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# *Assessment of Differential Blockade by Amitriptyline and Its* N*-Methyl Derivative in Different Species by Different Routes*

*Peter Gerner, M.D.,* Anna E. Haderer, M.D.,† Mustafa Mujtaba, Ph.D.,† Yukari Sudoh, M.D.,† Sanjeet Narang, M.D.,‡ Salahadin Abdi, M.D., Ph.D.,§ Venkatesh Srinivasa, M.D.,† Christof Pertl, M.D., Ph.D.,‖ and Ging Kuo Wang, Ph.D.#*

***Background:*** **Increasing the duration of local anesthesia and/or creating greater differential blockade (*i.e.*, selective block of pain-transmitting nerve fibers) has been attempted by modifying currently available agents. Most drugs show a different profile depending on the model or species studied. This study was designed to investigate the differential nerve-blocking properties of amitriptyline and its quaternary ammonium derivative in rats and sheep.**

***Methods:*** **The $Na^+$ channel–blocking properties of *N*-methyl amitriptyline were determined with the patch clamp technique in cultured $GH_3$ cells. Various functions (motor, nociception, proprioception–ataxia) were compared in rats (spinal and sciatic nerve blockade) and sheep (spinal blockade) with amitriptyline, *N*-methyl amitriptyline, lidocaine, and bupivacaine (partially from historical data).**

***Results: In vitro*** **testing revealed *N*-methyl amitriptyline to be a potent $Na^+$ channel blocker similar to amitriptyline but with a much longer duration of action. All drug concentrations tested in both the sciatic nerve model and the spinal block model produced no significant differential blockade in rats. Three of six rats in the 20-mM *N*-methyl amitriptyline group showed residual blockade 4 days after sciatic nerve injection. However, in the sheep spinal model, amitriptyline and in particular *N*-methyl amitriptyline displayed significant differential blockade at most time points. Sheep data for lidocaine and bupivacaine seemed to be more comparable to the clinical experience in humans than did rat data.**

***Conclusions:*** **Amitriptyline and *N*-methyl amitriptyline are potent $Na^+$ channel blockers and show greater differential blockade in sheep than in rats. This differential blockade in sheep is greater than that produced by lidocaine or bupivacaine.**

IN earlier work, amitriptyline was found to be a more potent local anesthetic than bupivacaine for rat sciatic nerve blockade, probably because of its greater potency to block $Na^+$ channels.[1,2] In that rat model, amitriptyline displayed some differential blocking properties, *i.e.*, selective block of specific (pain-transmitting) nerve fiber groups,[3] whereas no differential blockade was found with bupivacaine. This is in contradiction to the clinical impression that bupivacaine is the drug of choice when a more sensory-selective action over motor blockade is desired. We hypothesize that species differences account for the different amounts of differential blockade seen previously. We therefore investigated the differential block phenomenon *via* two different routes of administration in two different species (peripheral sciatic nerve block in rats and intrathecal block in rats and sheep).

In addition, because adding a methyl group to amitriptyline has been shown to increase blocking properties for $K^+$ channels in isolated rat sympathetic neurons,[4] we investigated whether this held true for $Na^+$ channels in $GH_3$ cells. We also investigated whether the addition of the methyl group to amitriptyline increased the amount of differential blockade. We studied this *N*-methyl derivative of amitriptyline because it has interesting physicochemical properties, *i.e.*, it is more soluble than amitriptyline and *N*-phenylethyl amitriptyline.[5] Preliminary electrophysiologic studies revealed that this drug is trapped within the cell and its block of sodium channels remains even after prolonged washoff.

## Materials and Methods

The protocol was approved by the Harvard Medical Area Standing Committee on Animals and by the Austrian Federal Ministry of Education, Science, and Culture. Female sheep (*Pecus montis austriacus*) weighing 40–50 kg were purchased from Biomedical Research (Graz, Austria); male rats (Sprague Dawley) weighing 250–300 g were purchased from Charles Rivers Laboratories (Cambridge, MA) and handled for a period of several days to familiarize them with the experimenter and the protocol.

### *Cell Culture and Electrophysiologic Experiments*

Rat clonal pituitary $GH_3$ cells were purchased from the American Type Culture Collection (Rockville, MD), split twice a week, and maintained in Dulbecco's modified Eagle's medium (Hyclon Laboratories, Logan, UT) supplemented with taurin (1%), penicillin–streptomycin (1%), HEPES (20 mM), and heat-inactivated fetal bovine serum (10%) as described previously.[6] For $Na^+$ current recording, cells were grown in a 35-mm culture dish, which was then used as a recording chamber. The whole-cell variant of the patch clamp method was used to measure $Na^+$ current in rat clonal $GH_3$ cells.[6] The

* Assistant Professor of Anesthesia, † Research Fellow, ‡ Instructor in Anesthesia, # Associate Professor of Anesthesia, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, and § Assistant Professor of Anesthesia, Department of Anesthesia and Intensive Care, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. ‖Professor of Oral Surgery, Department of Oral Surgery, University of Graz, Austria.

