Uninjured C-Fiber Nociceptors Develop Spontaneous Activity and \( \alpha \)-Adrenergic Sensitivity Following \( L_6 \) Spinal Nerve Ligation in Monkey


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Ali, Z., M. Ringkamp, T. V. Hartke, H. F. Chien, N. A. Flavahan, J. N. Campbell, and R. A. Meyer. Uninjured cutaneous C-fiber nociceptors develop spontaneous activity and \( \alpha \)-adrenergic sensitivity following \( L_6 \) spinal nerve ligation in the monkey. J. Neurophysiol. 81: 455–466, 1999. We investigated whether uninjured cutaneous C-fiber nociceptors in primates develop abnormal responses after partial denervation of the skin. Partial denervation was induced by tightly ligating spinal nerve \( L_6 \) that innervates the dorsum of the foot. Using an in vitro skin-nerve preparation, we recorded from uninjured single afferent nerve fibers in the superficial peroneal nerve. Recordings were made from 32 C-fiber nociceptors 2–3 wk after ligation and from 29 C-fiber nociceptors in control animals. Phenylephrine, a selective \( \alpha_2 \)-adrenergic agonist, and UK14304 (UK), a selective \( \alpha_2 \)-adrenergic agonist, were applied to the receptive field for 5 min in increasing concentrations from 0.1 to 100 \( \mu \)M. Nociceptors from in vitro control experiments were not significantly different from nociceptors recorded by us previously in in vivo experiments. In comparison to in vitro control animals, the afferents found in lesioned animals had 1) a significantly higher incidence of spontaneous activity, 2) a significantly higher incidence of response to phenylephrine, and 3) a higher incidence of response to UK. In lesioned animals, the peak response to phenylephrine was significantly greater than to UK, and the mechanical threshold of phenylephrine-sensitive afferents was significantly lower than for phenylephrine-insensitive afferents. Staining with protein gene product 9.5 revealed an \( \sim 55\% \) reduction in the number of unmyelinated terminals in the epidermis of the lesioned limb compared with the contralateral limb. Thus uninjured cutaneous C-fiber nociceptors that innervate skin partially denervated by ligation of a spinal nerve acquire two abnormal properties: spontaneous activity and \( \alpha \)-adrenergic sensitivity. These abnormalities in nociceptor function may contribute to neuropathic pain.

INTRODUCTION

Peripheral nerve injury often leads to a neuropathic pain condition characterized by ongoing pain and hyperalgesia. In some neuropathic pain patients, an anesthetic block of the sympathetic ganglia leads to a decrease in both ongoing pain and hyperalgesia. Doupe et al. (1944) and Nathan (1947) suggested that this sympathetically maintained pain (SMP) is because of a functional connection between the sensory and sympathetic nervous system such that primary afferent fibers become responsive to ongoing activity in the sympathetic nervous system.

Multiple sites for an interaction between the sympathetic and sensory nervous systems have been suggested, including the site of nerve injury (e.g., Devor and Janig 1981; Haber et al. 1987; Korenman and Devor 1981; Scadding 1981) and the dorsal root ganglion supplying the injury site (Abulla and Smith 1997; Chung et al. 1993; Devor et al. 1994a; McLachlan et al. 1993; Michaelis et al. 1996; Petersen et al. 1996; Xie et al. 1995; Zhang et al. 1997). Several lines of evidence suggest that the skin may also be an important site. 1) An increase in the number of adrenergic receptors has been reported in the skin of patients with neuropathic pain (Drummond et al. 1996). 2) Administration of adrenergic agonists to the skin of SMP patients produces pain (Davis et al. 1991; Torebjörk et al. 1995; Wallin et al. 1976). 3) In animal models of neuropathic pain, cutaneous nociceptive afferent fibers are activated following stimulation of sympathetic efferent fibers and following application of adrenergic agonists to their receptive fields (Koltzenburg et al. 1994; Sato and Perl 1991).

Because phentolamine reduces pain and hyperalgesia in patients with SMP (Arner 1991; Raja et al. 1991), this sympathetic-sensory coupling is likely \( \alpha \)-adrenergically mediated. Whereas, based on clinical studies, the \( \alpha_2 \) adrenoceptor appears to be the culprit (e.g., Ali et al. 1996; Campbell et al. 1992; Davis et al. 1991; Drummond et al. 1996; Wesselmann et al. 1996), the \( \alpha_2 \) adrenoceptor appears to be important in subprimate models of neuropathic pain (e.g., Chen et al. 1996; Peterson et al. 1996; Sato and Perl 1991; Tracey et al. 1995; Xie et al. 1995).

