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## ***The Role of Focal Nerve Ischemia and Wallerian Degeneration in Peripheral Nerve Injury Producing Hyperesthesia***

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**Background:** A new model of pain associated with an experimental peripheral mononeuropathy has stimulated interest in mechanisms of pain and their structural correlates in peripheral nerve, the site of the experimental lesion.

**Methods:** The pathology of the neuropathy was studied and the results correlated with alterations in nerve blood flow and with the behavioral response to heat applied to the foot. The focal neuropathy was created by loosely tying several ligatures around rat sciatic nerve, which produces hyperesthesia in the ligated limb in 3-5 days. The neuropathology was striking with epineurial and endoneurial vascular stasis, edema, and extensive nerve fiber injury in the ligated segment noted at 1 week after ligation.

**Results:** Nerve blood flow was reduced significantly in the ligated segment during the development of the hyperesthesia response, suggesting that changes in nerve blood flow caused by the ligature compression of the epineurial vessels contributes to the nerve fiber injury and pathophysiology of the model. To further test this hypothesis, the epineurial vasculature was removed from 1-cm lengths of rat sciatic nerve, which reduces nerve blood flow by 58%, and by ligation of the ipsilateral femoral artery, which focally reduces nerve blood flow by 70%, and the behavioral response to heating of the paw was evaluated at 1 week. Crush injury was used as a positive control creating Wallerian degeneration without a substantial reduction in nerve blood flow.

**Conclusions:** The results suggest that ischemia is an important initial pathogenic mechanism in the hyperesthesia associated with the loose ligature pain model, in so far as it produces Wallerian degeneration and axonal injury. Modest

degrees of ischemia producing only demyelination did not produce significant hyperesthesia. (Key words: Neuropathy. Neuropathology: pain.)

NEWLY developed techniques for testing thermal escape responses, in combination with the development of reliable techniques for creating chronic compression injury of nerves, have demonstrated that a behavioral hyperesthesia can be modeled in the rodent.<sup>1-4</sup> The model has focussed interest in identifying the mechanisms of hyperesthesia including the pathogenic mechanisms linking peripheral and central neuropathologic changes with abnormal behavioral function, the role of peripheral and central pathophysiologic processes in modifying the encoding of the pain message, and the identification of pharmacologic agents that can interrupt the pain. An important consideration is that studies already have suggested that pathologic factors at the site of injury can evoke structural changes in central terminals of primary afferents and in spinal neurons.<sup>5,6</sup> Such alterations argue for important changes in the substrate that processes afferent input.

The original model described by Bennett and Xie<sup>1</sup> consisted of the placement of four loose ligatures around rat sciatic nerve after surgical exposure. The ligatures are tightened until the nerve is slightly compressed, causing a twitch in the distal muscles and local blanching of the epineurium. The wound is closed and daily behavioral testing is performed by recording the latency to withdrawal of the hind feet from a focal heat source. Since each animal has only one nerve lesioned, with the contralateral, sham-operated nerve serving as control, the difference in latency scores between withdrawal of each hind foot is used as a measure of hyperesthesia in the receptive field of the lesioned nerve.

In this report, we corroborate the utility of this model and measure the changes in nerve blood flow after ligation in the compressed segment and in nerve segments proximal and distal to the ligation. Neuropathologic findings are presented at the light and ultra-

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structural levels. We then show that other forms of ischemic injury to rat sciatic nerve produce similar histologic and behavioral changes and discuss the pathogenic mechanisms common to these injuries that may have a role in the pathogenesis of hyperesthesia.

## Methods

### *Animal Preparation*

Seventy-nine adult Sprague-Dawley rats of either sex were used in several different experimental protocols approved by the local Animal Use Committee. In the present studies, five interventions were carried out that unilaterally involved the sciatic nerve at a space between the ischial notch and the popliteal fossa. The different protocols included groups of animals we identified as: control, loose ligature, epineurial devascularization, femoral artery ligation, and crush. Anesthesia for surgical procedures was accomplished with 2% halothane by mask or by the intraperitoneal injection of 0.3 ml/100 g body weight of a solution containing sodium pentobarbital (50 mg/ml), diazepam (5 mg/ml), and saline in volume proportions of 1:1:2, respectively.