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Address reprint requests to Dr. Gerner: Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Address electronic mail to: pgerner@partners.org. Additional article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

external solution contained 150 mM choline Cl and 10 mM HEPES adjusted to pH 7.4 with tetramethylammonium hydroxide. Micropipettes were fabricated and had a tip resistance of approximately 1 MΩ when filled with a $Na^+$ current solution containing 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with CsOH. These solutions create an outward $Na^+$ gradient and current, further reducing potential problems associated with series resistance errors.

The junction potential of electrodes was nulled before seal formation. After the patch membrane ruptured, the cell was allowed to equilibrate with the pipette solution for at least 15 min at a holding potential of −100 mV. For the electrophysiologic experiments, amitriptyline and *N*-methyl amitriptyline were dissolved in dimethyl sulfoxide at 100 mM, diluted shortly before the experiments, and applied at appropriate concentrations to cells with a flow rate of approximately 0.12 ml/min through a series of narrow-bored capillary tubes positioned within 200 μm of the cell. Drugs were washed out with a tube containing the external solution. Voltage clamp protocols were created with pClamp software (Axon Instruments, Foster City, CA) and delivered by a patch clamp amplifier (EPC7; List-Electronic, Darmstadt/Eberstadt, Germany). Leak and capacitance currents were subtracted by the P/−4 protocol. All experiments were conducted at room temperature (21 ± 2°C). At the end of the experiments, the drift in the junction potential was generally less than 2 mV.

*Nerve Blocks and Surgical Preparation*

Sciatic nerve block in rats anesthetized with sevoflurane was performed with a 27-gauge hypodermic needle attached to a tuberculin syringe at the sciatic notch as described previously.[7]

For rats in the intrathecal catheter group, a PE-10 catheter was inserted during xylazine–ketamine anesthesia by the direct lumbar catheterization method[8] (catheter-through-needle method). The catheter was inserted approximately 2 cm beyond the tip of the guide cannula into the subarachnoid space to reach the level of the caudal ribs, which corresponds to the lumbar enlargement of the spinal cord. The needle was carefully withdrawn, avoiding displacement of the catheter. The catheter was then tunneled under the skin, exiting at the occipital region, and sutured in place.

Because it is very difficult to perform and evaluate the efficacy of a sciatic nerve block in sheep, we opted to perform only intrathecal injections in these animals. These injections were performed in awake sheep as described previously,[9] with the sheep in the standing position with the front legs elevated approximately 30 cm to increase the cerebrospinal fluid pressure in the lumbar region. After infiltration of the skin with 2 ml of 0.5% lidocaine at the $L_6$–$S_1$ interspace, a 22-gauge Quincke spinal needle (Becton Dickinson and Co., Franklin Lakes, NJ) was advanced under sterile conditions to locate the subarachnoid space. Once free flow of cerebrospinal fluid was observed, the drug was injected. The animal was held in this position for an additional 10 min so that the possible baricity-dependent spread of the drug would be similar in all animals. Rats with neurologic deficits after insertion of the catheter and animals that did not achieve bilateral blocks were excluded from the study.

*Neurobehavioral Examination*

In rats, neurobehavioral examination consisted of evaluation of motor function, proprioception, and nocifensive response immediately before injection and at various intervals after injection. Changes of function were graded and presented as percentage of maximal possible effect as described previously.[7] In brief, motor function was evaluated by measuring the "extensor postural thrust" of the hind limbs by holding the rat upright with the hind limb extended so that the distal metatarsus and toes supported the animal's weight and measuring the extensor thrust as the gram force applied to a digital platform balance. Proprioception evaluation was based on the resting posture and postural reaction ("tactile placing" and "hopping"). This test was performed by lifting the front half of the animal off the ground and lifting one hind limb at a time off the ground so that the animal was standing on just one limb. Then the animal was moved laterally, which normally evokes a prompt hopping response with the weight-bearing limb in the direction of movement to prevent the animal from falling. A predominantly motor block would cause a prompt but weaker-than-normal response. Conversely, a predominantly proprioceptive block causes delayed hopping followed by greater lateral hops to prevent the animal from falling. In the case of full blockade, there would be no hopping maneuvers. Nocifensive reaction was evaluated by the withdrawal reflex or vocalization to pinch of a skin fold over the lateral metatarsus (cutaneous pain) and of the distal phalanx of the fifth toe (deep pain).