We sought to determine whether abnormal activity and adrenergic sensitivity develop in cutaneous nociceptors following tight ligation of the \( L_6 \) spinal nerve ligation in monkeys. This lesion was chosen because 1) injury to the spinal nerve in monkeys produces behavioral signs of neuropathic pain (Carlton et al. 1994; Kirk and Denny-Brown 1970), 2) a similar injury in rats produced behavioral signs of hyperalgesia that were reported to be reversed by surgical sympathectomy and by systemic administration of phentolamine (Kim and Chung 1991, 1992; Kinman and Levine 1995; but see Fontana et al. 1996; Lee et al. 1997; Ringkamp et al. 1999a,b), 3) preliminary in vivo experiments in our laboratory indicated that C-fiber nociceptors in monkey responded to close-arterial injection of norepinephrine after a spinal nerve injury (Selig et al. 1993), 4) the procedure to produce a spinal nerve ligation is standardized and thus results in a reproducible lesion, and 5) this lesion enables the investigation of uninjured nerve fibers in peripheral nerves that innervate the tissue via adjacent nerve roots.
METHODS

A nerve injury was induced in monkeys by tight ligation of the L₆ spinal nerve, which innervates the dorsum of the foot (Sherrington 1893). This procedure was chosen to induce a partial denervation in the skin area innervated by the superficial peroneal nerve from which final recordings were made. The peroneal nerve arises from spinal nerves L₃–L₅ (Howell and Strauss 1971). Two to three weeks after the ligation, an in vitro skin-nerve preparation was used to test α-adrenergic sensitivity of cutaneous C-fiber nociceptors from the innervation territory of the superficial peroneal nerve. The Johns Hopkins University Animal Care and Use Committee approved all procedures in this study.

Tight ligation of the left L₆ spinal nerve

Monkeys (Macaca fascicularis, 4.5–13.5 kg) were initially sedated by (10 mg/kg im) ketamine and maintained under halothane anesthesia (0.5–1.5% halothane-50% N₂O) and artificially ventilated (end tidal pCO₂ maintained at 32–40 Torr). Adequacy of anesthesia was checked by ensuring the absence of motor responses to noxious mechanical stimuli and by monitoring heart rate with an electrocardiogram. Hydration was maintained by intravenous infusion of 5% dextrose in saline (4–6 ml·kg⁻¹·h⁻¹). Core temperature was measured using a rectal probe connected to a thermal controller and maintained close to 38°C with the use of circulating water heating pads. At the beginning of the experiment, penicillin G (450,000 U) was administered for prophylaxis against infection.

With the monkeys in a prone position, a rostra-caudal incision was made into the skin. The incision was made 3–5 cm to the left of the midline and was centered at the level of the L₃/L₅ lumbar vertebrae subjacent to the iliac crest. The muscle layers were reflected by blunt dissection and retraction until the left transverse processes of the L₆ and L₇ vertebrae were exposed. The connective tissue just caudal to the transverse process of L₆ was carefully dissected until the L₆ spinal nerve could be visualized with the aid of a surgical microscope. The spinal nerve was dissected free of surrounding connective tissue and twice, tightly ligated, using 5–0 silk suture. The wound was closed in layers. The monkeys were maintained under close observation following the procedure to verify that they did not have any signs of infection, severe pain, or paralysis. The position of the ligation site was confirmed at autopsy.

Harvesting of skin-nerve preparation

To avoid the confounding effects of adrenergic agents on perfusion, the electrophysiological studies were carried out in an in vitro preparation, which also allows better control of drug concentrations. The superficial peroneal nerve and its innervation territory on the top of the foot were harvested in terminal experiments. The monkeys were initially sedated with (10 mg/kg) ketamine and maintained under pentobarbital sodium (continuous intravenous infusion, 6 mg·kg⁻¹·h⁻¹). Adequacy of anesthesia and core temperature were checked and maintained in the manner mentioned previously. Animals were paralyzed every 2 h with (0.1 mg/kg) pancuronium bromide and were artificially ventilated. Hydration was maintained by intravenous infusion of 5% dextrose in normal saline (4–6 ml·kg⁻¹·h⁻¹).

With the monkeys in a supine position, the superficial peroneal nerve of the left leg was exposed, distal to the knee joint. The skin incision was continued distally, allowing the nerve to be exposed proximal to where it becomes subcutaneous. Using a dissecting microscope, the nerve was carefully freed from the surrounding connective tissue. Synthetic interstitial fluid was regularly applied to the nerve to prevent dryness.

To minimize bleeding during dissection of the skin, the limb was elevated and an elastic bandage was applied in a distal to proximal direction. After a sphygmomanometer was placed at the thigh and inflated to a level above systolic pressure, the elastic bandage was removed. The hairy skin from the dorsum of the foot was freed from the underlying subcutaneous tissue, taking care to avoid cutting the branches of the superficial peroneal nerve running in the skin. The dissection was continued until the skin from the dorsum of the foot and superficial peroneal nerve could be removed and transferred to the in vitro perfusion and recording chamber (Fig. 1). The animal was then killed with an overdose of (40 mg/kg) pentobarbital.

Control animals

In vitro control experiments were performed on the skin of uninjured monkeys. The protocols were the same as those for the lesioned animals except that approximately one-half of the data from control monkeys was obtained from a preparation of the superficial radial nerve that innervates the dorsum of the hand.

In vivo control data were obtained from a retrospective analysis of previous data collected from our laboratory (e.g., Campbell and Meyer 1983; Meyer and Campbell 1981; Meyer et al. 1985, 1988; Treede et al. 1990). In these experiments, the animals were maintained under pentobarbital anesthesia, and a cutaneous nerve that innervated hairy skin was dissected for single nerve fiber recordings (Campbell and Meyer 1983).