**Control:** Control measurements were made in 10 rats in which both sciatic nerves were exposed by lateral incision of the thigh, and a 1-cm length of the nerve was mobilized by gentle dissection to free the epineurium from surrounding tissue. This procedure defines the sham operation that was used in all other groups of animals as the control lesion.

**Loose Ligature:** In another group of animals ( $n = 13$ ), loose ligatures were placed around one sciatic nerve, and the contralateral nerve received a sham operation. On the experimental side, four 4-0 chromic gut ligatures were placed 1.5 mm apart and tightened until a twitch was noted in the distal musculature. This was associated with minor visible compression of the nerve under the ligature and blanching in the adjacent epineurial circulation. In all animals, the wound was closed in two layers with 3-0 silk to approximate the muscle layer and metal staples to close the skin wound. The animals were observed closely until they recovered completely from anesthesia. Animals in obvious distress were euthanized, as were those who subsequently autotomized tissue distal to the lesion, although this was rarely necessary. In six additional animals, two ligatures placed approximately 3 mm apart were used instead of four ligatures to test the behavioral deficit of this

variation of the Bennett and Xie<sup>1</sup> model. This was the model used for measurement of nerve blood flow (see below), and we wanted to verify that it produced thermal hyperesthesia and nerve fiber injury similar to the four-ligature model.

**Epineurial Devascularization:** In 10 other animals, one sciatic nerve was exposed, and the epineurium with its associated circulation was removed over a 2-cm length. This was done by microdissection using two pairs of microforceps and a binocular operating microscope with fiber-optic illumination. Stretching or compression of the nerve was avoided, and the epineurium was left intact in the spaces between the three fascicles rather than risk injury to the nerve by manipulating it extensively to facilitate complete epineurial removal. We have shown previously that this procedure locally reduces nerve blood flow by approximately 58% in the subperineurial region, causing subperineurial ischemic damage to the nerve with significant demyelination.<sup>7</sup> The contralateral nerve received a sham operation.

**Femoral Artery Ligation:** One nerve in each of six additional animals was made ischemic by ligating the ipsilateral femoral artery at its exit from the abdomen. This produces a 70% decrease in sciatic nerve blood flow in a focal area 22–30 mm distal to the proximal edge of the tendon of the quadratus femoris muscle (Nukada and Myers, unpublished). The contralateral nerve received a sham operation.

**Crush:** One sciatic nerve in each of 10 rats was crushed in the mid-thigh region by smooth forceps for two 5-s periods separated by 5 s. This is an established method for producing Wallerian degeneration.<sup>8</sup> The contralateral nerve received a sham operation.

### *Nerve Blood Flow*

In 24 additional animals, nerve blood flow measurements were made by laser Doppler flowmetry in the area between two loose ligatures and in the areas immediately proximal and distal to the ligatures. Two loose ligatures were used because the flow probe would not fit between the ligatures in the four-ligature model. Since there were no radicular anastomotic vessels supplying the epineurial circulation between the ligatures, it was assumed that the four-ligature model would have a similar blood flow reduction to that recorded in the two-ligature model. Measurements were made before placement of ligatures (control), immediately after placement of the two ligatures, and in groups of eight animals each, at 24 h, 48 h, and 7 days after placement

of the ligatures. Nerve blood flow was measured in three adjacent 4-mm segments of nerve with a Perimed Periflux PF3 laser Doppler flowmeter (Piscataway, NJ) using a 0.5-mm diameter flow probe placed 1 mm above the exposed sciatic nerve. After control measurements, one loose ligature was placed at the proximal end and one at the distal end of the central segment, and measurements in all three segments were repeated. Measurements were repeated again in subgroups of animals ( $n = 8$ ) at 24 h, 48 h, and 7 days after ligation. To record nerve blood flow, five separate measurements were made over 5 min from slightly different locations along the nerve segment and averaged together to obtain a single value for that segment. Output of the laser Doppler device is given in proprietary flow units; since *in vivo* calibrations have not been made for peripheral nerve, it is not possible to express the results in absolute flow terms.<sup>7</sup> Instead, the results are reported as a percentage of a baseline value for nerve blood flow, determined separately for each animal. Data are expressed as mean  $\pm$  SD. Statistical comparisons were made with Student's independent samples, two-tailed *t* test.

