**AMITRIPTYLINE** is a tricyclic antidepressant drug that has been given orally or intravenously for the management of neuropathic pain.1-3 Amitriptyline has multiple complex pharmacologic actions that contribute to its analgesic activity: It inhibits norepinephrine and serotonin reuptake4 and blocks α2-adrenergic, nicotinic, muscarinic, cholinergic, N-methyl-D-aspartate, and histaminergic receptors.5

Despite these biologic effects, the clinical efficacy of amitriptyline for pain control remains controversial. It was recently reported in a human controlled trial with an “active” placebo (i.e., a placebo inducing similar adverse effects) that amitriptyline used orally was not effective in reducing chronic pain in patients with spinal cord injury.6 This might be because of its variable intestinal absorption.7,8 Amitriptyline also has significant potential adverse effects. In the central nervous system, these effects include sedation, seizures, and coma. Cardiologic toxicity includes QRS complex widening and cardiac arrest.9

Nevertheless, amitriptyline is an appealing analgesic and local anesthetic agent because it is a more potent Na+ channel blocker than bupivacaine when used for sciatic nerve block,10 and it has been reported to block voltage-gated K+ and Ca2+ channels.11,12 Experimentally, amitriptyline seems to have a more efficacious analgesic effect after peripheral administration13 than after intrathecal14,15 spinal,15 or intrathecal administration.1,14

Recent studies reported a use-dependent blockade and the prolongation of peripheral nerve blockade by amitriptyline or various derivative amitriptyline solutions (N-phenylethyl or N-methyl amitriptyline).16-18 This work has led to the suggestion that amitriptyline with epinephrine could be clinically useful for infiltration and postoperative analgesia.5 A recent clinical study and editorial in *Regional Anesthesia and Pain Medicine* provides cautious endorsement for this application of the drug.18,19

However, these experimental demonstrations and recommendations for the use of amitriptyline as a local anesthetic have apparently occurred without detailed preclinical neuropathologic studies. Because amitriptyline has significant neurotoxic potential, it is imperative that the drug be formally tested for peripheral neurotoxicity using established neuropathologic techniques with greater sensitivity and resolution than the paraffin hematoxylin and eosin preparations that have already been performed.20 Although useful in assaying gross histologic change, this latter method of tissue processing introduces artifacts in neurologic structures that can obscure early or subtle alterations in the relationships among axons, Schwann cells, and myelin that provide insights into neurotoxicologic mechanisms. Even if the drug is to be applied as a cream on the skin or injected subcutaneously, at some time it will surely come in contact with peripheral nerves in concentrations that were not intended. Because of the rapidly escalating use of amitriptyline as a local anesthetic without these data, we undertook the current study to determine the neuropathologic effect of extraneural administration of am-
Bupivacaine).5,17,21 Other local anesthetics (1–25 mg/ml, 39.8 mM or 8.0 nmol), 1.25 mg (6.25 mg/ml, 19.9 mM or 4.0 nmol), and 0.625 mg (3.125 mg/ml, 10.0 mM or 2.0 nmol). The pH of the solutions ranged from 4.12 (5-mg preparation) to 5.72 (0.625-mg preparation). We determined in preliminary histology experiments that this pH difference was not significant in the in vivo setting where a small volume is neutralized by tissue pH. Each group contained 12 rats. The experimental doses were dissolved in 0.2 ml saline immediately before application: 5 mg (25 mg/ml, 79.6 ms or 16 nmol), 2.5 mg (12.5 mg/ml, 39.8 ms or 8.0 nmol), 1.25 mg (6.25 mg/ml, 19.9 ms or 4.0 nmol), and 0.625 mg (3.125 mg/ml, 10.0 ms or 2.0 nmol). The pH of the solutions ranged from 4.12 (5-mg preparation) to 5.72 (0.625-mg preparation). We determined in preliminary histology experiments that this pH difference was not significant in the in vivo setting where a small volume is neutralized by tissue pH. Each group contained 12 rats. The experimental doses were determined to be compatible with doses used in previous rodent models of transdermal,21 subcutaneous,5,22,23 intrapertioneal,14,24 and intrathecal13 application or for its use in sciatic nerve block experiments10,17 for comparison with other local anesthetics (1–5% lidocaine and 0.5% bupivacaine).5,17,21