In vitro perfusion and recording chamber

The in vitro chamber was a scaled up version of a chamber previously developed for the rat skin (Reeh et al. 1986). A schematic of the in vitro perfusion and recording chamber is shown in Fig. 1. The skin was placed corium side up into the perfusion chamber and was continuously perfused with fresh, gassed, synthetic interstitial fluid. The synthetic interstitial fluid (SIF) consisted of (in mM) 107.7 NaCl, 3.48 KCl, 0.69 MgSO₄, 26.2 NaHCO₃, 1.67 NaH₂PO₄, 1.53 CaCl₂, 9.64 sodium gluconate, 5.5 glucose, and 7.6 sucrose (Bretag 1969; Reeh et al. 1986). The reservoir of SIF was continuously bubbled with a 95% O₂-5% CO₂ mixture to obtain a pH of 7.4. A roller pump (Gilson, model M312) was used to control the flow of SIF at a rate of 750 ml/h from the reservoir through a heat-exchanger to the perfusion chamber. The heat exchanger was used to raise the temperature of the SIF to 33°C and consist of a straight stainless steel tube through which the SIF passed. The tube was surrounded by a tightly wound coil of flexible tygon tubing through which water from a temperature-controlled water bath flowed. Several bubbling stones were placed within the perfusion chamber to keep the perfusate saturated with the 95% O₂-5% CO₂ mixture. The stones were arranged to establish a continuous circulation of fluid within the perfusion chamber, which helped to minimize thermal and pH gradients. The temperature of the chamber was maintained between 32 and 34°C by means of a second temperature-controlled water bath that circulated water through a loop of stainless steel tubing within the perfusion chamber.

The proximal end of the nerve was passed through a hole into a separate, mineral-oil-filled recording chamber. The nerve was placed on a platform and the hole was sealed with petroleum jelly. The epineurium and perineurium were removed from the nerve, and small filaments were teased from bundles cut from the nerve. A small filament was placed on a gold electrode for extracellular recording. The gold return electrode was placed under the nerve trunk within the recording chamber.

Electrophysiological and pharmacological studies

The mechanical receptive fields of single nociceptive afferents in the skin were identified. Nociceptive afferents were initially
identified by their preferential and slowly adapting response to firm prodding of the skin. The mechanical threshold for the nociceptive afferents was determined by applying von Frey filaments to the most sensitive part of the receptive field. The smallest von Frey probe that produced a response in \( \geq 50\% \) of the trials was taken as threshold. The conduction velocity of the fiber was determined by electrical stimulation of the receptive field with a constant voltage stimulus. The stimulus intensity was increased until an action potential with a similar shape to that generated mechanically could be recorded. The voltage was increased fourfold to verify that no other fibers of the same action potential shape could be stimulated from this site. The conduction velocity was calculated by dividing the conduction distance by the latency of action potential to a suprathreshold stimulus. In all cases, suprathreshold mechanical stimuli were applied throughout the mechanical receptive field to confirm that only one fiber was being recorded. In this study we concentrated on isolating C-fiber nociceptors, and faster conducting fibers were discarded.

For drug administration, a Plexiglas ring of adequate diameter to surround the receptive field was placed on the skin and sealed with petroleum jelly. The fluid within this ring was removed to verify that no leakage from the surrounding fluid occurred. The well formed by this ring was filled with the solutions of interest. During times when drugs were not being applied, the SIF within the well was refreshed every 5 min to maintain adequate control of pH and oxygenation. Before drug application, each fiber was tested for cold sensitivity by evacuating the SIF from the well and replacing it with ice cold (\(< 4^\circ C\)) SIF for 20 s. For some fibers heat testing was also carried out. The beam of a halogen projector lamp was projected through the bottom of the Plexiglas chamber onto the epidermal side of the receptive field. A needle thermocouple (Omega, HYP-0) was placed between the skin and the bottom of the chamber to monitor stimulus temperature. The stimulus was applied for up to 15 s (max temperature \( = 48^\circ C \)) and, to avoid sensitizing the fiber, was terminated as soon as the fiber started to respond.

A 5-min predrug interval was used to determine the level of ongoing activity of the fiber in the absence of any stimuli. The SIF within the well was refreshed at the beginning of this interval. At the end of the 5-min predrug interval, the well was evacuated and filled with the appropriate drugs. The drugs used in this study were the \( \alpha_1 \)-adrenergic agonist phenylephrine (1, 10, and 100 \( \mu M \)) and \( \alpha_2 \)-adrenergic agonist UK14304 (0.1, 1, 10 \( \mu M \)); a gift from Pfizer Pharmaceuticals, Sandwich, UK). UK14304 is also known as brimonidine.

Phenylephrine and UK14304 were chosen for this study because of their high efficacy and high selectivity for \( \alpha_1 \)- and \( \alpha_2 \)-receptors, respectively. The doses of phenylephrine (1–100 \( \mu M \)) and UK14304 (0.1–10 \( \mu M \)) were chosen based on their potency and selectivity for activating \( \alpha_1 \)- and \( \alpha_2 \)-adrenergic receptors, respectively (Flavahan et al. 1984).

All drugs were dissolved in SIF and warmed to 34°C. The drugs were bubbled with the 95% O\( _2 \)-5% CO\( _2 \) mixture just before administration. For both phenylephrine and UK14304, all doses were applied for up to 15 s (maximum temperature \( = 48^\circ C \)) SIF into the bath and replaced with the next highest dose. When the dose series for a drug was completed, the well was rinsed with SIF at least five times. A minimum of 10 min elapsed before the ascending series of the next drug was applied. Most fibers were tested to both phenylephrine and UK14304; one-half of the fibers were tested first to the ascending dose series of phenylephrine and then UK14304; in the remaining fibers the order was reversed.