In sheep, neurobehavioral examination was similar to that reported in earlier work.[9] Nociceptive reaction was assessed by graded pinches with an Allis forceps to the skin between the hooves, and the animal was observed for nocifensive behavior (escape actions, increased respiratory rate, defecation, restlessness). Motor function was assessed as 100% maximal possible effect (no motor function at all and/or no muscular tone), 75% (animal is able to stand when lifted up), 50% (able to walk but requires help to get up from the recumbent position), 25% (able to get up without help, walks, but can be pushed down by applying pressure to the back), and 0% (animal strongly resists being pushed to the ground; baseline). Proprioceptive testing as outlined above for rats was replaced by observation and grading of any gait disturbance or lack of muscular coordination (ataxia).

For consistency, the experimenter was the same for each study group. Although experimenters were initially blinded, it soon became clear whether the animal belonged to a study or control group, low or high dose, respectively. However, the experimenter was unaware of the results of the preceding evaluation.

*Drugs and Their Administration for* In Vivo *Experiments*

Amitriptyline (Sigma Chemical Co., St. Louis, MO), lidocaine (AstraZeneca, Inc., Waltham, MA), and *N*-methyl amitriptyline (Sigma) were dissolved in NaCl 0.9% for the sciatic nerve injections or in dextrose 7.5% for the spinal injections. After injection, the low pH of these plain solutions (ranging from pH 5.0 to 6.5) is likely to be buffered quickly by the tissue fluid, which has a pH of 7.4. *N*-Methyl amitriptyline was custom synthesized, the purity was greater than 99% by high-performance liquid chromatography, and the molecular weight was 372.3.

For rat sciatic nerve blockade, 0.2 ml of *N*-methyl amitriptyline was injected at concentrations of 5, 10, and 20 mM (n = 6 or 7 per group). The control consisted of amitriptyline and lidocaine, which were administered in our laboratory under conditions identical to those reported previously.[1,10]

For rat spinal blockades, the intrathecal catheter was injected with 90 μl of either amitriptyline or *N*-methyl amitriptyline at their respective concentrations of 5 and 10 mM (n = 6 or 7 per group). Comparison was made with lidocaine at 40 mM, which had been reported previously in our laboratory.[11] The relatively high volume of 90 μl was necessary because amitriptyline is very hydrophobic and did not spread sufficiently to provide a block to the lower thoracic levels in pilot studies at lower volumes. *N*-Methyl amitriptyline, conversely, is much more hydrophilic and therefore appears to spread to cervical levels when used at this high volume. Because pilot studies with the 20-mM concentration caused several animals in the *N*-methyl amitriptyline group to suffer respiratory arrest at this concentration and volume, we ceased investigating with this concentration.

Sheep spinal blockade was performed with 2.5 ml of amitriptyline at the same concentrations as for rat intrathecal injections or *N*-methyl amitriptyline at 5 and 10 mM and compared with lidocaine at 40 mM (n = 4–7 per group). Similar to the rat studies, pilot studies with an *N*-methyl amitriptyline concentration of 20 mM revealed an extensive spread to the cervical levels, with ensuing blockade of the front legs and respiratory compromise; therefore, sheep were euthanized, and study of this group was abandoned.

*Statistical Analysis*

An unpaired Student *t* test was used to detect significant differences among the motor and nociceptive functions of the animals after amitriptyline, *N*-methyl amitriptyline, or lidocaine injection (Origin, Microcal Software, Inc., Northampton, MA). Data are presented as mean ± SEM. Statistical significance was defined as a value of $P < 0.05$.

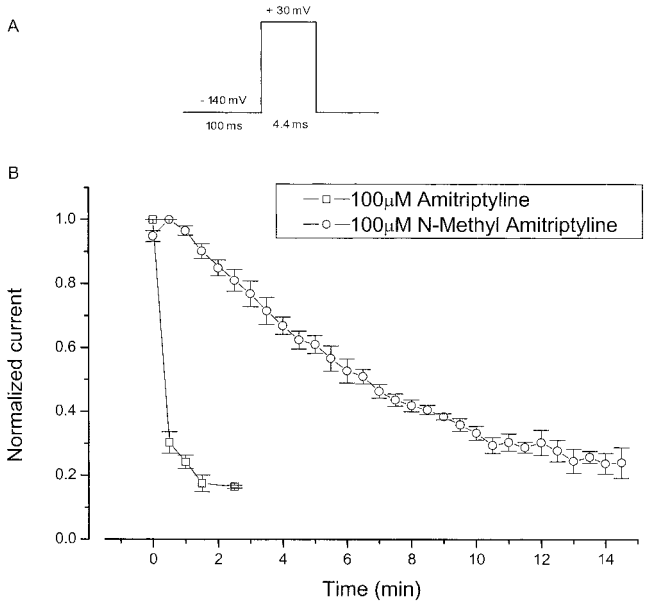


**Fig. 1. Tonic inhibition of $Na^+$ currents by amitriptyline and its permanently charged derivative *N*-methyl amitriptyline. The $Na^+$ channel–blocking effects of these drugs were studied with a reverse $Na^+$ gradient. (*A*) Pulse protocol. Holding potential was −140 mV, and duration of the test pulse at 30 mV was 4.4 ms. Pulse interval was 30 sec. (*B*) Steady-state concentration was achieved with amitriptyline within approximately 2 min but still had not occurred with *N*-methyl amitriptyline at approximately 15 min.**

## Results

*Voltage Clamp Experiments*

*N*-Methyl amitriptyline is a potent $Na^+$ channel blocker with a slow wash-in rate (fig. 1), reaching a blockade of approximately 80% at 15 min. Wash-in of amitriptyline was much faster, reaching its maximal blockade (approximately 85%) at 2 min.