#### *Behavioral Testing*

The thermal nociceptive threshold was measured with a device similar to one previously described.<sup>9</sup> The rats were placed in a plastic cage with a clear glass floor below which was a focal radiant heat source (halogen projector lamp CXL/CXP, 50W, 8v, Ushio, Tokyo). The heat source was movable beneath the floor and had a radiant beam approximately 3 mm in diameter that impinged on the glass floor and the plantar surface of the hind paw. The voltage to the thermal source was controlled by a constant voltage supply and was adjusted so that the average response latency in normal rats was 10 s. To initiate a test, the rat was placed in the cage and allowed 5–10 min to acclimate. The under-floor heat source was then positioned so that it was beneath the plantar surface of one hind paw. The heat source was activated until the paw was withdrawn. The time interval between the application of the heat and the hind paw withdrawal was measured to the nearest 0.1 s. In the absence of a response, the trial was terminated at 20 s. This maximum value was then assigned as the withdrawal latency. Each animal was its own control since there was only one lesioned nerve per animal. The difference in latency between the lesioned and sham-operated nerves, measured in seconds, was used as an indicator of hyperesthesia. Thus, a negative difference value corresponded to a

relative hyperesthetic response of the lesioned paw. A large, positive difference score suggests an anesthetic foot that was not sensitive to the heat stimulus. No difference or little difference in latency to withdrawal was characteristic of control animals or abnormal readings from the control paw. Although there is some variation in response between different animals to the heat stimulus, variations in latency between normal paws in the same animal was usually small. Statistical comparisons of the behavioral data to the control group were made by first using Fisher analysis of variance tests with 95% confidence levels to compare the means of the different experimental groups. Student's independent samples, two-tailed *t* test, corrected for multiple comparisons, was then used to assess the significance of the altered response to heat stimuli.

#### *Histology*

To examine the neuropathologic changes in the peripheral nerve, sciatic nerves were harvested in anesthetized animals at intervals after the placement of the ligatures. The nerve segments were fixed by immersion in phosphate-buffered 2.5% glutaraldehyde for 48 h, after which the ligatures were removed by fine dissection and the tissue cut into 2-mm blocks for processing for electron microscopy. Following dehydration, osmium infiltration, and embedding in araldite, 1- $\mu$ -thick sections were cut for light microscopy. Ultra-thin sections for electron microscopy were cut subsequently from selected blocks. Light microscopic sections were stained with pararphenylenediamine; EM sections were stained with uranyl acetate and lead citrate and viewed in a Siemens 101 electron microscope (Madison, WI) operating at 80 KeV.

Semiquantitative histologic evaluation of nerves from animals in the epineurial devascularization group was performed to correlate the behavioral deficit with the severity of pathologic change. A four-point scale, based on similar analyses used in previous studies,<sup>10,11</sup> was used and ranged from 0 for normal nerves to 3 for nerves with edema, demyelination, and axonal injury involving more than one-half of a major fascicle in the sciatic nerve.

#### **Results**

Nerve blood flow measurements were made immediately before and after the placement of two loose ligatures 3 mm apart around the sciatic nerve. The values recorded before placement of the ligatures were



tissue in response to irritation from a foreign body (the sutures). This abnormal tissue was not further disturbed during blood flow measurements and may have obscured the true endoneurial blood flow reading.