**Data collection and analysis**

The signal from the recording electrode was differentially amplified, filtered, and displayed on an oscilloscope. In addition, the amplified and filtered signal was recorded using a computer-based data acquisition system (Microstar Laboratories data acquisition board, DAP 3200e315). A customized data acquisition and analysis software (DAPSYS) (Turnquist 1995) was used to digitize and store all action potential waveforms as well as to perform real-time and postshock spike discrimination based on multiple time-amplitude window criteria. The software was also used to control
the timing of the heat stimuli, to record temperatures during the
heat stimulus, to control timing of the drug administration, and to
time stamp the action potentials relative to stimulus delivery.

Criteria for drug responders

The total number of action potentials generated during the 5-
min incubation of each dose of a drug was determined by a posthoc
analysis of each fiber. A fiber was considered to have given a
positive response during drug application only if 1) more than five
action potentials above predrug activity were evoked and 2) the
magnitude of the response during drug application was ≥30%
greater than predrug activity. To avoid counting action potentials
evoked by mechanical stimulation rather than due to drug effects,
action potentials that were generated during the time of drug ex-
change were not counted.

Protein gene product 9.5 staining and counting of
epidermal nerve fibers

Skin punches (3-mm diam) were obtained from the dorsal sur-
faces of both feet (lesioned side as well as nonlesioned side) of
L4 lesioned monkeys after completion of the electrophysiological
experiments. The skin punches were fixed with a cold mixture of
2% paraformaldehyde, lysine, and sodium periodate (McClean and
Nakane 1974) and kept in 0.05 M tris (hydroxymethyl)amiono-
methane-buffered saline (TBS) until sectioning. Before sectioning,
the skin was cryoprotected in 10% sucrose in TBS and snap-frozen
in melting isopentane that was cooled with liquid nitrogen.

Vertical sections of skin (20 μm) embedded in optimal cutting
temperature (OCT) medium were mounted on poly-lysine-coated
glass slides. After drying for 2 h, sections were rehydrated for
staining or stored at −80°C for future use. The sections were
washed with TBS and treated with 3% hydrogen peroxide in TBS
for 30 min and then incubated for 30 min in 10% normal goat
serum (Vector Laboratories, Burlingame, CA) and 1% Triton-
X100 (Sigma, St Louis, MO) in TBS. The slides were then incu-
bated overnight at 4°C with polyclonal anti-ubiquitin carboxyl ter-
ninal hydrolase (protein gene product 9.5, 1:1000) (Wilkinson et
al. 1989) antibody to stain epidermal nerve fibers. The sections
were then incubated with a biotinylated secondary antibody (Vec-
tor, 1:300) followed by streptavidin-biotin-peroxidase solution
(Dako, Carpentaria, CA) for 30 min. The slides were developed
with an SG chromogen substrate kit (Vector) with dark-blue reaction
product, and counterstained with Mayer’s hematoxylin, dehy-
drated, and mounted.

The slide sections were submitted to an investigator who was
blinded to the experimental protocol and the number of epidermal
nerve fibers in sections from both the lesioned as well as the
nonlesioned (control) side was counted. A priori counting rules
were established to count only single intraepidermal fibers and not
multiple branches of the same fiber. The total length of epidermis
was measured for each section, and the number of intraepidermal
fibers per mm length was determined.

Statistical analysis

A paired t-test was used to compare the number of epidermal
nerve fibers on ligated side versus the contralateral side. Conduc-
tion velocities and von Frey thresholds were compared between
different groups of nociceptors (e.g., lesioned vs. control) using
the t-test and Mann-Whitney U tests, respectively. Receptive field
areas were compared using a t-test. A χ² test was used to compare
the incidence of spontaneous activity, heat sensitivity, cold sensi-
tivity, phenylephrine responsiveness, and UK14304 responsiveness
in nociceptors recorded from ligated monkeys compared with con-
trol monkeys. Nociceptors tested with phenylephrine and UK14304
were separated into responsive and nonresponsive fibers, respec-
tively. Drug effects on the responsive fibers were tested using a
Friedman analysis of variance (ANOVA). When the test indicated
a significant difference, posthoc analysis was made between the
predrug interval and each of the doses of drug using a Wilcoxon
matched pairs test with Bonferroni correction. For fibers tested with
both phenylephrine and UK14304, the peak responses (minus
the baseline) were compared using a Mann-Whitney U test. Data
are presented as means ± SE or as medians (quartiles). Signifi-
cance was accepted at the 0.05 level.

Results

The left L4 spinal nerve was tightly ligated in 11 monkeys. An
in vitro skin-nerve preparation was used to record from 32 C-fiber nociceptors in the superficial peroneal nerve. Twenty-nine C- fiber nociceptors were also studied from nine nonlesioned (control) monkeys. In nine lesioned mon-
keys, skin biopsy punches were obtained from the dorsal skin of both feet (ipsilateral and contralateral to the ligation) after completion of the electrophysiological experiments, and the number of nerve fibers in the epidermis stained with protein gene product 9.5 (PGP9.5) was counted.

Epidermal nerve fiber counts

In skin from the right, nonlesioned foot, PGP9.5-stained
fibers could be visualized in both epidermal as well as sub-
epidermal tissue. The morphological appearance of PGP9.5
labeled fibers in monkey skin (Fig. 2, A and B) was similar
to what has previously been described for human skin (Mc-
carthy et al. 1995). Individual fibers and small bundles
crossed the dermis to join the subpapillary plexus. The sub-
papillary fibers, in general, lay parallel to the surface of the
skin in bundles. In addition to innervating specialized end-
ings in the dermis and dermal papillae, many subpapillary
fibers turned toward the surface and entered the epidermis.
In the epidermis, these thin, varicose fibers passed between
keratinocytes and often branched in passage. Many fibers
reached the stratum corneum.

