Inflammation Induces Ectopic Mechanical Sensitivity in Axons of Nociceptors Innervating Deep Tissues

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INTRODUCTION

Primary sensory neurons are considered to “sense” only at their endings, within the structure they innervate. Sensory quality and localization are normally initiated by the activation of modality-specific sensory transducers in the tissue being stimulated, resulting in action potentials that are carried by axons, bundled in nerves, to the CNS. However, in many human patients, movements of intact, apparently uninjured nerves far from the innervated tissue can elicit radiating pain (e.g., “sciatica”). Such movement-induced radiating pain could be explained by the induction of ectopic sensory function along the axon. Whereas cut axons of injured nerves seem to regain their original sensory function at their tips (Koschorke et al. 1998; Zager et al. 1998), reports of mechanical responses of intact sensory axons seem to be limited to discharge unrelated to stimulus intensity (Eliav et al. 1999). Injury discharge (Wall et al. 1974), and conductance changes (Julian and Goldman 1962). Furthermore, these observations are limited to invertebrate axons and to rapidly conducting axons that normally give rise to the sensation of touch, not pain. There is no report addressing the possibility of mechanical transduction in intact mammalian nociceptor axons, which normally subserve the sensation of pain. Yet, this possibility could explain the vast number of cases of radiating pain in the absence of overt nerve injury (Laslett et al. 1991; Loeser 2001; Riihimaki et al. 1994), common among patients with diseases such as back and neck pain, compressive neuropathies, diabetes, and endometriosis (Dyck et al. 2000; Loeser 1985; Waddell 1987; Woertgen et al. 1998; Zager et al. 1998).

Models of neuritis have recently been developed that evoke an immune-mediated inflammation (Eliav et al., 1999; Chacur et al., 2001) but do not cause disruption of the axons. Using extracellular recording techniques in a model of neuritis, we now report that inflammation of intact axons leads to axonal mechanical sensitivity. We found that this phenomenon was limited to slowly conducting axons innervating noncutaneous structures. We also found that the time course of the axonal mechanical sensitivity was not the same as the time course of the hypersensitivity that occurs with neuritis.

METHODS

All experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Neuritis model

Rat sciatic nerves were inflamed using complete Freund’s adjuvant (CFA) placed on the nerve 1 to 32 days before electrophysiological recordings, as previously described (Eliav et al. 1999). Left sciatic nerves were exposed in the mid-thigh of barbiturate-anesthetized male Wistar rats (225–250 g). Nerves were freed from underlying tissue for 6–7 mm and a 5 × 5 × 10-mm piece of absorbable gelatin sponge saturated in the test compound [CFA or incomplete Freund’s adjuvant (IFA), approximately 150 µl] was wrapped around the nerve. The dose of mycobacterium tuberculosis in the CFA experiments was 75 µg. This resulted in a robust inflammation that started within hours, was fully established in days, and caused residual abnormal gross morphology for ≥2 mo. Control experiments were performed on previously unoperated animals and 7 days after application of IFA to the nerve. Treatment with IFA did not lead to gross inflammation of the nerve at any time point.

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Electrophysiology

Single-unit recordings were made from subdivided dorsal rootlets to isolate neurons with axons passing through the inflamed sciatic nerve (Fig. 1A). One to 32 days after neuritis induction, animals were anesthetized with urethane, and a lumbar laminectomy was performed to expose the contents of the spinal column. The L5 dorsal root was cut close to the dorsal root entry zone and placed over a bipolar stimulating electrode. Single shock electrical stimuli of the dorsal root using increasing intensity (≤30 V, 0.05 ms; <0.5 Hz) were used to identify C- and Aδ-axons. Because electrical stimuli of this intensity causes electrical blockage of axons with faster conduction velocities, recordings of neurons with Aδ-axons were performed in separate experiments. In these experiments noxious and innocuous mechanical stimuli of the foot and calf were used to initially identify the neurons, followed by electrical stimulation of the sciatic nerve to assess isolation. Only filaments containing clearly identifiable single waveforms were studied.