The behavioral data are depicted in figure 2A. Each animal is represented by a single data point recorded 7 days after operation. The control animals are clustered around a difference score of approximately zero; the average value is  $0.45 \pm 0.67$  s (SD). Animals in which four loose ligatures had been tied around the sciatic nerve had a hyperesthetic behavioral response to heat stimuli applied to the ipsilateral footpad. The average difference score was  $-1.63 \pm 1.74$  s and was significantly different from the control group ( $P < .002$ ). A smaller group of animals with two ligatures were also hyperesthetic to heat stimuli, having an average difference score of  $-4.58 \pm 1.10$  s ( $P < .001$ ) 7 days after surgery. There was no significant difference between the difference scores for the two- and four-ligature groups. Crush injuries and femoral artery ligations also produced a significant hyperesthetic response ( $P < .002$  and  $P < .001$ , respectively) with average difference scores of  $-2.04 \pm 2.08$  s and  $-1.29 \pm 0.84$  s, respectively. The average difference score for animals whose nerves had been stripped of the epineurial vessels was  $-0.38 \pm 1.43$  s and was not significantly different from the control value, although the majority of

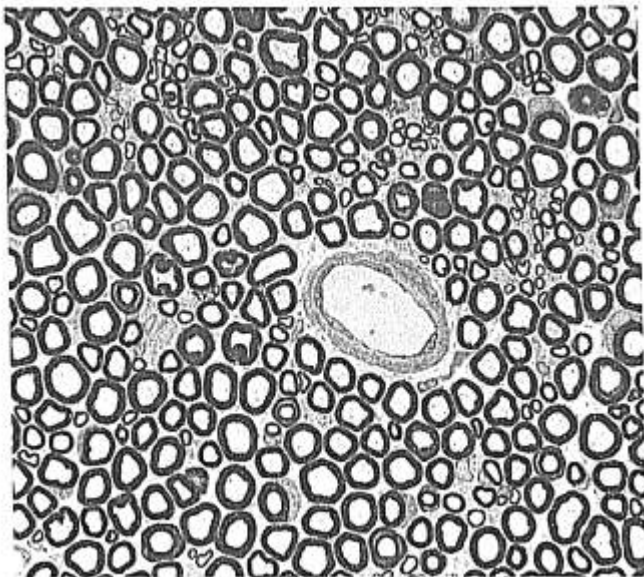


Fig. 3. Sham-operated control nerve showing the normal compact appearance of closely grouped myelinated fibers and an arteriole from the central region of the fascicle. Stain = paraphenylenediamine. Original magnification = 100X.

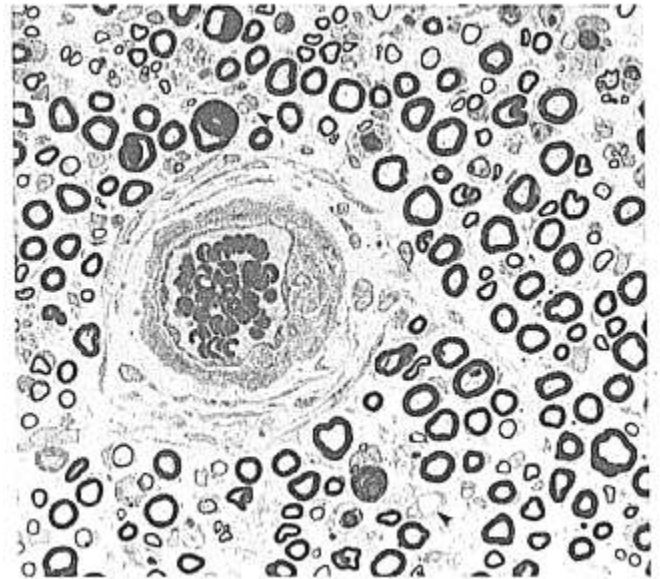


Fig. 4. Interstitial edema, vascular mural thickening, and nerve fiber injury are apparent 23 days after placement of four loose ligatures around the sciatic nerve. Thinly myelinated and demyelinated (arrowhead) fibers are observed. Stain = paraphenylenediamine. Original magnification = 100X.

difference scores were negative. In these hyperesthetic animals, there was a histologic correlation between the severity of the injury and the difference score (fig. 2B). Animals with mild degrees of ischemic injury that was limited to demyelination of subperineurial fibers had low difference scores, whereas animals with more severe ischemia producing axonal injury had a hyperesthetic response to heat stimuli.