The morphological appearance of epidermal fibers on the
side ipsilateral to the L4 spinal nerve ligation (Fig. 2B) was
not different from fibers on the contralateral side (Fig. 2A).
However, the density of PGP stained fibers on the lesioned
side [11.2 ± 2.5 (SE) fibers/mm] was significantly less than
on the control side (28.2 ± 2.2 fibers/mm; P < 0.001, paired
t-test). Thus the L4 lesion resulted in a significant decrease
in the cutaneous innervation on the dorsum of the foot.

Comparison of nociceptors from in vitro and in vivo
experiments

The properties of 299 C-fiber nociceptors recorded in vivo
from nerves that innervate the hairy skin were obtained from
a retrospective analysis of previous data from our laboratory.
As indicated in Table 1, most of the properties of the afferents
recorded during the control and lesion in vitro experiments
were not significantly different from the control in vivo data.
There was no significant difference between the conduction velocities or von Frey thresholds of C-fibers recorded from L6 lesioned monkeys and C-fibers recorded from nonlesioned control monkeys (Table 1). In addition, the receptive field sizes were not significantly different, which would suggest that sprouting was not substantial or that the sprouts were not mechanically sensitive. The incidence of cold sensitivity was similar in control and lesioned animals. Most fibers tested with heat responded.

In contrast, the incidence of spontaneous activity in C-fiber nociceptors from the lesioned monkeys was significantly greater (17 of 25) than in C-fibers recorded from control monkeys (3 of 24, \( P < 0.001, \chi^2 \)). In addition, the magnitude of spontaneous activity was significantly higher in C-fibers recorded from lesioned monkeys (median = 1 action potentials/5 min; range = 0–147 action potentials/5 min) than in control C-fibers (median = 0 action potentials/5 min; range = 0–4 action potentials/5 min; \( P < 0.01 \) Mann-Whitney U test; Fig. 3). However, the mechanical threshold of spontaneously active fibers (2.2 ± 0.4 bar) was not significantly different from fibers without spontaneous activity (2.6 ± 0.9 bar).

Response to the selective \( \alpha_1 \)-adrenergic agonist phenylephrine

LESIONED ANIMALS. Phenylephrine evoked a response in 9 of 17 C-fiber nociceptors tested in L6 nerve lesioned monkeys. There was a large variability in the magnitude and duration of the phenylephrine evoked response between the different fibers. The responses of two fibers are shown in Fig. 4. For fiber 1 (Fig. 4, A and C) phenylephrine evoked a vigorous response that increased in a dose dependent manner. For fiber 2 (Fig. 4, B and C) phenylephrine evoked a moderate response that did not increase in a dose related manner.

Figure 5 summarizes the median response to different doses of phenylephrine from the nine fibers that responded to phenylephrine application. The responses to all doses of phenylephrine were significantly greater than the responses during the predrug period (\( P < 0.05 \), Friedman ANOVA followed by Wilcoxon matched pairs posthoc analysis and Bonferroni correction).

**TABLE 1. Comparison of nociceptor properties**

<table>
<thead>
<tr>
<th>In Vitro Ligation (32)</th>
<th>In Vitro Control (29)</th>
<th>In Vivo Control (299)</th>
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<tbody>
<tr>
<td>Conduction velocity, m/s</td>
<td>0.73 ± 0.08* (22)</td>
<td>0.73 ± 0.06* (23)</td>
</tr>
<tr>
<td>Mechanical threshold, bar</td>
<td>2.0 [1.2–3.6] (30)</td>
<td>2.2 [0.9–4.3] (29)</td>
</tr>
<tr>
<td>Receptive field area, mm²</td>
<td>51 ± 13 (24)</td>
<td>61 ± 13 (21)</td>
</tr>
<tr>
<td>Incidence of spontaneous activity</td>
<td>17 of 25§</td>
<td>3 of 24</td>
</tr>
<tr>
<td>Incidence of heat sensitivity</td>
<td>15 of 15</td>
<td>11 of 12</td>
</tr>
<tr>
<td>Incidence of cold sensitivity</td>
<td>14 of 25</td>
<td>4 of 11</td>
</tr>
<tr>
<td>Incidence of PE sensitivity</td>
<td>9 of 17†</td>
<td>2 of 16</td>
</tr>
<tr>
<td>Incidence of UK14304 sensitivity</td>
<td>6 of 18</td>
<td>1 of 14</td>
</tr>
</tbody>
</table>

Values in Conduction velocity, Mechanical threshold, and Receptive field area are means ± SE; numbers in parentheses are numbers of fibers; numbers in brackets are quartiles with median. I.D., insufficient data; PE, phenylephrine. *Significantly different from in vivo control (\( P < 0.001, t \)-test). †Significantly different from in vitro control (\( P = 0.001, \chi^2 \)). §Significantly different from in vitro control (\( P = 0.02, \chi^2 \)).
pared with the response evoked by any of the doses of UK14304 \((P > 0.05,\ \text{Friedman ANOVA followed by Wilcoxon Matched pair with Bonferroni correction})\). In addition, there was not a significant difference between the conduction velocities, von Frey thresholds, receptive field size, and incidence of heat and cold sensitivities of C-fiber nociceptors responding to UK14304 and those of fibers that did not respond to UK14304.

