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Group IV nociceptors develop axonal chemical sensitivity during neuritis and following treatment of the sciatic nerve with vinblastine

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Abstract

We have previously shown that nerve inflammation (neuritis) and transient vinblastine application lead to axonal mechanical sensitivity in nociceptors innervating deep structures. We have also shown that these treatments reduce axonal transport, and proposed that this leads to functional accumulation of mechanically sensitive channels in the affected part of the axons. While informing the etiology of mechanically induced pain, axonal mechanical sensitivity does not address the common report of ongoing radiating pain during neuritis, which could be secondary to the provocation of axonal chemical sensitivity. We proposed that neuritis and vinblastine application would induce sensitivities to noxious chemicals, and that the number of chemosensitive channels would be increased at the affected site. In adult female rats, nerves were either untreated, or treated with complete Freund’s adjuvant (to induce neuritis) or vinblastine. After 3-7 days, dorsal root teased fiber recordings were taken from Group IV neurons with axons within the sciatic nerve. Sciatic nerves were injected intraneurally with a combination of noxious inflammatory chemicals. While no normal sciatic axons responded to this stimulus, 80% and 38% of axons responded in the neuritis and vinblastine groups, respectively. In separate experiments, sciatic nerves were partially ligated and treated with complete Freund’s adjuvant or vinblastine (with controls), and after 3-5 days were immunolabeled for the histamine 3 receptor. The results supported that both neuritis and vinblastine treatment reduce transport of the histamine 3 receptor. The finding that nociceptor axons can develop ectopic chemical sensitivity is consistent with ongoing radiating pain due to nerve inflammation.
New & Noteworthy

Many patients suffer ongoing pain with no local pathology or apparent nerve injury. In this manuscript, we show that nerve inflammation and transient application of vinblastine induce sensitivity of Group IV nociceptor axons to a mixture of endogenous inflammatory chemicals.

We also show that the same conditions reduce the axonal transport of the histamine 3 receptor. The results provide a mechanism for ongoing nociception from focal nerve inflammation or pressure without overt nerve damage.
Keywords

Histamine 3 receptor

Neuritis

Radicular pain

Radiating pain

Neuropathic pain
Introduction

Many patients with limb pain have no detectable pathology associated with their painful area, and no overt nerve pathology. Typical presentations include pain at rest and pain evoked by movements, especially movements that also move the nerves that innervate their painful area, implying altered neuronal function. These symptoms accompany a broad spectrum of disorders, such as back pain and lower limb pain with radiculopathy (Bove et al. 2005; Waddell 1987), upper limb pain with neck injuries, thoracic outlet syndrome, and radiation plexopathy (Christo and McGreevy 2011; Greening et al. 2005; Olsen et al. 1993), and endometriosis (Dhote et al. 1996; Missmer and Bove 2011).

Normal sensory neurons transmit information from their target organ to the central nervous system, but previous work from our laboratory has demonstrated that nerve inflammation, or neuritis, leads to axonal mechanical sensitivity of Group IV nociceptor axons, which are not normally mechanically sensitive (Bove et al. 2003; Dilley and Bove 2008b; Dilley et al. 2013). This is consistent with movement-induced radiating pain. However, this sensitivity does not address ongoing pain without movement arising from fully intact nerves, which suggests ectopic chemical sensitivity. Normal axons were reported to be insensitive to inflammatory mediators (Zimmermann and Sanders 1982), but sensitive to tumor necrosis factor-α in untreated rats (Leem and Bove 2002; Sorkin et al. 1997). There are no published data on chemical sensitivities of axons exposed to a pathological environment.