After surgical exposure, the epineurial surfaces of rat sciatic nerve in both the two and four loose ligature models appeared thickened and hyperemic. Light and electron microscopic sections of nerves removed between 7 and 23 days after placement of loose ligatures showed a vigorous response by connective tissue elements on the epineurial surface consisting chiefly of thickening and proliferation of fibroblasts. Endoneurial edema was prominent at this time and could be seen in the subperineurial region, in perivascular spaces, and between nerve fibers (figs. 3, 4). Changes in endoneurial vessels also were noted (figs. 4-7). Specific changes in precapillary arterioles and capillaries included endothelial proliferation and mural thickening. In the larger vessels, this was due to an increase in the layers of smooth muscle cells. Fibroblasts were also prominent in the endoneurial interstitium, becoming more conspicuous because of increased cytoplasmic

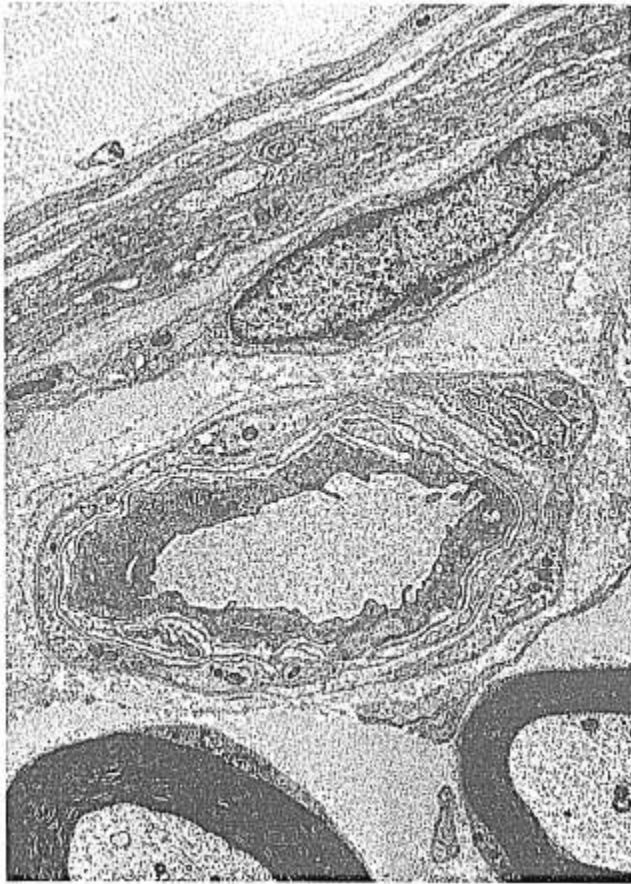


Fig. 5. Electron micrograph showing a normal-appearing capillary in the subperineurial space of a control rat. Stain = uranyl acetate and lead citrate. Original magnification = 3,000 $\times$ .

volume and prominent rough endoplasmic reticulum. Mast cells appeared throughout the endoneurium, as did macrophages in which the cytoplasmic areas were packed with lamellar debris and lipid droplets. Both axonal degeneration and demyelination were observed. Demyelination was noted especially in the subperineurial region but could be detected in other regions of the fascicle (fig. 8). Schwann cells appeared hypertrophied (fig. 8) and sometimes contained phagocytosed debris.

Compared to the epineurial devascularization model of nerve injury,<sup>7</sup> the loose ligature models were noteworthy for the striking changes elicited in both epineurial and endoneurial vessels in which mural thickening and endothelial proliferation were observed (figs. 4–7) and the degree of Wallerian degeneration distal to the lesion. The flattened, scaphoid appearance of normal endothelial cells gave way to rhomboid config-

urations with prominent polygonal nuclei instead of the spindle-shaped nuclear profiles characteristic of this cell type. Endothelial cytoplasm projected into the narrow lumens of small vessels. Enlargement of endoneurial vessels and mural thickening appeared due to proliferation of pericytes, and the cytoplasm of both these and endothelial cells contained masses of closely packed filaments. All of these changes are within the repertoire of vascular reactions to injury.