*Rat Experiments*

For the rat sciatic nerve block experiments, the time course of recovery of blockade for *N*-methyl amitriptyline is shown in figure 2. At the 20-mM concentration, three of six rats had not recovered fully at day 4 (approximately 30% residual blockade for motor and proprioceptive function and approximately 45% for nociception) and were therefore euthanized.

Similar results regarding differential blockade were obtained in the rat intrathecal catheter groups with both *N*-methyl amitriptyline (fig. 3) and amitriptyline (fig. 4), as well as with lidocaine and bupivacaine in earlier work.[7] Actually, in the amitriptyline 5 mM group, although all functions tested recovered fully at the same time, proprioceptive blockade was even slightly greater

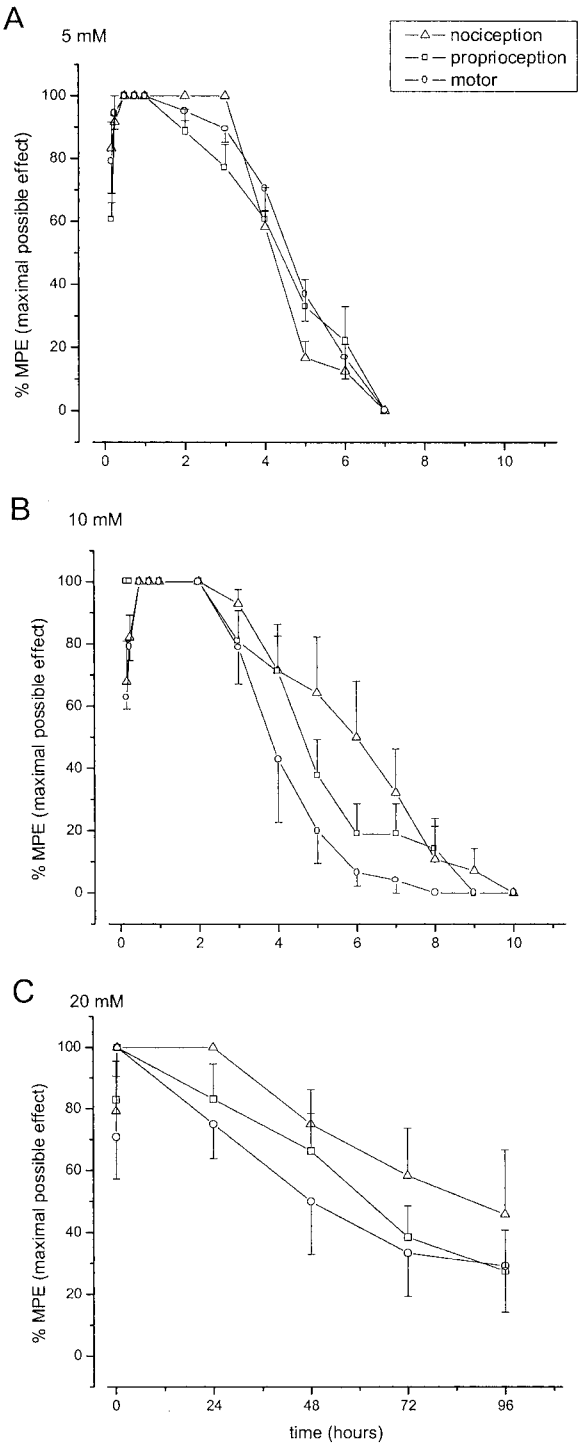


**Fig. 2. *N*-Methyl amitriptyline in rat sciatic nerve blockade. A volume of 0.2 ml of various concentrations was injected *via* the sciatic notch. Because this is a permanently charged drug, onset of block is accordingly relatively slow, but full nerve block was achieved by approximately 30 min. (*A*) *N*-Methyl amitriptyline 5 mM (n = 7) revealed a time to full recovery of approximately 7 h. (*B*) *N*-Methyl amitriptyline 10 mM (n = 7) revealed a time to full recovery of approximately 10 h. Although nociceptive blockade is more pronounced, no significant difference was found in motor blockade. (*C*) *N*-Methyl amitriptyline 20 mM (n = 6): This concentration appears to be toxic, because three animals (50%) had residual neural blockade at day 4 and were euthanized. Please note the different time scale on the *y* axis.**