The present study expands on previous work from our laboratory that has examined inflammation- and vinblastine-induced axonal mechanical sensitivity (Bove et al. 2003; Dilley and Bove 2008a; Dilley et al. 2013), by investigating the possibility that these conditions induce axonal chemical sensitivity. We tested this hypothesis by applying endogenous noxious
chemicals to normal axons and to axons previously treated with complete Freund’s adjuvant (CFA) or vinblastine. We also tested the hypothesis that CFA and vinblastine alter the amount of receptors for histamine, which was included in the noxious chemical combination. Our results demonstrate that C-fibers of Group IV nociceptors can develop ectopic chemical sensitivity, which is likely associated with ongoing deep radiating pain, and that the same treatments affect the amount of the histamine H3 receptor.

Methods
Experiments were carried out in strict accordance with the Animal Care and Use Committee of the University of New England. A total of 40 adult female Sprague Dawley rats (175-250g, Charles River Laboratories, Wilmington, MA) were used in this study. Female rats were for consistency with previous reports, because of the subjective perception that there is less connective tissue within the dorsal roots, and because there has been no difference found between sexes in our studies in similar parameters (Bove et al. 2003; Dilley and Bove 2008a; Dilley et al. 2013).

Dorsal Root Recordings
Surgery. Neuritis induction and vinblastine treatment were the same as previously published (Bove et al. 2003; Dilley and Bove 2008a). Rats were anesthetized with isoflurane in pure oxygen. The fur over the left posterior thigh was clipped, the skin cleaned with surgical scrub, and the area draped with sterile plastic. A small incision was made posterior to the femur, and the muscles separated to expose the sciatic nerve, which was then cleared of connective tissue for ~10 mm using only epineurial fascia to mobilize the nerve. In some animals, a 4 X 4 X
10 mm piece of GelFoam saturated with ~150 µl CFA (Sigma, emulsified 1:1 with sterile buffer) was gently wrapped to surround the nerve. In other animals, a cone was formed with Parafilm and placed around the nerve prior to placing cotton wool soaked in 0.1 mM vinblastine in sterile buffer around the nerve (Dilley and Bove 2008a; Fitzgerald et al. 1984). After 15 minutes, the vinblastine was removed and copiously rinsed with sterile buffer. The incision was closed in layers with 4-0 nylon sutures, and the rats moved to a clean cage for recovery. Because we have not seen differences between surgical sham and unoperated groups using these methods (Bove et al. 2003; Dilley and Bove 2008b), and because we have two distinct procedures, we used unoperated rats as controls. Because vinblastine is anti-inflammatory (Norris et al. 1977), it can be considered a control group for the inflammation caused by the application of CFA.

*Electrophysiology and experimental protocol.* Rats were anesthetized to areflexia with isoflurane and prepared for electrophysiology 3-4 days following vinblastine treatment or 6-7 days following the induction of neuritis. These endpoints were chosen because they are when the agents show their greatest effects and to be consistent with our previous reports (Dilley and Bove 2008a; b; Dilley et al. 2013). Body temperature was maintained at 37°C using a feedback controlled thermal pad with a rectal probe (FCH-Inc.) and a circulating warm water flexible pad (Gaymar) folded over the upper body. Electrophysiological methods and neuronal isolation were performed as previously described (Fig. 1; also see Bove et al. 2003). A laminectomy was performed from L2 - L5 to expose the spinal cord. The skin was glued to a metal ring and the pool filled with 37°C mineral oil. The dura mater was incised and the L5 and L4 dorsal roots were cut at the dorsal root entry zone. Dorsal roots were draped over a bipolar stimulating electrode and placed on a small glass plate. Fine filaments (~6-12 µm) were separated from the dorsal roots using honed forceps, and draped over a bipolar recording electrode made with fine
gold wires. The distance between the stimulating and recording electrodes ranged from 11-14 mm. Electric stimuli were delivered to the dorsal root at intensity suprathreshold for C-fiber activation (0.05-0.1 ms, 20-30V) using an isolated constant-voltage stimulator (Grass, USA) to identify neurons and determine conduction latencies. Action potentials were amplified, band-pass filtered (50–5,000 Hz), and monitored with an oscilloscope. Neuronal activity was digitized and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, United Kingdom) for off-line analysis. Conduction velocities were determined by dividing the conduction distance by the latency of individual units. When clear single-neuron waveforms were obtained, receptive fields (RFs) in deep structures (Group IV) were searched for distal to the knee using noxious stimuli applied with the fingers and/or forceps. Cutaneous RFs were distinguished from deep RFs by using forceps to carefully maneuver and pinch the skin only, as previously described (Bove and Light 1995). If the responsiveness moved with the skin, it was concluded that the RF was within the skin. Only Group IV neurons with high threshold RFs were recorded further (Figs. 2A - B). Group III neurons are far less common when recording from the dorsal roots (Bove et al. 2003) and were not encountered. Neurons with cutaneous RFs were not characterized, as they have shown to not develop axonal mechanical sensitivity.