Receptive fields (RFs) for isolated neurons were located using pinching stimuli of the lower limb and foot using forceps and fingers. The loose property of skin was exploited to carefully discriminate cutaneous versus deep RFs. Cutaneous neurons had RFs that remained associated with the skin regardless of the skin excursion. If the neuron responded to pinching a fold of skin, and maintained similar responsiveness when this fold was displaced, it was concluded that the RF was associated with the skin. Deep RFs were identified through the skin and proved by moving the overlying skin and repeating the effective stimulus to the same underlying spot, through a different portion of skin. Neurons were included for study if they had mechanically sensitive RFs in the lower limb located distal to and remote from the exposed part of the sciatic nerve. Deep and/or cutaneous neurons were recorded in each experiment. Data from neurons with unclear RFs were not collected. After determining the location of the RF, the sciatic nerve was electrically stimulated immediately distal to the neuritis granuloma to determine whether the distal axon of the recorded neuron traveled through the inflamed nerve. The conduction velocity of the axon was used to classify the axon as C, Aδ, or Aβ (Lawson et al. 1997). For C- and Aδ-axons, the mechanically and electrically evoked action potentials were determined to be the same by stimulating the RF mechanically while stimulating the axon electrically. When the electrical stimulus occurred during the relative refractory period of the axon, it would not result in an action potential (Fig. 1B). For Aβ-axons the mechanically and electrically evoked action potentials were compared by visual inspection of overlying traces on the oscilloscope. Data were collected and further analyzed off-line using a PC and waveform discrimination software (Forster and Handwerker 1990).

Mechanical stimulation of the sciatic nerve

It was critical in these experiments to prevent axonal damage and retain electrical through-conduction of the axons being tested. Mechanical stimulation was manually applied to the nerve in a semi-quantitative manner using stimulators with 5 × 10-mm conical tips (Fig. 1A). The contacting tips were molded from silicone (Sylgard 184, Dow Corning; durometer 50). While one probe provided backing, another was pressed onto the nerve. The experimenter controlled the amount of force applied, and forces were presented only relative to other forces used in the particular experiment. Forces of ≤4 N can be applied with this probe; in these 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 mm². In preliminary studies, using the probes in this manner activated nervi nervorum but did not elicit nor interrupt conduction of action potentials from axons in passage.

Pain behavior

To assess whether sciatic neuritis caused paw hypersensitivity, and to determine the time course of these changes, a graded, noxious stimulus was applied to the soles of the hindpaws. The methods were previously described (Wallas et al. 2003). Briefly, a 0.1-mm wire was attached perpendicular to a calibrated spring gauge (Correx, Bern, Switzerland). Rats were placed in an enclosure with a perforated metal floor, and the wire was advanced onto the glabrous skin of the foot. The force necessary to cause a withdrawal was recorded 5 times per foot, bilaterally, at 1-min intervals. There was never any other behavior before the consistently brisk withdrawal. Eleven animals were tested before application of CFA and periodically to 21 days postoperatively. Differences between operated and unoperated sides were compared using paired t-test. All animals were subsequently used for electrophysiology.

Immunocytochemistry

Sciatic nerves were harvested bilaterally 7–8 days after neuritis induction. Barbital-anesthetized animals were transcardially perfused with buffered saline and the sciatic nerves were harvested, flash frozen in isobutane, and sectioned at 8 μm using a cryostat. Sections were fixed with 4% paraformaldehyde for 8 min, and rinsed with PBS. Some sections were stained with H&E. Sections for immunocytochemistry were rinsed in immunohistostaining chambers (Thermo Shandon) 5 times over 20 min in PBS, blocked for 1 h with 4% normal goat serum, 0.3% Triton X-100, and 20 mg/ml bovine-globulin in PBS, and incubated with the primary antibody (ED1 [Serotec] or...
TCRβ (BD PharMingen) at room temperature overnight. Slides were rinsed 5 times over 20 min and incubated for 1 h in the dark with the secondary antibody [Cy3 (Chemicon) or FITC (Jackson Immunoresearch)], rinsed 5 times over 20 min, dried, and coverslipped with Prolong (Molecular Probes). Digital images were collected on a Nikon E600 fluorescence microscope fitted with a SPOT RT camera.