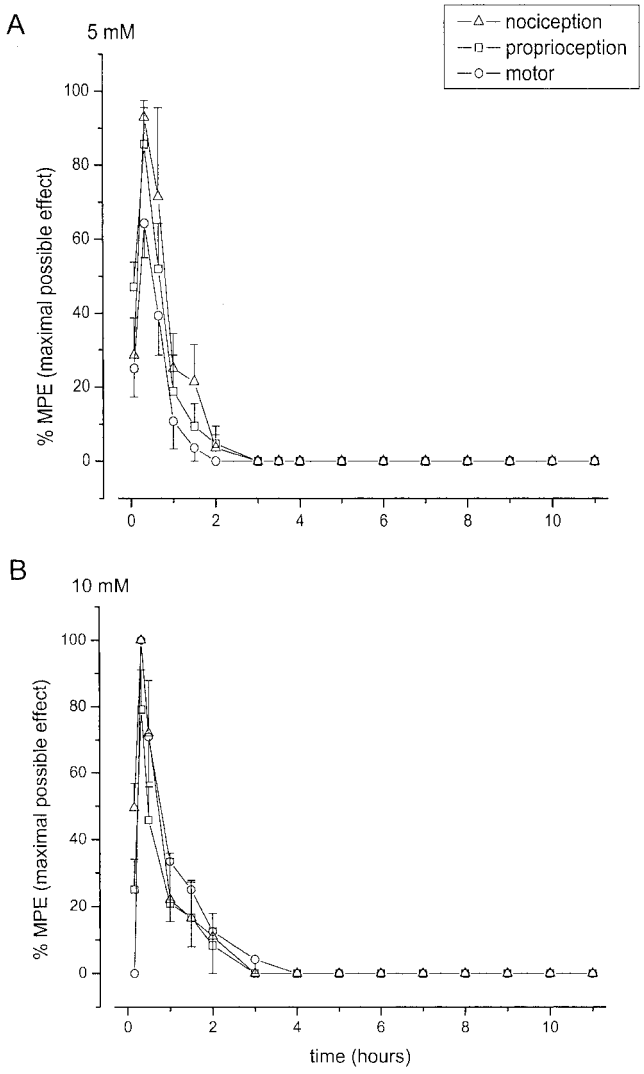


**Fig. 3. *N*-Methyl amitriptyline in rat spinal blockade. A volume of 90 μl at various concentrations was injected through an intrathecal catheter inserted at the L5–L6 interspace and advanced 2 cm cranially. (*A*) At a concentration of 5 mM (n = 7), no complete blockade was observed for all functions tested. (*B*) At a concentration of 10 mM (n = 6), motor function is even more blocked than antinociception at most time points. At a concentration of 20 mM, because of the extensive cranial spread of this drug, rats suffered respiratory arrest; data are therefore not included in the graph but are discussed in detail in the text.**

than nociceptive blockade at the preceding time points. In the 10-mM group, although at some time points nociceptive function was blocked to a significantly greater extent than motor function, because of the short duration, this result was clinically insignificant. The results for higher concentrations (25 and 50 mM) of intrathecal amitriptyline in rats displaying no significant differential blockade have been reported previously.[12] Although the blockade of tested functions with amitriptyline and *N*-methyl amitriptyline seems rather short, it is nevertheless longer than with lidocaine (40 mM) and bupivacaine (15 mM) under comparable conditions.