In early experiments, we determined that the latency in response to topical application of “inflammatory soup” [IS; bradykinin, serotonin, histamine, and prostaglandin, all at 10^{-5} M in observation medium (OM; Light et al. 2008)] was 30-60 minutes. We attributed this to the diffusion barrier and positive intrafascicular pressure that the perineurium presents (Peltonen et al. 2013). We attempted to disrupt this barrier using sodium deoxycholate (Todd et al. 2000a; b), but this did not reduce the response latency. Because these recordings are often time-limited, we chose to inject substances subperineurally using a 30 ga bent needle.
Once peripheral RFs were identified, the following protocol was followed:

1) Recording of ongoing activity for 5+ minutes;

2) Mechanical stimulation of the RF;

3) Exposure and mechanical stimulation of the nerve, accomplished by pressing the nerve between a silastic probe and a hooked spatula;

4) Subperineural injection of 100-150 µl of OM into the nerve with 5+ minutes recording;

5) Subperineural injection of 100-150 µl of IS into the nerve with 5+ minutes recording;

6) Injection of IS into the distal mechanically sensitive RF with 5+ minutes recording.

The mechanical stimulation of the nerves was previously described in detail (Bove et al. 2003). In brief, forces of 4 N can be applied with this probe; in these experiments as in previous experiments the forces used on the nerve were limited to 2 N or less (measured earlier on an electronic scale), on a footprint of 6–8 mm. As in our previous studies, using the probes in this manner does not interrupt conduction of action potentials from axons in passage, confirmed by mechanically activating the natural RF following nerve stimulation. When the ectopic mechanically sensitive RF responds, it does so similarly to the natural RF. These observations confirm that the responses are not injury discharge.

Because it is unknown whether previous exposure to noxious chemicals would induce sensitivity changes, we limited our data collection to the first Group IV neurons that were appropriately identified. This primarily resulted in 1 neuron per experiment; however in 3 experiments, 2 neurons with distinctively identifiable waveforms were recorded from the same filament.
Immunohistochemistry

*Model and Surgery.* We hypothesize that slowing the flow of channels and receptors will facilitate functional expression on the axons. We previously reported that partial tight ligation of the sciatic nerve led to kinesin accumulation, and that this accumulation was attenuated during neuritis and by vinblastine application (Dilley et al. 2013). These observations supported the prediction that these treatments reduce fast axoplasmic flow. We repeated this approach to evaluate kinetics of the histamine H3 receptor (H3R). Sixteen rats were operated on as above, using 4 rats per group (CFA and control, vinblastine and control). During each surgery, we added a partial (~50%) tight ligation of the sciatic nerve, using 7-0 nylon suture. Non-ligated axons were present in all nerves, and served as a control, though not used as a comparison group.

CFA and vinblastine rats had the agent applied as described above to cover 8 - 10 mm of the nerve proximal to and including the ligation. Control rats had their sciatic nerve partially ligated with no other procedure performed.