### Discussion

These findings corroborate previous reports that focal nerve injury in the rat produces hyperesthesia<sup>1–4</sup> and demonstrates that ischemia can be an important early

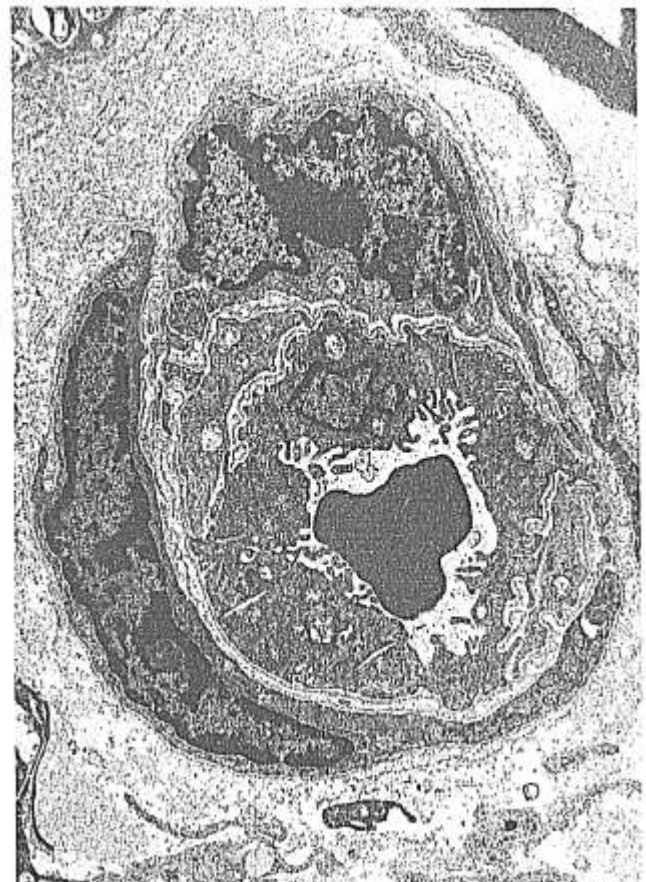
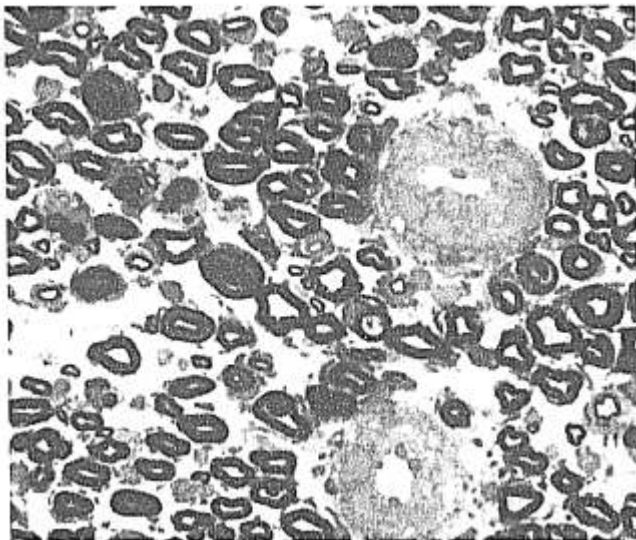


Fig. 6. Endothelial proliferation and vascular mural thickening are noted 7 days following four loose ligature constrictions of the sciatic nerve. Endothelial cells have numerous cytoplasmic projections in their luminal surfaces and have lost their normal scaphoid appearance. Stain = uranyl acetate and lead citrate. Original magnification = 3,000 $\times$ .