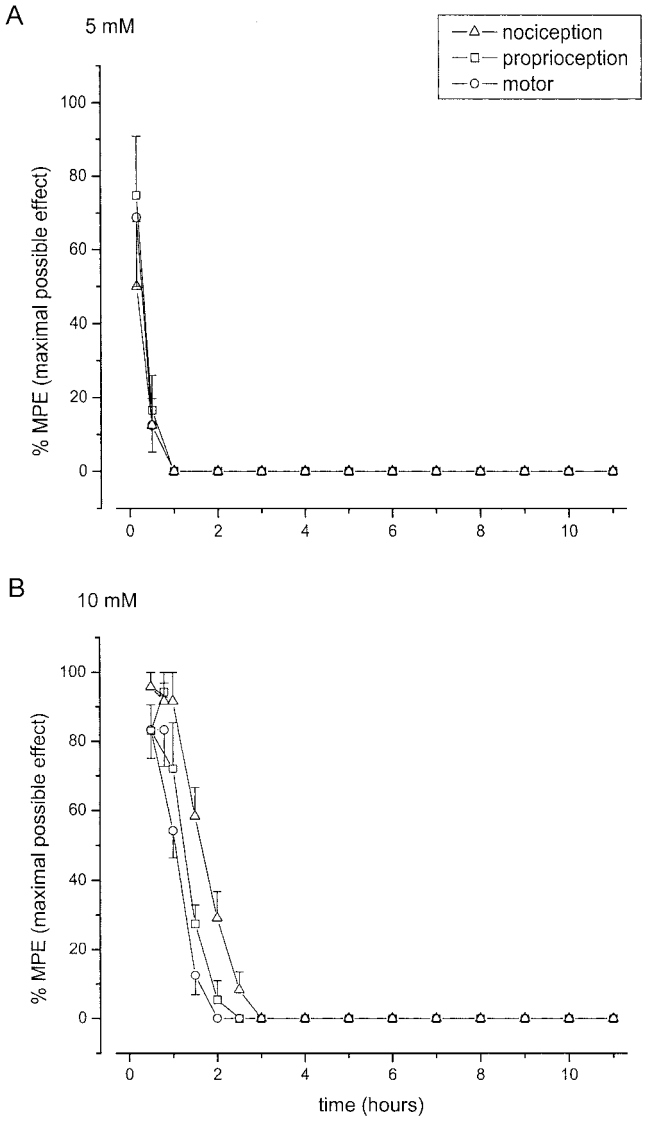


**Fig. 4. Amitriptyline in rat spinal blockade. A volume of 90 μl at a concentration of 5 and 10 mM was injected intrathecally. (*A*) At the 5-mM concentration (n = 4), only brief and mild antinociception and motor and proprioceptive blockade were observed. The durations of both functions were indistinguishable from each other. (*B*) At the 10-mM concentration (n = 6), nociceptive blockade becomes more prominent but is of very short duration overall.**

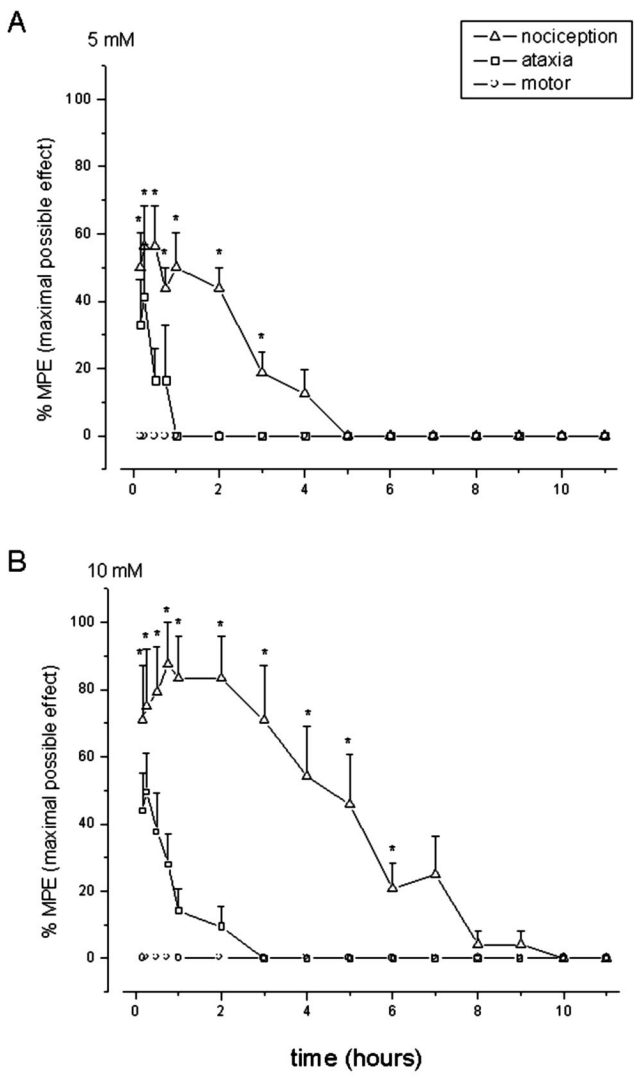


**Fig. 5. *N*-Methyl amitriptyline in sheep spinal blockade. A volume of 2.5 ml of drug at various concentrations dissolved in 7.5% dextrose was injected at the L6–S1 level. *$P < 0.05$ for motor blockade *versus* nociceptive blockade. (*A*) No complete blockade of nociception was achieved at a 5-mM concentration (n = 5). There was moderate ataxia and absence of motor blockade. (*B*) At a 10-mM concentration (n = 7), nearly complete blockade of nociception was present for several hours. Moderate ataxia but not motor blockade was detectable. Similar to rats, at a concentration of 20 mM, some sheep suffered respiratory arrest because of "high spinal"; data are therefore not included in the graph but are discussed in detail in the text.**

*Sheep Experiments*

A somewhat striking difference regarding differential block was found in the *N*-methyl amitriptyline and to some extent in the amitriptyline intrathecal groups at all concentrations tested in the sheep. As shown in figure 5, both *N*-methyl amitriptyline groups (5 and 10 mM) displayed no detectable motor blockade and only varying degrees of ataxia.

A similar relationship was found in the amitriptyline 5 mM (fig. 6, *A*) and 10 mM (fig. 6, *B*) groups, but at the 20-mM (fig. 6, *C*) concentration, there was complete motor blockade for a relatively short period. Conversely, with lidocaine, no differential block was found, and the time course of spinal block appears similar to that seen in the clinical setting (fig. 7).