After 3 days (vinblastine) and 6 days (CFA), treated and control rats were terminally anesthetized with sodium pentobarbital (200 mg/kg, intraperitoneally) and perfused transcardially with heparinized 0.1 M phosphate-buffered saline. The affected segments of their sciatic nerves were removed and immersion-fixed in 4% paraformaldehyde for 4 hours while pinned straight, and then placed in 30% sucrose for cryoprotection. Nerves were sectioned longitudinally at 10 µm using a cryostat and processed for H3R immunoreactivity using standard methods. Histamine 3R was chosen because histamine is known to be involved in nociceptor activation and sensitization (Mense and Schmidt 1974; Pongratz et al. 2002; Zhang et al. 2007) and a well-characterized antibody was available. Sections were incubated in rabbit anti-H3R
antibodies [Sigma Aldrich H7038, 30 μg/ml (Cannon et al. 2007; Chen et al. 2015)] overnight at 4°C, rinsed, and incubated with donkey anti-rabbit IgG DyLight 488 (Jackson Immunoresearch, 711-485-152, 1:200) for 60 min at room temperature. The pancreas was used as a positive control for the anti-H3R (Nakamura et al. 2014); sections incubated without the primary antibody displayed no positive signal.

**Imaging.** Sections were photographed and montaged at 20X using a Nikon upright microscope fitted with Prior motorized stage and a Ds-Qi1 MC camera, both controlled by NIS Elements (Nikon). A background correction image was taken using the appropriate fluorescent slide (Chroma USA) and was applied to all images.

**Image Analysis.** Montaged images were renamed using random numbers (by GMB) and analyzed in one session by the same person (by RMG), using NIS Elements. A region of interest (ROI) was defined as the part of the ligated nerve starting 20-30 μm proximal to the ligation and extending another 500 μm proximally (Fig. 3xx). ROIs were analyzed from 4-6 sections per nerve. The positive signal within the ROI was subjectively determined using the thresholding tool within NIS Elements (Fig. 3A), used to select pixels of light intensities that matched those chosen by the experimenter as “positive.” We measured the fraction of the total ROI area that was above threshold for each section, and refer to it as the “H3R signal.”

**Data Analysis**

Data were analyzed using GraphPad Prism 7 and expressed as Mean ± SEM unless otherwise noted, with statistical significance set at ≤ 0.05. Conduction velocities were analyzed using a one-way ANOVA. Proportions of axonal mechanical and chemical sensitivities, and ongoing activity, were analyzed using Chi-square (χ²) tests. The H3R signal in treatment and control groups were compared using un-paired t-tests.
Results

Dorsal root recordings were obtained from a total of 27 neurons in 24 rats (9 naive rats, 9 CFA treated rats, and 6 vinblastine treated rats). There was no difference in conduction velocities by group (F3, 24 = 0.77, p = 0.8). The mean conduction velocity for all recorded neurons was 0.83 m/sec ± 0.20 (SD; Table 1). All neurons had a non-cutaneous mechanically responsive RF in the lower limb or foot (Fig. 2B).

Receptive field responses to IS. Sixty-six percent of normal neurons responded to injection of IS directly into their mechanically sensitive RFs (Table 1 and Fig. 2D). Following treatment with CFA or vinblastine, 29% and 50% responded to IS injection into their RFs, respectively. There was no statistical difference between these proportions. These results are consistent with previous reports of cutaneous C-fibers (Kessler et al. 1992) and muscle nociceptors (reviewed by Mense 2009). The discharge patterns were similar among the groups, consisting of a mechanical artifact or response of the axon due to the needle insertion and pressure of the fluid, followed by a true response (Fig. 2D). The response latencies and durations were highly variable among groups, ranging from 0.5 – 171 sec and 16 – 143 sec, respectively.

