Neosaxitoxin in Rat Sciatic Block

Improved Therapeutic Index Using Combinations with Bupivacaine, with and without Epinephrine

Jay S. Templin, M.S., Matthew C. Wylie, M.S., M.D., Joseph D. Kim, M.A., Katherine E. Kurgansky, M.P.H., Grzegorz Gorski, M.S., John Kheir, M.D., David Zurakowski, Ph.D., Gabriel Corfas, Ph.D., Charles Berde, M.D., Ph.D.

ABSTRACT

Background: Neosaxitoxin (NeoSTX) is a site-1 sodium channel blocker undergoing clinical trials as a prolonged-duration local anesthetic. Rat sciatic block and intravenous infusion models were used to assess efficacy and local and systemic toxicities for NeoSTX in saline (*NeoSTX-Saline*), bupivacaine (*Bup*), and their combination (*NeoSTX-Bup*). Exploratory studies evaluated the effects of addition of epinephrine to *NeoSTX-Bup* (*NeoSTX-Bup-Epi*).

Methods: Rats received percutaneous sciatic blocks with escalating doses of *NeoSTX-Saline* or *NeoSTX-Bup*. Sensory-nocifensive block was assessed using modified hotplate and Von Frey filaments. Motor-proprioceptive function was assessed by extensor postural thrust. Nerves were examined histologically after 7 days and scored on the Estebe–Myers scale. Median lethal dose was estimated for *NeoSTX-Saline* and in combinations. Accidental intravenous overdose was simulated in isoflurane-anesthetized, spontaneously breathing rats receiving *NeoSTX-Saline* (n = 6), *Bup* (n = 7), or *NeoSTX-Bup* (n = 13), with respiratory, hemodynamic, and electrocardiographic endpoints. Additional groups received blocks with *NeoSTX-Bup-Epi* (n = 80). Investigators were blinded for behavioral and histologic studies.

Results: *NeoSTX-Bup* produced more prolonged sensory and motor block compared with *NeoSTX-Saline* or *Bup*. *NeoSTX-Bup-Epi* further prolonged median time to near-complete recovery for 3 μg/kg *NeoSTX-Bup* (hotplate: 48 *vs.* 6 h, $P < 0.001$). With sciatic injections, addition of *Bup* did not worsen the systemic toxicity (median lethal dose) compared with *NeoSTX-Saline*. Intravenous *NeoSTX-Saline* infusion had significantly longer times to apnea, first arrhythmia, and asystole compared with *Bup* ($P < 0.001$ for each). Histologic injury scores overall were low for all groups, with median scores of 0 (interquartile range, 0 to 0) on a 5-point scale.

Conclusion: *NeoSTX-Bup and NeoSTX-Bup-Epi* hold promise for prolonged-duration local anesthesia. **(ANESTHESIOLOGY 2015; 123:886-98)**

NEOSAXITOXIN (NeoSTX) is a site-1 sodium channel blocker under clinical development as a prolonged-duration local anesthetic (see accompanying article).[1–4] Site-1 blockers are a family of molecules long recognized for their potent and specific blockade of specific subtypes of voltage-gated sodium channels.[5,6] Combinations of the site-1 blocker tetrodotoxin with bupivacaine produced long-duration sciatic nerve blockade in rats without increased systemic toxicity compared with tetrodotoxin alone.[7] Subsequent study examined rat sciatic nerve blockade with several members of the saxitoxin series, including NeoSTX.[1] Phase 1 studies of subcutaneous infiltration in human volunteers showed that NeoSTX caused cutaneous hypoesthesia and

What We Already Know about This Topic

- Neosaxitoxin, a site-1 sodium channel blocker with long duration, neither has previously undergone preclinical safety testing, nor has its interactions with traditional local anesthetics and epinephrine been examined

What This Article Tells Us That Is New

- In rats, combination of Neosaxitoxin with bupivacaine for sciatic nerve block resulted in motor and sensory block, which was longer than either agent alone and was up to 48 h when epinephrine was added
- Histologic examination showed no evidence of neural toxicity, and intravenous injection of Neosaxitoxin resulted in cardiotoxicity with longer delays than bupivacaine

Corresponding article on page 741. The first three authors contributed equally to this article.