Materials and Methods

Drug

A commercially available, preservative-free preparation of amitriptyline was used in this study (amitriptyline-hydrochloride, Laroxyl®; Laboratoires Roche, Neuilly-sur-Seine, France). Four doses were evaluated, each dissolved in 0.2 ml saline immediately before application: 5 mg (25 mg/ml, 79.6 ms or 16 nmol), 2.5 mg (12.5 mg/ml, 39.8 ms or 8.0 nmol), 1.25 mg (6.25 mg/ml, 19.9 ms or 4.0 nmol), and 0.625 mg (3.125 mg/ml, 10.0 ms or 2.0 nmol). The pH of the solutions ranged from 4.12 (5-mg preparation) to 5.72 (0.625-mg preparation). We determined in preliminary histology experiments that this pH difference was not significant in the in vivo setting where a small volume is neutralized by tissue pH. Each group contained 12 rats. The experimental doses were determined to be compatible with doses used in previous rodent models of transdermal,21 subcutaneous,5,22,23 intraperitoneal,14,24 and intrathecal13 application or for its use in sciatic nerve block experiments10,17 for comparison with other local anesthetics (1–5% lidocaine and 0.5% bupivacaine).5,17,21

Animals and Surgery

Adult female Sprague-Dawley rats (weight, 200–250 g; Harlan Labs, Indianapolis, IN) were maintained in an animal care facility approved by The Association for Assessment of Laboratory Animal Care and handled in accordance with National Institutes of Health Guidelines for Animal Use. The protocol was approved by the local animal research committee (San Diego, California). Rats were briefly anesthetized with isoflurane (induction box and maintained via a facemask; 3–4%). The sciatic nerves were exposed by lateral incision of the thighs and retraction of superficial fascia and muscle. Under magnification, 0.2 ml of the test dose was injected with a 25-gauge needle directly beneath the clear fascia surrounding the nerve but outside the epineurium and perineurium, proximal to the sciatic bifurcation. The perineurium was not breached. The superficial muscle layer was sutured with 4-0 silk, and the wound was closed with metal clips.

Neurobehavioral Evaluation

After recovery from general anesthesia, sciatic nerve function was evaluated by observing the rat’s ability to hop and to place weight on its hind limb and by the rat’s ability to grasp with its hind paw when suspended by the tail, using a scale that reflected the presence or absence of the result. Resting posture and postural reactions (‘‘hopping’’ and ‘‘tactile’’ placing) were also recorded. The contralateral side was used as control. Animals were tested every 10 min during the first hour, then every 30 min until 7 h. Animals that had not recovered by that time were evaluated twice per day until the day of sacrifice.

Pincching behaviors (lifting, shaking, or rippling of the haunch) and biting/licking behaviors were recorded. Vocalization and the presence or absence of a withdrawal reflex to pinching of the hind limbs were also recorded. These tests were performed two times each day before sacrifice (days 1–7). Grooming, food intake, responsiveness to environmental stimuli, and exploratory activity were also monitored.

Tissue Processing

For the four dose groups, animals were killed at 1, 3, and 7 days after amitriptyline application (n = 4 at each day). The two sciatic nerves (i.e., treated and contralateral side as control) were excised under the anesthesia protocol used for surgery; then, the rat was euthanized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg). For fixation, the nerves were immersed in 2.5% phosphate-buffered glutaraldehyde for 24 h. They were then rinsed with phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated in serial concentrations of alcohol, and embedded in araldite according to the recommended procedure for neurotoxicologic tissue evaluation.20 One-micrometer-thick semithin sections from the central 2-mm block of each 6-mm-long segment were cut for light microscopy and stained with methylene blue, azure II. Tissue sections were evaluated by an observer (R. R. M.) who was unaware of the experimental groupings.

Semiquantitative evaluation of neuropathologic change was performed in animals receiving saline and 0.625–2.5 mg amitriptyline. The nerves receiving 5 mg amitriptyline were too severely damaged to process for morphologic analysis. This was done to determine if there were statistically significant changes in neuropathology. A value was assigned for each histologic slide based on a scale with one-half unit increments from 0 to 4 in which 0 represented normal nerve and 4 represented a finding of Wallerian degeneration extending throughout the nerve bundle.