**RESULTS**

**Neuritis-induced mechanical sensitivity limited to the axons of deep nociceptors**

A total of 194 neurons with mechanically sensitive RFs were recorded in 41 experiments, at various time points postoperatively. In unoperated and IFA control experiments, mechanical stimulation of the sciatic nerve along the inked portion was not effective in eliciting action potentials from through-conducting axons, regardless of the tissue innervated or conduction velocity (1/72; Table 1). The stimulus was also ineffective during CFA neuritis for all neurons with cutaneous RFs or Aαβ-axons (0/128). In contrast, the axons of 45/111 (41%) of the neurons with noncutaneous musculoskeletal RFs were mechanically sensitive over 1–3 mm of the nerve within the granuloma (Table 1). The RFs of all these neurons responded preferentially to mechanical stimulation that was judged by the experimenter to be noxious. The proportions of mechanically sensitive axons of neurons with C- and Aδ-axons (37/92) and Aδ-axons (8/19) were not different ($P = 0.94$, by chi-square test). Importantly, the axons consistently responded to increasing mechanical stimuli with increasing discharge, indicating that the axons were encoding the relative intensity of the stimuli (Fig. 2, A–C). There was no sustained discharge after the mechanical stimulation. The response to axon stimulation was repeatable, demonstrating that the stimuli did not cause gross damage to the axon, which would have resulted in transient, irregular, and nonreproducible injury discharge (Howe et al. 1977; Wall et al. 1974). Furthermore, the subsequent response of the distal RF after the axonal stimuli demonstrated that the stimuli did not disable the axon’s normal function of transmitting action potentials (Fig. 2, A–C). In contrast, the only response that could be elicited from 3 of the Aαβ-axons was during very intense stimuli (approximately 4 N, using the probe), which resulted in 1–3 action potentials and subsequently interrupted through-conduction.

To establish a time course of the incidence of the axonal mechanical sensitivity (AMS) during neuritis, later experiments focused on characterizing deep neurons. The time course of the percentages of AMS (Fig. 3A) best fit a curve of the form

$$%\text{AMS} = k t e^{\alpha t}$$

where $k = 22.91$, $\alpha = 1.112$, and $\beta = -0.1467$ ($t =$ time in days; $P = 0.0001$). This curve shows that after applying CFA, 72% of the axons became mechanically sensitive by 7.6 days. The time to reach $\frac{1}{2} %\text{AMS}_{\text{max}}$ was 1.9 days, and the time to decline to $\frac{1}{2} %\text{AMS}_{\text{max}}$ after reaching maximum was 19 days.

**Neuritis-induced ongoing activity in deep nociceptors**

Action potentials in the absence of evident or applied stimuli are termed ongoing activity (OA). Ongoing activity was recorded for 2 min after the RF was located, and after mechanical stimulation of the nerve. During CFA neuritis, more deep neurons with C- and Aδ-axons had OA (19/111) than did cutaneous neurons with C- and Aδ-axons recorded during the same experiments [24/3; $P < 0.05$ (chi-square)], or than did deep neurons with C- and Aδ-axons recorded during the control experiment [1/27; $P = 0.05$ (Fisher’s exact)]. No neurons with Aδ-axons expressed OA (when muscle spindles or cutaneous units were characterized, which often have OA, there was always a physiological position in which the OA would cease). When present, the OA was always irregular, with rates ranging from 0.3 to 2 Hz (mean 0.9 ± 0.43 Hz). Of the 19 deep neurons with OA, axonal mechanical sensitivity was observed in 7. Mechanical stimulation of the nerve induced OA in one neuron. During neuritis, OA occurred with equal frequency among neurons with AMS (7/45) and neurons with insensitive axons (12/66; $P = 0.94$). These findings suggest that the capacities to develop axonal mechanical sensitivity and OA are independent features of these sensory neurons.

**Neuritis led to cutaneous hypersensitivity**

Animals did not exhibit postural abnormalities or autotomy after the surgery. The withdrawal thresholds of the paws on the side of CFA neuritis (left) decreased during the first 4 days, and then increased to a similar value to the contralateral, unoperated side (right) by day 14 (Fig. 3B). The right paw thresholds decreased slightly but not significantly during the 21-day test period. The respective left and right thresholds were significantly different by paired t-test ($P = 0.001$), demonstrating that the neuritis resulted in hypersensitivity of the ipsilateral, but not contralateral paw, for days 2–10. Sensitivity changes did not occur after surgical control experiments, where the sciatic nerve was exposed and mobilized (data not shown).