Axonal chemical sensitivity during neuritis and following transient application of vinblastine. Following treatment with CFA or vinblastine, 80% and 38% of axons, respectively, responded to injection of IS (Table 1 and Figs. 2E and G). No normal axons or treated axons responded to intraneural OM, and no normal axons responded to intraneural IS (Fig. 2B). The proportions of responsive axons differed significantly by group [$\chi^2$ (2, n = 26) =11.51, p = 0.005]. The discharge pattern to IS varied, with latencies ranging from 6 to 88 seconds. The duration of the responses to IS also varied, lasting from 16 to 148 sec.
Axonal mechanical sensitivity. Axonal mechanical sensitivity was not present in any normal axons, but was present in 33% and 50% of axons treated with CFA or vinblastine, respectively (Table 1 and Fig. 1D). While the contingency test with these data was not statistically significant [$\chi^2 (2, n = 25) = 5.16, p = 0.08$], there was a statistically significant trend towards more axonal mechanical sensitivity in treated axons [$\chi^2 (1, n = 25) = 11.51, p = 0.03$], and the proportions are consistent with our previous reports (Bove et al. 2003; Dilley and Bove 2008a; b). The normal RFs of all these neurons were responsive to noxious mechanical stimulation (Fig. 2C). There was no sustained discharge after the mechanical stimuli were removed (Figs. 2C and D).

Ongoing activity. Few neurons in this series of experiments had ongoing activity (1 of 9 control, 2 of 10 during neuritis, and 0 of 8 after vinblastine treatment). There were no statistical differences between the proportions, which is consistent with our previous reports (Bove and Dilley 2010; Dilley and Bove 2008a).

Neuritis and vinblastine reduce H3R transport. In sections of sciatic nerve that underwent partial ligation with no treatment, there was a robust accumulation of the H3R proximal to the ligation (Fig. 3B). In sections of sciatic nerve that underwent partial ligation and treatment with either CFA or vinblastine, a reduction in the accumulation of H3R was clearly visible (Figs. 3C - E). When quantified, the differences were statistically significant for the vinblastine experiment ($p < 0.05$; Fig 3E). This reduction of accumulation is indicative of reduced axoplasmic flow induced by the treatments.
Discussion

Using single neuronal recordings, we have demonstrated that while normal Group IV nociceptor axons are chemically insensitive, CFA-induced neuritis and treatment with vinblastine induce ectopic axonal sensitivity to chemicals normally found in the inflammatory milieu (bradykinin, histamine, serotonin, and prostaglandin). We have also shown that these same conditions impair the axonal transport of H3R.

We chose the combination and concentration of chemicals for our IS because many studies have been published using this combination (Becerra et al. 2017; Kessler et al. 1992; Lang et al. 1990). The initial descriptions of this combination (Steen et al. 1995) were based on concentrations found in various tissues, and the chemicals were applied to skin nociceptors. We do not know the concentrations and proportions of these chemicals that are present in the CFA model. Our observations of little to no ongoing activity in the CFA model but consistent responses to intraneural application of IS suggests that the concentrations of these chemicals inside the nerve are too low to evoke activity. Our combined observations remain consistent with the concept that inflammatory mediators are not required to lead to ectopic sensitivities, but that reduced axonal transport, such as induced by vinblastine, is sufficient.

While others and we have shown that CFA-induced neuritis reduces axonal transport (Armstrong et al. 2004; Dilley et al. 2013), there remains limited insight on the mechanism of this phenomenon. Our previous studies (Bove et al. 2003; Dilley and Bove 2008a) and the current report show a parallel between axonal transport and ectopic sensitivities, but are methodologically unable to directly correlate the phenomena. Our reports of similar effects of neuritis and vinblastine on kinesin transport suggest a common mechanism of reduced axonal transport, independent of the effects of inflammation, especially since vinblastine is anti-
inflammatory (Norris et al. 1977). While we hypothesize that reduced axonal transport facilitates the insertion of functional receptors into the axonal membrane, such as H3R, we cannot test this prediction using the methods presented here.