Submitted for publication November 24, 2014. Accepted for publication May 15, 2015. From the Department of Anesthesiology, Perioperative, and Pain Medicine, Boston Children's Hospital, and Department of Anaesthesia, Harvard Medical School, Boston, Massachusetts (J.S.T., M.C.W., J.D.K., K.E.K., D.Z., C.B.); F.M. Kirby Neurobiology Center, Boston Children's Hospital, and Harvard Medical School, Boston, Massachusetts (G.G.); Department of Cardiology, Boston Children's Hospital, and Harvard Medical School, Boston, Massachusetts (J.K.); F.M. Kirby Neurobiology Center, and Department of Neurology and Department of Otology and Laryngology, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts (G.C.). Current affiliations: Department of Psychology, University of Massachusetts, Boston, Massachusetts (J.S.T.); and Kresge Hearing Research Institute, Department of Otolaryngology, Head and Neck Surgery, University of Michigan, Ann Arbor, Michigan (G.C.).

 Anesthesiology 2015; 123:886-98

that combination of NeoSTX with bupivacaine resulted in more prolonged analgesia compared with NeoSTX or bupivacaine alone.[3]

Conventional local anesthetics are associated with local tissue toxicities to nerve[8,9] and muscle[10] and profound cardiovascular toxicity[11–14] in overdose or with inadvertent intravascular injection. Site-1 sodium channel blockers have shown a benign histologic profile after peripheral[15] and neuraxial[16] administration. In a sheep model, deliberate IV injection of NeoSTX produced minimal cardiovascular effects.[17] Bupivacaine cardiovascular toxicity may involve multiple cellular targets[18–21] but is likely mediated at least in part by the cardiac sodium channel Nav1.5, which is much more resistant to binding and inactivation by site-1 sodium channel blockers.[22] Site-1 sodium channel blockers produce dose-dependent respiratory and skeletal muscle weakness. In anesthetized sheep receiving subcutaneous injection of NeoSTX, coinjection of bupivacaine did not worsen surrogate measures of respiratory or neuromuscular toxicity from NeoSTX.[17]

The current study further investigates the dose–response of NeoSTX and NeoSTX-bupivacaine combinations on neurobehavioral measures of rat sciatic nerve blockade, as well as on local and systemic toxicities of NeoSTX combinations. A separate IV infusion model further assessed the systemic toxicity using several physiologic endpoints. These experiments were performed as preclinical studies for an Investigational New Drug Application by using NeoSTX formulations manufactured for clinical use in the accompanying phase 1 clinical trial.

Our hypotheses were the following.

1. At fixed NeoSTX doses, addition of bupivacaine increases the intensity and duration of rat sciatic nerve blockade.
2. In the presence or absence of bupivacaine, intensity and duration of block increase with NeoSTX dose.
3. Addition of epinephrine to NeoSTX-bupivacaine combinations in rat sciatic blocks produced further prolonged block durations.
4. In a model of rapid accidental IV infusion, NeoSTX and bupivacaine and their combinations generate respiratory and electrocardiographic endpoints with different time courses.
5. The histologic effects of NeoSTX (in saline or in combination with bupivacaine) on rat sciatic nerve are benign over the intended dose range and not statistically different from those of vehicle or untreated nerves.

Materials and Methods

Drugs

For the remaining discussion and the figures, the terms *Bup*, *NeoSTX-Saline*, *NeoSTX-Bup*, and *NeoSTX-Bup-Epi* refer to injections performed with bupivacaine, NeoSTX-saline, NeoSTX-bupivacaine, and NeoSTX-bupivacaine-epinephrine, respectively. In all cases where bupivacaine or epinephrine was used for sciatic nerve blocks, their final concentrations were fixed at 2 mg/ml (0.2%) and 5 μg/ml (1:200,000), respectively. NeoSTX concentrations varied as described in each section.