**CONTROL ANIMALS.** In control animals only 1 of 14 C-fibers responded to UK14304. Interestingly, this fiber also responded to phenylephrine. There was a tendency of an increased incidence of UK14304 responses in L6 spinal nerve lesioned monkeys compared with control animals \((P \approx 0.08, \chi^2 \text{test})\).

**FIG. 3.** Spontaneous activity recorded during the 5-min interval before administration of the first adrenergic agonist. Each point corresponds to a different fiber; incidence of spontaneous activity was significantly higher in nerve fibers recorded from L6 lesioned monkeys than in control monkeys.

The von Frey thresholds of phenylephrine responsive fibers \([1.1 (0.9–1.6) \text{ bar}; \text{median (quartiles)}]\) were significantly lower than those of phenylephrine nonresponsive fibers \([3.2 (1.4–5.1) \text{ bar}; P < 0.02, \text{Mann-Whitney } U \text{ test})\]. In contrast, the receptive field size, conduction velocities, and incidence of heat and cold sensitivities were not significantly different. Interestingly, two of the fibers with a high initial level of spontaneous activity \((>20 \text{ action potentials in 5 min, Fig. 3})\) exhibited a marked decrease in spontaneous activity during phenylephrine administration, but had no change in spontaneous activity following administration of UK14304.

**TACHYPHYLAXIS TO REPEATED PHENYLEPHRINE.** Four of nine C-fibers that responded to phenylephrine were subsequently tested with a second application of phenylephrine. The second application elicited a much weaker response (Fig. 6), indicating a marked tachyphylaxis in the phenylephrine-evoked response. This tachyphylaxis prohibited a study of the effects of selective antagonists.

**CONTROL ANIMALS.** The incidence of phenylephrine evoked activity was significantly lower in C-fibers of control monkeys \((2 \text{ of } 16)\) when compared with the C-fibers of L6 nerve lesioned monkeys \((9 \text{ of } 17; P < 0.02, \chi^2 \text{ test})\). The peak response was 180 action potentials in one fiber and 9 action potentials in the other.

**Response to the selective \(\alpha_2\)-adrenergic agonist UK14304**

**LESIONED ANIMALS.** The \(\alpha_2\)-adrenergic agonist UK14304 evoked a response in 6 of 18 C-fiber nociceptors recorded from lesioned monkeys. As with the phenylephrine, the duration and magnitude of the response varied between fibers. Figure 4, D and E, illustrates the responses of two fibers to UK14304. These are the same two fibers whose responses to phenylephrine are illustrated in Fig. 4, A and B.

Figure 7 summarizes the median response to different doses of UK14304 from the six fibers that responded to UK14304 application. There was not a significant difference between the response evoked during the predrug period compared with the response evoked by any of the doses of UK14304 \((P > 0.05,\ \text{Friedman ANOVA followed by Wilcoxon Matched pair with Bonferroni correction})\). In addition, there was not a significant difference between the conduction velocities, von Frey thresholds, receptive field size, and incidence of heat and cold sensitivities of C-fiber nociceptors responding to UK14304 and those of fibers that did not respond to UK14304.

**CONTROL ANIMALS.** In control animals only 1 of 14 C-fibers responded to UK14304. Interestingly, this fiber also responded to phenylephrine. There was a tendency of an increased incidence of UK14304 responses in L6 spinal nerve lesioned monkeys compared with control animals \((P = 0.08, \chi^2 \text{test})\).

**Comparison of the response to phenylephrine and to UK14304 in lesioned animals**

Fifteen fibers were tested with both phenylephrine and UK14304 (Fig. 8). Of these, eight fibers responded to phenylephrine and five responded to UK14304. Four fibers that responded to phenylephrine did not respond to UK14304, whereas only one fiber that responded to UK14304 did not respond to phenylephrine. The response was greater during phenylephrine administration in three of four fibers that responded to both phenylephrine and UK14304. Two of these fibers are illustrated in Fig. 4. The median peak response to phenylephrine \([35 (12–133) \text{ imp/5 min}]\) was significantly greater than the median peak response to UK14304 \([7 (6–13) \text{ imp/5 min}; P < 0.05, \text{Mann-Whitney } U]\). In control skin, only 2 of 13 fibers tested with both agonists responded (Fig. 8); one responded to both phenylephrine and UK14304, and the other to just phenylephrine.

The maximum response to UK14304 never exceeded 30 action potentials during the application of any dose of UK14304. By contrast, the maximum response to phenylephrine exceeded 30 action potentials for five of nine responding fibers (Fig. 8). It is unlikely that the doses of UK14304 that were chosen were too low, as the threshold dose for UK14304 responding fibers was 0.1 \(\mu\text{M}\) for four fibers and 1 \(\mu\text{M}\) for the remaining two fibers. Furthermore, three fibers were also tested with an additional 100 \(\mu\text{M}\) dose of UK14304. Two of three fibers did not respond to UK14304, whereas the third fiber showed no increase in response compared with effects evoked by lower doses. The lower incidence of UK14304-sensitive fibers relative to phenylephrine-sensitive fibers is probably not caused by cross tachyphylaxis because 1) UK14304 was presented before phenylephrine for one-half of the fibers, 2) a UK14304 response was observed in three of six responding fibers after first being tested with phenylephrine, and 3) the magnitude of the response to UK14304 was not significantly different for those fibers tested before phenylephrine compared with those tested after phenylephrine.