Clinical Implications. We have shown that neuritis and transient vinblastine application lead to ectopic axonal sensitivity to a mixture of endogenous chemicals. This finding has possible clinical diagnostic relevance for cases presenting with ongoing radiating pain, especially when combined with our previous reports of ectopic axonal mechanical sensitivity induced by the same means (Bove et al. 2003; Dilley and Bove 2008a). Inflammation and mechanical pressure are interrelated (Schmidt et al. 2013), reduce axonal transport (Armstrong et al. 2004; Dahlin et al. 1984; Dilley et al. 2013; Gallant 1992), and can result in ectopic mechanical and, as we have shown here, chemical sensitivity. This could manifest as movement-induced and ongoing nociception, respectively. The site of mechanical pressure can often be appreciated with current diagnostic imaging methods, but similar methods are limited in terms of revealing neuritis. Clinicians can use “neurodynamic tests” that have been designed to specifically move and tension the major nerves of the limbs (Butler 2000; Shacklock 2005), and have shown moderate reliability in identifying the involved nerve by reproducing the presenting symptoms (Greening et al. 2005; Schmid et al. 2009). Palpation of an involved nerve is relatively straightforward and can lead to the identification of the site of pathophysiology, again by reproducing the symptoms (Greening et al. 2005; Schmid et al. 2009). In cases of deep radiating pain, clinicians are advised to search for areas along the entire path of the involved nerve for tenderness, which could identify a site of inflammation and lead to an accurate diagnosis.
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References


**Figure 1. Experimental schematic and nociceptor isolation.** A. Recording methods schematic. The sciatic nerves were treated with complete Freund’s adjuvant (CFA) or vinblastine (shaded portion of nerve) and recordings were later performed through the dorsal root from singularly identifiable Group IV nociceptors. The key characterization in this experiment was to inject the nerve with a combination of inflammatory mediators and evaluate ectopic sensitivities. Injections and mechanical stimuli were delivered within the treated zone. B. To identify that a receptive field is from a specific neuron, the dorsal root was electrically stimulated (A) while the receptive field (RF) was mechanically stimulated. When the electrical stimulus occurs during the refractory period of the axon, it fails to evoke an action potential (arrow). C. Noxious mechanical stimulation of a Group IV neuron’s RF, applied using the fingers (arrowheads are application and removal of the stimulus). D. Mechanical stimulation of the sciatic nerve using a soft silicone probe during neuritis, at the treated site, evoking a response from the axon of the neuron being recorded.

**Figure 2. Responses of Group IV nociceptors to chemical stimuli.** Representative responses of Group IV nociceptors to chemical stimuli of their natural RFs (A, C, and F) and of their axons passing through the sciatic nerve (B, E, and G). A and C show a robust response to inflammatory mediators injected into the RF, but not when applied to the axon of the same neuron. C and E (CFA), and F and G (vinblastine), show responses to injections to both RFs and their axons. D. Response to vehicle injection into the nerve of a mechanically sensitive axon. Note immediate response rather than the delay in E. There were no responses in C-F due to the injections after the time frames of the graphs.
Figure 3. Immunohistochemical quantification method and results. A. Regions of interest consisted of the area of nerve between 20-30 μm (small arrow) and 500 μm proximal (large arrow) to the sutures (*). B. Same image as A, showing histamine 3 receptor (H3R) receptor signal 5 days after ligation surgery. C and D. Representative samples from inflamed and vinblastine-treated nerves, respectively showing little H3R signal. ** = unligated axons exposed to vinblastine, showing a relative lack of signal. Scale bar (for all panels) = 100 μm. E. Nerves treated with either CFA or vinblastine showed less H3R signal than non-treated nerves. CON = control nerves (ligated but not treated), n = 4 per group.
Table 1. Axonal Chemical Sensitivity develops following induction of Neuritis. CV = conduction velocity (m/sec). AMS = axonal mechanical sensitivity. IS in RF = injection of inflammatory soup into the identified peripheral receptive field. ACS = axonal chemical sensitivity. OA = ongoing activity. * = neuron stopped responding prior to stimulus.

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