 16 (no IM); 24, 21, 10 (+IM). C) Quantification of vesicular velocity. Number of vesicles analyzed (in order of control, 25 nM PTX, and 250 nM PTX): 306, 386, 121 (no IM); 567, 506, 167 (+IM). * p < 0.05, ** p < 0.01. *** p < 0.001; Students t-test or Mann-Whiney U-test.

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