Statistical Analysis

The data were analyzed by one-way analysis of variance with Tukey-Kramer multiple comparison tests.
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Results

Rat Sciatic Nerve Blockade

After the animals had recovered from general anesthesia, no adverse clinical systemic effects were recorded, i.e., no overt cardiac impairment, seizures, or sedation was observed in any of the rats. Three minutes after the end of surgery and general anesthesia, neurologic evaluation could be performed. All of the rats in the experimental groups receiving 5, 2.5, and 1.25 mg amitriptyline (79.6, 39.8 and 19.9 ms, respectively) had complete motor blockade at this time. In contrast, complete motor blockade was delayed in animals receiving the 0.625-mg (10-ms) dose until 20 ± 5 min after injection, and the duration of complete blockade was 4 ± 0.5 h in this group. All animals in this group recovered complete motor function and had normal behavior evaluations thereafter. However, for the animals receiving doses of 1.25 and 2.5 mg amitriptyline, the durations of block were 7 ± 1.5 and 24 ± 5 h, respectively, and 50% of the these animals (four of eight in each group) did not completely recover by day 2. Some of the animals in this group showed weakness in motor function (i.e., unilateral hind-limb gait deficit) and increased flinching and biting/licking behaviors until day 7 (two of four in each group). Animals in the 5-mg dose group had no recovery of motor function during the 7 days of evaluation. By day 3, clear hyperalgesic and allodynic behaviors (i.e., avoiding use of the injected leg, biting/licking activity, vocalization when rats were held or when the hind limb was touched) were observed in all rats in this group, which persisted until the animals were killed through day 7.

Neuropathologic Findings

Macroscopic Evaluation. When the surgery was performed for sciatic excision, considerable sciatic edema was observed in animals receiving 5, 2.5, or 1.25 mg amitriptyline, as manifested by gross enlargement of the nerve bundle and a change in gross fascicular color to pale gray from a normal pink. These changes were associated with extensive hypervascularization of the epineurial tissue. The appearance of the muscle also seemed altered because it was pale in the animals receiving 5- or 2.5-mg doses of amitriptyline. In animals receiving the 0.625-mg dose of amitriptyline, the muscle seemed to be normal; however, the sciatic nerve seemed slightly edematous compared to the contralateral control side.

Microscopic Evaluation. All nerves exposed to amitriptyline displayed neuropathologic changes. These changes ranged from relatively mild and reversible in the 0.625-mg dose group to severe and irreversible in the 5-mg dose group, based on the degree of Wallerian degeneration and the likelihood for successful regeneration. It was clearly evident that amitriptyline caused a dose-related injury to the axon and supporting tissue that was manifested as the neuropathologic process called Wallerian degeneration. The data are consistent with and parallel in magnitude the motor and behavioral findings described above. That is, low doses of amitriptyline injured relatively few axons and support cells, such that neurologic function was not significantly affected after recovery from the local anesthetic effect of the drug. High doses of the drug caused immediate and lasting injury to the peripheral nervous system that was accompanied by neuropathic pain-like behaviors.

Figure 1 is taken from animals receiving the 1.25-mg (intermediate) dose of amitriptyline and depicts aspects of the neurotoxic injury that can be seen to variable degrees in every animal studied. In these animals, by day 1, there was significant endoneurial edema, particularly evident in the subperineurial region adjacent to the application of the drug, but also evident in the perivascular region and between individual nerve fibers that were tightly packed in contralateral nerves used for control or in vehicle-treated nerves previously studied. There was also dark-staining axoplasm, indicating axonal injury. This finding was more prevalent in the subperineural region. By day 3, these findings were more severe, with increased edema and axonal pathology throughout the fascicles. Numerous Schwann cells were activated, and myelin lamellae had begun to disintegrate; there was fibroblast proliferation and immune cells adherent to the lumen of intraneuronal vascular endothelia. By day 7, endoneurial edema was reduced, but there was clear evidence of significant ongoing Wallerian degeneration with nerve fibers exhibiting the processes described above intermixed with degenerated nerve fibers undergoing phagocytosis by invaded macrophages.

Figure 2 shows these same processes in the 0.625-, 1.25-, and 2.5-mg groups, but the magnitude of the changes are dose-related. In the 2.5-mg group and 5-mg group (not illustrated), Wallerian degeneration was extreme, with essentially all nerve fibers affected.