**Incidence of adrenergic sensitivity across animals**

Successful adrenergic studies were performed on a total of 20 afferents from eight lesioned animals. In three animals, none of the 7 fibers tested responded to adrenergic agents, whereas in the remaining five animals, 11 of 13 fibers tested...
responded to one or both agonists. This suggests that adrenergic sensitivity may vary between animals just as the occurrence of sympathetically maintained pain varies across patients with neuropathic pain.

DISCUSSION

We demonstrate that spontaneous activity and adrenergic sensitivity develop in uninjured cutaneous C-fiber nociceptors in primate skin following ligation of the L6 spinal nerve. More fibers responded to the selective $\alpha_1$-adrenergic agonist phenylephrine than to the selective $\alpha_2$-adrenergic agonist UK14304, and the maximum response to phenylephrine was significantly greater than the maximum response to UK14304. Thus cutaneous nociceptors in monkeys develop $\alpha_1$-adrenergic sensitivity after nerve lesion.

Comparison of in vivo and in vitro data from normal skin

In vitro recordings were obtained from cutaneous afferents in monkey skin with the use of a modification of the technique developed for rat skin by Reeh (1986). An in vitro technique was chosen to control better the concentrations of the applied drugs as well as to avoid potential artifacts associated with the vasoactive properties of the adrenergic agents. The properties of afferents from control skin recorded in vitro were similar to those from normal skin recorded in vivo, except that the conduction velocities were slower. We attribute this difference to the cooler
temperatures in the in vitro chamber compared with the limb temperature in vivo. Thus the in vitro technique appears to be valid for investigating the properties of primate nociceptors.

Cutaneous nociceptors exhibit $\alpha_1$- and $\alpha_2$-adrenergic sensitivity after nerve lesion

The incidence of adrenergic sensitivity was significantly higher in nociceptive afferents from the L6 lesioned monkeys compared with nociceptive afferents from normal animals. Our data demonstrate that cutaneous nociceptors develop $\alpha_1$-adrenergic sensitivity after nerve lesion. Several lines of evidence suggest that the $\alpha_1$-adrenoceptor is also a culprit in human patients suffering from SMP. 1) Intradermal injection of phenylephrine can evoke pain in SMP patients (Ali et al. 1996; Davis et al. 1991). 2) The number of $\alpha_1$-adrenoceptors in hyperalgesic skin of patients with SMP is significantly greater than in the skin of normal subjects (Drummond et al. 1996). 3) Topical administration of the $\alpha_2$-adrenergic agonist, clonidine, did not evoke pain in patients with sympathetically maintained pain but actually led to a decrease in pain and hyperalgesia (Davis et al. 1991; Wessellmann et al. 1996).

A marked tachyphylaxis in the response was observed in the units tested with a second administration of the phenylephrine ascending dose sequence. Tachyphylaxis may have contributed to the nonmonotonic dose-response relationship observed in some fibers. The mechanism and physiological significance of this tachyphylaxis remain unclear. Marked tachyphylaxis has also been reported for other noxious chemicals (e.g., Lang et al. 1990; Manning et al. 1991). This tachyphylaxis prohibited an investigation of the effects of selective adrenergic antagonists.

We do not rule out a role of $\alpha_2$-adrenoceptors in primates. Because UK14304 also activates imidazoline receptors, which are in general inhibitory, the low incidence and low response observed by us to UK14304 could be the result of opposing effects of UK14304 at the imidazoline receptor and the $\alpha_2$-adrenoceptor. In addition, studies in subprimates suggest that $\alpha_2$-adrenoceptors contribute to cutaneous adrenergic sensitivity (O’Halloran and Perl 1997; Sato and Perl 1991; Tracey et al. 1995). However, recent studies in rats indicate that $\alpha_1$-adrenoceptors also contribute to adrenergic sensitivity (Chen et al. 1996; Choi et al. 1996; Lee and Chung 1997).

**Increased spontaneous activity in lesioned animals**

The incidence of spontaneous activity was significantly higher in nociceptive afferents from the L6 lesioned monkeys.
compared with nociceptive afferents from normal animals. Because we recorded centripetally conducted action potentials, this spontaneous activity must originate along the course of the peripheral axon or at the peripheral cutaneous terminal. Koltzenburg et al. (1994) also found a significant increase of spontaneous activity in saphenous nerve fibers after a chronic constriction injury of that nerve; this spontaneous activity could be abolished by application of lidocaine to the cutaneous receptive field. Thus the receptor terminal is likely a site for generation of spontaneous activity.

The spontaneous activity in cutaneous nociceptors observed in this study differs from that previously reported in neuroma and following a chronic constriction nerve injury for two reasons: 1) the spontaneous activity was observed in fibers that were not injured by the nerve injury and 2) the spontaneous activity was observed in cutaneous fibers, whereas the spontaneous activity from neuroma appears to be mainly from motor afferents (Proske et al. 1995). The incidence of spontaneous activity in neuroma of cutaneous nerves is relatively low (Blumberg and Janig 1984; Meyer et al. 1985). The development of spontaneous activity in uninjured C-fiber nociceptors may be specific to primates because spontaneous activity was rarely observed in presumably uninjured C-fiber nociceptors following a partial injury of the auricular nerve in rabbits (O’Halloran and Perl 1997; Sato and Perl 1961).