## Discussion

We have found that amitriptyline displays some differential blockade at some time points, but its quaternary ammonium derivative *N*-methyl amitriptyline displays significant differential blockade when injected intrathecally in sheep. Both drugs failed to show significant differential blockade when injected intrathecally or in a

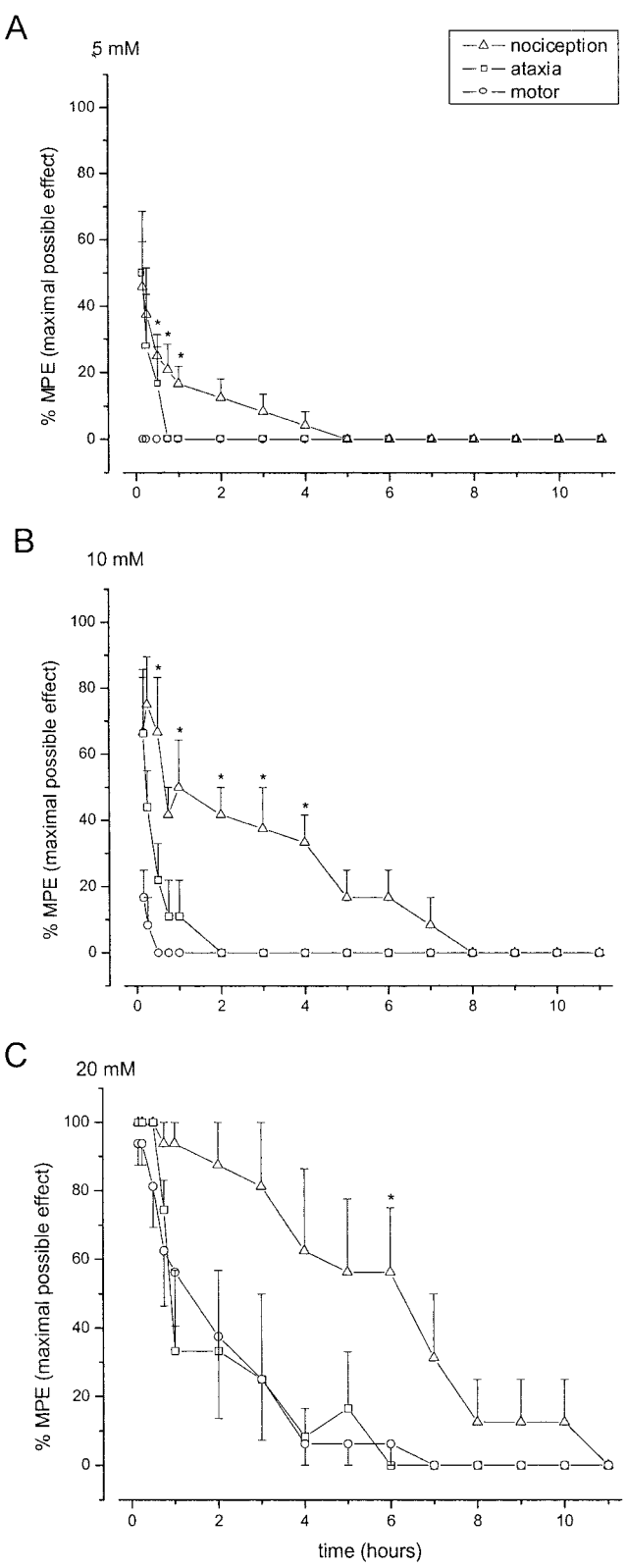


**Fig. 6. Amitriptyline in sheep spinal blockade. A volume of 2.5 ml of drug at various concentrations dissolved in 7.5% dextrose was injected at the L6–S1 level. *$P < 0.05$ for motor blockade *versus* nociceptive blockade. (*A*) Mild antinociception and minimal ataxia were present at a 5-mM concentration (n = 6). (*B*) Near complete antinociception, moderate ataxia, and minimal motor blockade were found at the 10-mM concentration (n = 4). (*C*) At a concentration of 20 mM, there was more nociceptive than motor blockade at most time points, with initial complete motor blockade. Although nociceptive blockade is overall considerably greater than motor blockade, the *P* value did not reach statistical significance (except at one time point), because the power of the two-sample *t* test used in this case is very low because of the small number of animals (n = 4).**