Figure 3 depicts in bar graph form the degree of nerve fiber injury, using a semiquantitative scale for the extent of Wallerian degeneration. All treatment groups displayed were statistically separate from the control group at the level of $P < 0.01$. All treatment groups were also statistically different from each other at the level of $P < 0.01$ except for the 1.25- and 2.5-mg groups, which were significantly different at a level of $P < 0.05$.

Discussion

Amitriptyline is neurotoxic to peripheral nerve fibers, causing direct injury to axons that produce Wallerian degeneration of the nerve fibers. This occurs even though the drug is applied outside the peripheral nerve bundle in relatively low doses. An increased neurotoxic
The pathologic process invoked by amitriptyline is Wallerian degeneration and was first described by Augustus Waller in 1850 in the context of nerve transection. It is a complex process that begins with injury to the axon and results with degeneration of the axon and its support cells (collectively termed the nerve fiber) from the site of axonal injury distally to the terminal tissue. The axon initially becomes electron dense or dark staining, and this is rapidly followed by clumping of organelle and other particles in the axoplasm, swelling of the axon, and disintegration. During this time, axon–Schwann cell communication is altered, and Schwann cells become activated (expressing proinflammatory cytokines) and undergo mitosis. Myelin is disintegrated and phagocytosed by Schwann cells and by invading hemogenous macrophages, which are recruited in large numbers several days after the start of Wallerian degeneration in response to upregulation of tumor necrosis factor α and other proinflammatory chemoattractant proteins. This process occurs in a proximal–distal direction from the site of injury throughout the distal neurologic tissue in a sequential temporal pattern and is often the intended neurolytic process associated with cryolysis or the injection of alcohol. In these cases, it is intended to cause essentially irreversible change in the function of the nervous system rather than the short-term and more reversible changes caused by neurotoxic agents injuring Schwann cells or myelin and sparing the axon.

Although the exact molecular mechanism of amitriptyline neurotoxicity is unclear, it is known that other concentrated local anesthetic agents and severe ischemia can cause Wallerian degeneration, whereas control (vehicle) solutions, such as the saline used in these...
It is interesting to note the relation of drug concentration to neurotoxic injury, as indicated by the susceptibility of nerve fibers near the periphery of the nerve bundle in the subperineurial space, which are closest to epineural administered drugs. With even low doses (0.625 mg) of amitriptyline administered in the epineural space (in a 0.2 ml volume), we observed some nerve fibers undergoing Wallerian degeneration in the subperineurial space. With higher doses (delivered in the same volume) the number of degenerating fibers was increased, as was their spatial distribution.

The functional consequences of Wallerian degeneration are severe, as axonal communication with muscles/sense organs is completely interrupted from/to their neurons. Fortunately, peripheral nerve fibers attempt to regenerate in response to neurotrophic factors liberated by denervated and degenerating tissue. There is an important link between nerve degeneration and regeneration in that degeneration must be complete before regeneration can proceed. Both of these processes are driven by proinflammatory cytokines, which we believe are also the principal factors in orchestrating the development of neuropathic pain states. This relation is reinforced by the findings in this study, which link severe Wallerian degeneration with hyperalgesia.

In subcutaneous administration of amitriptyline, a dose of 10 nmol was reported to be inactive in formalin-evoked behaviors. However, at doses of up to 100 nmol administered by this route, amitriptyline induced tissue edema (i.e., increase of paw volume), which exhibited a long time course. This edema was not mediated by biogenic amines because it was not blocked by a histamine H1 receptor antagonist (mepyramine). Transcutaneous administration of amitriptyline at a high dose (500 mg) seems to be toxic to the skin. In comparison with the same concentration of bupivacaine administered subcutaneously, amitriptyline at a dose that was...
Finally, it has been reported that an axial segmental degeneration sometimes occurs with percutaneous administration of 5 mM N-phenylethyl amitriptyline, a molecule reported to be even more powerful than amitriptyline hydrochloride. This pathologic finding was correlated clinically with incomplete recovery of the block. Therefore, the demonstration of incomplete recovery or very long differential blocks with experimental local anesthetic agents (5–43 h for complete and full recovery for 2.5 mM, and 22–79 h for 5 mM N-phenylethyl amitriptyline; 3–4.5 h for 5 mM, and 4–10 h for 10 mM amitriptyline hydrochloride) should be interpreted cautiously with the suspicion that neurotoxic injury may be part of the mechanism for delayed recovery.

References