The increased incidence of spontaneous activity and of adrenergic sensitivity following the nerve injury suggests that they may be related. One possibility is that the factors that lead to an increase in spontaneous activity also lead to an increase in adrenergic sensitivity. For example, an increased density of sodium channels (e.g., Devor et al. 1994b) might lead to an increased membrane excitability. Our observation of a significantly lower mechanical threshold in phenylephrine-sensitive afferents compared with phenylephrine-insensitive afferents would be consistent with this possibility. Another possibility is that the enhanced adrenergic sensitivity results in spontaneous activity caused by the local endogenous leakage of norepinephrine. In this case, the unit with the highest spontaneous activity may not have responded to the exogenous agonists because it was already at its peak discharge frequency. A decrease of spontaneous activity was observed in two other units that had relatively high rates of spontaneous activity. The mechanisms involved in such an inhibition are not known, but a similar observation has been reported for cutaneous afferents from animals with a chronic constriction injury (Koltzenburg et al. 1994).

**Adrenergic activation of C-fibers in normal skin**

Most previous studies have found little evidence for sympathetic activation of nociceptors in normal skin (e.g., Barasi and Lynn 1986; Roberts and Elardo 1985; Shea and Perl 1985). We found that a small proportion of C-fiber nociceptors in normal skin could be activated by phenylephrine and UK14304. Although intradermal norepinephrine produces little pain in normal human subjects (Ali et al. 1996; Davis et al. 1991), cutaneous administration of adrenergic agonists can produce heat hyperalgesia in human subjects (Drummond 1995; Meyer and Raja 1996) and mechanical hyperalgesia in rats (Khasar et al. 1995). Sympathetic stimulation also appears to alter the propensity for C-fiber nociceptors in rabbits to become sensitized by repeated heating (Sato and Perl 1991). Adrenergic sensitivity has also been observed in nociceptors following acute injury (Roberts and Elardo 1985), injection of inflammatory mediators (Sanjue and Jun 1989), and chronic inflammation (Sato et al. 1993). α1-adrenergic receptors have been reported in dorsal root ganglion neurons and peripheral nerve fibers (Gold et al. 1997; Nicholas et al. 1993). Thus adrenergic receptors may normally be present in cutaneous afferents and, under certain conditions, have the capacity when activated to initiate responses in nociceptors.

One possible explanation for the increased incidence of adrenergically sensitive nociceptors is that nerve injury augments the ability of adrenergic receptors already in the skin to activate nociceptors. However, Drummond et al. (1996) reported an increase in α1-adrenergic receptors in the skin of neuropathic pain patients. This suggests that a nerve injury alters the synthesis of adrenergic receptors in either the dorsal root ganglion of the primary afferents innervating the skin or of other cells in the skin. Nerve injury may also alter the expression and relative distribution of different adrenergic receptor subtypes in dorsal root ganglion neurons (Birder and Perl 1996). Adrenergic supersensitivity could also develop as the result of the sympathetic denervation associated with a nerve injury. Nociceptors have been shown to develop...
adrenergic sensitivity following surgical sympathectomies (Bossut et al. 1996). However, chronic pain in the limb is rarely a complication of surgical sympathectomies for hyperhidrosis (Drott and Claes 1996; Lai et al. 1997; Zacherl et al. 1998).

Role of peripheral generators in neuropathic pain

The pain generator in human cases of nerve injury is generally thought to arise from spontaneous activity at the nerve injury site or perhaps more proximally from the dorsal root ganglion cells of fibers that have undergone axotomy. However, several lines of evidence suggest that pain may also arise from peripheral generators that are distal to the site of injury. 1) Spontaneous activity and catechol sensitivity develop in cutaneous nociceptors following nerve injury (this study; Koltzenburg et al. 1994; Sato and Perl 1991). 2) In patients with well-documented radiculopathy from lumbar disk herniation, a distal anesthetic block of the sciatic nerve eliminates the sciatic pain (e.g., North et al. 1996). 3) In tic douloureux, surgical lesions of the trigeminal nerve distal to the presumed trigeminal nerve compression near the brain stem abolish the pain (Quinn 1965; Stokey and Ranoshoff 1959). 4) Topical capsaicin is efficacious in treatment of neuropathic pain disorders caused by proximal nerve lesions (Ellison et al. 1997). 5) Topical clonidine attenuates hyperalgesia and pain in SMP (Davis et al. 1991). 6) Low-threshold mechanoreceptors are thought to be the peripheral generators for pain to light touch in the zone of secondary hyperalgnesia (LaMotte et al. 1991) as well as in patients with neuropathic pain (Campbell et al. 1988). Most likely the ectopic activity originating from the nerve injury site, the dorsal root ganglion, and the intact fibers may all contribute to the development and maintenance of central sensitization and spontaneous pain. The relative importance of each generator site may be influenced by a myriad of variables, including interval of time from the nerve lesion, species as well as individual differences, and type and location of the lesion.

This study establishes that C-fiber nociceptors in the skin develop a sensitization to adrenergic agents after nerve injury in primates. The role of A-fiber nociceptors in primates is not clear, but A-fiber nociceptors in rabbits appear to develop only a meager response to adrenergic agents after nerve neuroma by stimulation of the sympathetic supply in the rat.

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