**TABLE 1. General properties of characterized neurons**

<table>
<thead>
<tr>
<th>RF Locus</th>
<th>Fiber Type</th>
<th>CFA</th>
<th>IFA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N AMS CV</td>
<td>N AMS CV</td>
<td>N AMS CV</td>
</tr>
<tr>
<td>Deep</td>
<td>C</td>
<td>92 0.9 (0.2)</td>
<td>14 0.9 (0.2)</td>
<td>8 0.9 (0.3)</td>
</tr>
<tr>
<td></td>
<td>Aδ</td>
<td>19 2.5 (1.3)</td>
<td>3 1.8 (0.2)</td>
<td>2 3.6 (2.2)</td>
</tr>
<tr>
<td></td>
<td>Aαβ</td>
<td>16 25.1 (8.0)</td>
<td>na/a</td>
<td>18 28.6 (11.4)</td>
</tr>
<tr>
<td>Cutaneous</td>
<td>C</td>
<td>36 1.0 (0.2)</td>
<td>7 0.8 (0.1)</td>
<td>5 0.8 (0.1)</td>
</tr>
<tr>
<td></td>
<td>Aδ</td>
<td>7 3.2 (0.2)</td>
<td>na/a</td>
<td>1 0.6</td>
</tr>
<tr>
<td></td>
<td>Aαβ</td>
<td>24 25.0 (4.6)</td>
<td>na/a</td>
<td>14 29.1 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>194 24</td>
<td>48</td>
<td>194</td>
</tr>
</tbody>
</table>

Fiber type based on conduction velocity (Lawson et al. 1997). CFA, complete Freund’s adjuvant; IFA, incomplete Freund’s adjuvant; RF, receptive field; N, number of neurons recorded; AMS, number of neurons with axonal mechanical sensitivity; CV, conduction velocity (SD); na/a, not applicable. There were no significant differences in CVs of similar groups.
Immunocytochemistry of the inflamed nerve

At 7 days postoperatively, the affected section of the nerve was characterized by encasement with granulation tissue and hyperemia of the intrinsic vasculature. Histology of the lesion demonstrated epineurial edema, increased lymphocytes, and a massive infiltration of macrophages within the epineurium and the granuloma (Fig. 4, B, D, and F). Axonotmesis resulting from this procedure is reported to be minimal (Eliav et al. 1999, 2001), and this is suggested by the paucity of immune cells within the fascicles (Fig. 4, D and E).

DISCUSSION

These data demonstrate that neurons innervating deep structures and having properties of nociceptors (i.e., slow conduction velocities and high mechanical activation thresholds) developed axonal mechanical sensitivity during neuritis. The other types of neurons that we studied were not affected. Furthermore, in control nerves, no axons were mechanically sensitive. It is likely that our recordings included so-called silent nociceptors (Lewin and Mendell 1994; Meyer et al. 1991). We employed noxious stimuli in the search for receptive fields, and thus especially for deep neurons, the tissues containing the terminals were often swollen, and therefore inflamed, before identification. Such inflammation is likely to

TABLE 2. Postoperative timing of recordings and proportions of mechanically sensitive axons

<table>
<thead>
<tr>
<th>RF Locus</th>
<th>Postoperative Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep</td>
<td></td>
</tr>
<tr>
<td>Cutaneous</td>
<td></td>
</tr>
<tr>
<td>Postoperative</td>
<td>0* 1 2 3 6 7 15 22 30</td>
</tr>
<tr>
<td>Cutaneous</td>
<td>0/13 0/11 0/3 0/5 0/4 0/2 — 0/13 0/5</td>
</tr>
</tbody>
</table>

Cells contain the proportion of mechanically sensitive axons to number of neurons recorded at the postoperative day, for neurons with C- and Aa-axons.

* Unoperated and incomplete Freund’s adjuvant control experiments combined.
reveal the latent receptive fields of otherwise silent nociceptors (Kress et al. 1992), making specific identification of such neurons improbable. Inflammation is also known to induce ongoing activity. Although we observed a statistically significant, increased incidence of ongoing activity in deep neurons during neuritis, these data must also be considered carefully because of the methodology used to identify the receptive fields. With these data, we cannot rule out that the axons of silent nociceptors from either deep or cutaneous tissues become mechanically sensitive during neuritis.