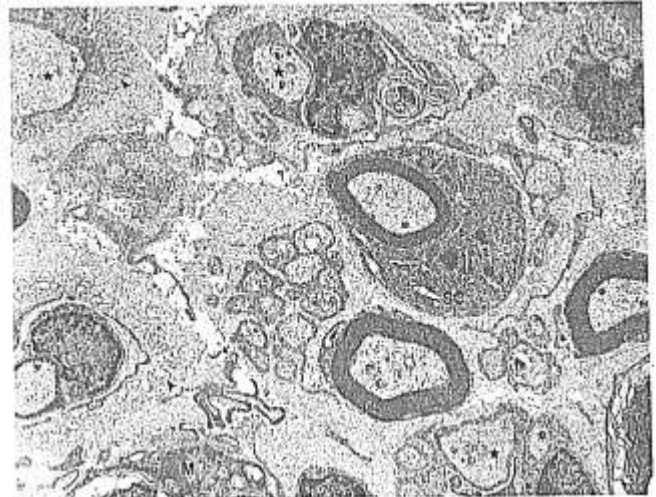


component of the mechanism of pain since nerve ischemia is sufficient to initiate the development of behavioral hyperesthesia in the ipsilateral limb if it produces Wallerian degeneration. Nerve blood flow was reduced significantly in each of the ischemic models as a part of the initial injury.<sup>7</sup> All of these ischemic injuries were associated with a qualitatively similar behavioral response characterized by hyperesthesia to heat stimuli applied to the foot pad; however, the severity of the injury was related more directly to the subsequent extent of the associated Wallerian degeneration.

The behavioral testing paradigm is central to the success of the loose ligature pain models, yet several potential problems can confound data interpretation. The test is based on the numerical difference in latency to withdrawal of the hind paws from a heat source. The data show that on the average there is little difference between the response of the right and left foot pads of individual control animals but the standard deviation of the results for all experimental animals dictates that relatively large groups of animals be tested to reduce the significance of the biologic and experimental variability. The sham operation does not alter the physiology or histology of the nerve and produces a behavioral response that is not significantly different from animals with no operation (unpublished observation).



**Fig. 7.** Interstitial edema, vascular mural thickening, and nerve fiber injury also are seen in nerves from the two loose ligature model 23 days after surgery. Other pathologic findings in this group were similar to those illustrated for the four loose ligature group. Stain = paraphenylenediamine. Original magnification = 100X.



**Fig. 8.** Several demyelinated fibers (stars) are visible in this electron micrograph. Ruffled, residual basal lamina (arrowheads) reflects loss of Schwann cell volume in animal from the four loose ligature model. Reactive changes of Schwann cells also can be seen with hypertrophic Schwann cell cytoplasm (SC). M = macrophage. Unmyelinated fibers appear within normal limits. Stain = uranyl acetate and lead citrate. Original magnification = 3,000X.

The reports of contralateral histologic changes in spinal cord secondary to nerve lesions<sup>6</sup> do not correlate with the behavioral data, suggesting that these changes, if present, are not sufficient to alter withdrawal latencies. Some variations in latency to withdrawal from heat are due to variations in room (plate) temperature and different skin temperatures,<sup>12</sup> while some are due to mechanical factors or are operator-induced including improper positioning of the heat source. The use of an under-plate, thermistor-controlled heater maintained at 30° C minimizes the influence of environmental temperature. Also of importance, given that the lesion may alter cutaneous skin temperature, is that the plate serves to condition the foot temperature. These variations also are minimized by the averaging of three separate readings for each latency score. Biologic and preparation variability in latency scores that cannot be controlled may be a factor in the wide standard deviation of the data and may account for our apparently aberrant results in which large positive difference scores were recorded in several of the ischemia (epineurial stripping) and crush animals. The data suggest that in some animals the limb was hypoesthetic or analgesic and, in fact, this is seen frequently in the experimental nerve immediately after injury. However, in our animals the primary data indicate a shorter than

## ISCHEMIA AND HYPERESTHESIA

normal latency on the control side that caused the difference score to be positive and which diminished the significance of the hyperesthesia recorded in the other animals of the group. More commonly, the variability in the behavioral scores was related to the extent of the nerve injury (fig. 2B). The correlation between the severity of pathologic findings and the average behavioral difference score was noteworthy in that the injuries producing extensive Wallerian degeneration were associated with hyperesthesia, whereas less severe injuries consisting primarily of demyelination resulted in a more moderate hyperesthetic response. The pathologic findings in the four loose ligature model were somewhat less severe than the crush model but greater than the femoral artery ligation and epineurial devascularization models and consisted primarily of demyelination with axonal degeneration distally as a consequence of focal ischemic injury. This order of the data correlates with the average hyperesthesia difference score. While this is consistent with our hypothesis, a definitive proof of the causative relationship between axonal injury and abnormal behavior will require detailed morphometric analysis in a larger series of animals.