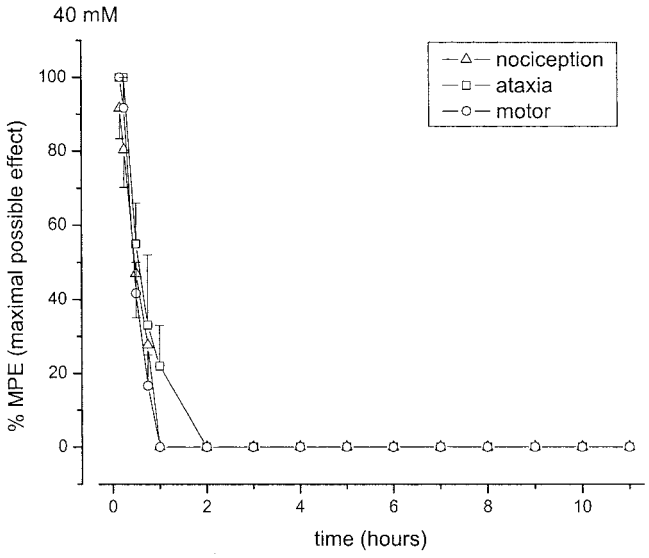


**Fig. 7. Lidocaine in sheep spinal blockade at 40 mM (approximately 1%). All functions tested recovered more or less at the same time (n = 4).**

sciatic nerve block model in rats at all concentrations tested.

One possible explanation for these results seems to be species difference, *i.e.*, a different amino acid sequence of $Na^+$ channels in rat and sheep. Second, the differential block observed with amitriptyline and *N*-methyl amitriptyline may be a result of the low concentrations used. From clinical practice, it is known that bupivacaine at lower concentrations demonstrates a varying degree of differential block that is not seen at higher concentrations. Similarly, amitriptyline at the higher concentration of 20 mM had significant motor blockade, although with relatively short duration. *In vitro* studies assessing the use-dependent block (additional block at high-frequency stimulation) of amitriptyline[13] and/or a quaternary ammonium derivative[5] have shown that a high level of additional block occurs at relatively low concentrations, which is not the case with lidocaine or bupivacaine.[10] The fact that there was little tonic block (block at relatively low stimulation frequencies) with amitriptyline and *N*-methyl amitriptyline at low concentrations may actually support the theory that discharge at a high rate from stimulated sensory–pain fibers will be blocked, whereas other nerve fibers will be left undisturbed. Therefore, it could be that amitriptyline and *N*-methyl amitriptyline preferentially block "firing" of high-frequency action potentials in sensory–pain fibers–

nociceptors and leave the normal action potential transmission intact.

We did not study bupivacaine in sheep or rats because there is ample literature for direct comparison. For example, spinal bupivacaine (2.5 ml of 0.5% using the same approach as ours) was found to provide relatively little differential blockade, although the time to full recovery of sensory function was much longer than motor blockade.[14] In their work (and in ours), no complete dose-response curves were provided. It is therefore possible to make a statement only for the concentrations tested. It might well be that at some concentrations, even lidocaine might provide more differential blockade than amitriptyline. Conversely, it appears that there is no differential block with lidocaine even at very low concentrations, because we studied a 40-mM concentration (which corresponds to the lower end of the clinically useful concentration of approximately 1%).

Addition of a methyl group to amitriptyline does not increase its potency in blocking nerve functions or $Na^+$ channels. *N*-Methyl amitriptyline and amitriptyline seem to be comparably potent. However, the wash-in of the quaternary ammonium derivative is much slower than that of amitriptyline in the *in vitro* but not in the *in vivo* experiments, consistent with the fact that a permanently charged drug needs a much longer time to achieve blockade of $Na^+$ currents but once inside the membrane–$Na^+$ channel has a much longer dwell time. This quaternary ammonium derivative would therefore have the potential to be a very long-acting local anesthetic, similar to the previously reported *N*-phenylethyl amitriptyline.[5] The cause of the short duration of *N*-methyl amitriptyline in the *in vivo* experiments (at least at the lower concentrations) seems to be that the concentrations specified do not provide a density of molecules high enough to effectively traverse the membrane before the charged molecules diffuse along the cerebrospinal fluid.

In an earlier study, amitriptyline administered intrathecally in rats had no effect on nociception,[15] an apparent contradiction to the effectiveness of amitriptyline in our rat spinal catheter model. But considering that these authors used a much lower dosage (60 $\mu$g of amitriptyline in 3 $\mu$l *vs.* our 140–280 $\mu$g in 90 $\mu$l), the differences may be explained by administered dosage and volume alone. Our pilot studies with lower dosages and volumes also displayed little to no effect.

Amitriptyline and particularly *N*-methyl amitriptyline appear to have statistically and clinically significantly more sensory-selective local anesthetic properties in sheep than in rats when applied intrathecally. Because amitriptyline is used orally in pain clinics in much higher dosages than for potential intrathecal application and worrisome changes in blood pressure or spinal cord blood flow were not found when amitriptyline was injected intrathecally in sheep,[16] systemic adverse effects appear relatively unlikely. However, detailed neurotoxicity studies in animals need to be performed before amitriptyline is tested in humans (*N*-methyl amitriptyline, being a new compound, would need more comprehensive testing).

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