The development of axonal mechanical sensitivity was induced by local inflammation, which was characterized by the recruitment of epineurial macrophages and lymphocytes. This cellular infiltrate may play a key role in the axonal mechanical sensitivity. These cells lead to increased levels of tumor necrosis factor alpha, which increases sodium conductance in mammalian cell membranes (Hribar et al. 1999) and induces spontaneous activity in nociceptors when applied to axons (Leem and Bove 2002; Sorkin et al. 1997). Coupled with two normal features of neurons and their axons, increased sodium conductance could lead to the observed mechanical sensitivity and ongoing activity. First, mechanical stimulation of normal axons causes a graded, Na⁺-dependent membrane depolarization, although this depolarization is insufficient to elicit more than a solitary action potential (Ganot et al. 1981; Gross et al. 1983; Julian and Goldman 1962; Petrov and Usherwood 1994). Increased sodium conductance could render the normally sub-threshold depolarizations attributed to mechanical stimuli sufficient to initiate action potentials. Second, the membrane potential in sensory neurons oscillates, dependent on Na⁺ channels, and normally approaches but does not reach the triggering threshold of the neuron (Amir et al. 1999). These oscillations become greater as the membrane is depolarized (Amir et al. 1999). Such oscillations would be potentiated by increased sodium conductance or sodium current, and could lead to spontaneous activity. Importantly, these oscillations are more pronounced in neurons innervating noncutaneous structures (Liu et al. 2002), which may be the basis for our observations that only the axons of noncutaneous neurons became mechanically sensitive.

Many nerve injury models lead to sensory changes that are detectable in the distribution of the nerve, that is, within the tissue that is innervated (Chacur et al. 2001; Eliav et al. 1999; Kim et al. 1997; Seltzer et al. 1990). In our model, there was altered cutaneous sensitivity in the innervation territory of the CFA-treated sciatic nerves, consistent with previous reports of CFA-induced neuritis (Clatworthy et al. 1995; Eliav et al. 1999; Wallas et al. 2003). The tests used rely on cutaneous

**FIG. 4.** Features of neuritis attributed to CFA. H&E-stained sections of normal (A) and treated nerves (B) from same animal at post-operative day 7 revealed epineurial edema in response to CFA. TCRβ (C and D) and ED1 (E and F) immunoreactivity demonstrated aggregation of T lymphocytes and macrophages, respectively, in epineurium, but not within fascicles of CFA-treated nerve (D and F), compared with normal nerve (C and E). Asterisks in A and B indicate epineurium; arrows indicate perineurium; measurement bar = 100 μm.
sensors as the afferent limb of a withdrawal reflex. However, in our electrophysiological experiments and in similar models, such neurons did not demonstrate ongoing activity (Michaelis et al. 2000) or mechanical sensitivity changes (Bulka et al. 2002). Moreover, cutaneous allodynia seems dependent on at least transient activation of slowly conducting axons (Vatine et al. 1998). These findings, and ours, suggest that the observed sensitivity change was related to changes in the sensitivity of higher-order neurons that receive convergent information from the noncutaneous peripheral neurons that were sensitized by the neuritis. Alternatively, the cutaneous sensitivity may have been secondary to changes in ongoing activity of silent nociceptors (not specifically recorded from, as discussed above), or transient changes in neurons with faster conducting axons, which were not tested before or at the peak of heightened cutaneous sensitivity.

The appearance of axonal mechanical sensitivity during neuritis is consistent with reports of typically deep pain radiation provoked by mechanical stimulation of inflamed human dorsal roots (Kuslich et al. 1991; Smyth and Wright 1958; G. M. Bove and Z. H. Bajwa, unpublished observations), and radiating deep pain in the apparent absence of nerve injury (Verdugo and Ochoa 1993). Neuritis has long been recognized as a common malady of the peripheral nervous system (Gowers 1886), and can occur as a result of direct nerve injury or by extension from other diseases with inflammatory components, such as diabetes and endometriosis (Dyck et al. 2000; Zager et al. 1998). Neuritis may be a common denominator in these otherwise seemingly unrelated disorders. The development of axonal mechanical sensitivity seems necessary to explain some clinical features of patients with radiating pain worsened by movements (e.g., “sciatica”), suffered by more than one-half of the population at some point in their lives (Frymoyer et al. 1983; Hult 1954). Furthermore, the appearance of mechanical transduction in a location that does not normally support such transduction may provide an opportunity for discovering the elusive mechanism of mechanical transduction.

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DISCLOSURES

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