Similar pathologic findings previously have been associated with the four loose ligature compression model.<sup>13,14</sup> Endoneurial edema was a prominent finding that presumably was associated with increased endoneurial fluid pressure.<sup>15</sup> Previous studies of Wallerian degeneration show that endoneurial fluid pressure is maximal at 6–7 days after injury,<sup>8</sup> which corresponds to the peak in hyperesthesia, and that endoneurial fluid pressure returns to normal values at a rate matching the decrease in hyperesthesia. The possibility exists that increased endoneurial fluid pressure may be reflected back to the dorsal root ganglia, providing a mechanical stimulus for evoking activity in sensory neurons.<sup>16,17</sup>

The loose ligature models affect nerve fibers throughout the fascicles, although the severity of the injury is variable. Epineurial devascularization also produces variable behavioral scores and pathologic findings that are a consequence of the surgical method that does not include removing epineurial tissue from between fascicles. Epineurial devascularization produces a predominantly demyelinating injury in the subperineurial region, which does not extend into the central core of the fascicle,<sup>7</sup> although in some animals, panfascicular injuries with Wallerian degeneration distally were ob-

served and correlated with greater behavioral hyperesthesia. We included a group of animals with crushed sciatic nerves to control for this extreme in compression nerve injury and expected these animals would have hypoesthetic or anesthetic difference scores if all nerve fibers were disrupted. We found, however, that they were hyperesthetic 7 days after injury. The pathologic findings were qualitatively as expected and previously reported.<sup>8</sup> However, at the crush site, some fibers were not injured. These remaining fibers may have been sufficient to mediate the nociceptive stimuli. The role of unmyelinated fibers in the pathogenesis of hyperalgesia is uncertain. It is suggested that myelinated fibers are in part responsible for the behavioral deficit and that unmyelinated fibers are less significantly involved.<sup>14</sup> Our test of intrathecal capsaicin<sup>18</sup> produced hypoalgesia to noxious heat stimuli in control nerves, but did not reduce the hyperesthesia in the four loose-ligature experimental nerves. However, Shir and Seltzer<sup>4</sup> report that neonatally capsaicinated rats did not develop thermal hyperalgesia following partial nerve injury. The actual mechanism of the changes underlying the alterations in sensory coding leading to a hyperesthetic response is not known, but it may be in the dorsal column of the ipsilateral spinal cord where degenerating neurons have been seen after nerve injury.<sup>5,6,16</sup> The processes leading to the central changes are not known, although ectopic electrophysiologic activity in the dorsal root ganglia may contribute.<sup>19</sup> Recent studies have suggested that factors generated at the site of injury may be necessary, as the hyperesthesia can be blocked by application of topical colchicine to the nerve proximal to the injury site.<sup>18</sup> Colchicine blocks retrograde axonal transport and presumably prevents the movement of a factor or factors signaling central awareness of injured nerve. It is known that retrogradely transported nerve growth factor alters sensory neuronal function.<sup>20,21</sup> Other growth factors or cytokines from hematogenous macrophages also may have a role in central nociceptive processing. The recruitment of hematogenous macrophages occurs within 1 week of injury and coincides with the peak in hyperesthesia. Also, the reduction of hyperesthesia temporally matches the elimination of macrophages from the endoneurial space. In addition to their phagocytic role, nonresident macrophages are potent sources of cytokines with varied biologic activities. The severe endothelial changes we report may be caused by tumor necrosis factor liberated from macrophages, since there



is a similarity in endothelial pathology between our results and experiments in other tissues with controlled application of this factor.<sup>22</sup>

Our report has emphasized the role of nerve ischemia as an initial pathogenic factor capable of producing hyperesthesia, but the principal mechanism of hyperesthesia remains unknown. Ischemia initiates a cascade of pathologic events including nerve edema, increased endoneurial fluid pressure, macrophage recruitment, and phagocytosis with related biochemical changes that are proportional to the degree of injury, and these latter changes may be more directly related to the development of hyperesthesia than the initial ischemic insult.

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