In the sciatic nerve injection model, injectates were prepared on the day of the experiment, and injectate volume was fixed at 0.3 ml. NeoSTX (Proteus SA, Chile) was transported and stored according to the Boston Children's Hospital safety standards, in compliance with the Harvard Committee on Microbiological Safety.

Drug substance was produced at Proteus SA. Drug product was packaged initially by Biosano Laboratories (Santiago, Chile) (denoted NeoSTX #4) and subsequently by Saval Laboratories (Santiago, Chile) (NeoSTX#5). NeoSTX#4 was used in the phase 2 clinical trial performed in Chile,[3] and NeoSTX#5 was used in the U.S. phase 1 studies reported in the accompanying articles. Methods of purification and packaging for NeoSTX#4 and #5 were the same, and bridging studies showed analytical and *in vivo* equivalence of these two batches. In both cases, NeoSTX drug product is packaged at a concentration of 20 μg/ml in sodium chloride solution, 0.9 mg/ml, at pH 4.5 in 1-ml sealed ampules. Drug assays for different manufacturing, toxicologic, and pharmacokinetic studies used multiple approaches, including high-performance liquid chromatography followed by fluorescence detection and high-performance liquid chromatography followed by tandem mass spectrometry. A series of studies confirmed sterility, stability, and nonpyrogenicity. Absence of cyanobacterial DNA was confirmed by a reverse transcriptase polymerase chain reaction method using positive and negative controls. Absence of cyanobacterial protein or peptides was confirmed by a combined approach using Bradford protein assays, proteomics (mass spectrometry), and amino acid analysis after acid hydrolysis. NeoSTX did not appear mutagenic or carcinogenic in Ames and chromosomal aberration tests. Preparative methods, analytical methods, and Good Laboratory Practices toxicologic studies in rats and sheep were submitted to the U.S. Food and Drug Administration as part of the Investigational New Drug application.

NeoSTX was diluted in 0.9% saline or bupivacaine hydrochloride (Sensorcaine®; APP Pharmaceuticals, USA) before injection. Depending on the intended final NeoSTX concentration, commercial vials of bupivacaine as either bupivacaine 5 mg/ml (0.5%) or bupivacaine 2.5 mg/ml (0.25%) were used to reach final bupivacaine concentrations of 2 mg/ml (0.2%) in the final injectates. In the add-on study, epinephrine was added separately from 1 mg/ml ampules on the morning of each experiment to yield a final concentration of 5 μg/ml.

In the IV overdose model, infusion concentrations were as follows: bupivacaine 2 mg/ml, NeoSTX 1.88 μg/ml, "full-concentration" combination: bupivacaine 2 mg/ml and NeoSTX 1.88 μg/ml, and "half-concentration"

combination: bupivacaine 1 mg/ml and NeoSTX 0.94 μg/ml. Infusion rates were adjusted according to animal weight to ensure constant weight-scaled drug delivery rates. Thus, as single drugs, bupivacaine was administered at a rate of 3.2 $mg \cdot kg^{-1} \cdot min^{-1}$ and NeoSTX at 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Full-dose combination animals received both bupivacaine 3.2 $mg \cdot kg^{-1} \cdot min^{-1}$ and NeoSTX as 3 $\mu g \cdot kg^{-1} \cdot min^{-1}$, whereas half-dose combination animals received bupivacaine at 1.6 $mg \cdot kg^{-1} \cdot min^{-1}$ and NeoSTX 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$, and all animals received a constant weight-scaled fluid administration at rate of 1.6 $ml \cdot kg^{-1} \cdot min^{-1}$. Infusion concentrations were chosen based on previous estimates of median lethal dose (LD50) for each drug from extravascular (sciatic perineural) injections.

Animal Care

Male Sprague–Dawley rats obtained from Charles River Laboratories (USA): young adults weighing 200 to 250 g were used for the sciatic injection model and 325 to 400 g animals for the IV overdose model. Animals were cared for and sacrificed in compliance with protocols approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital (Boston, Massachusetts). Handling procedures were developed to habituate animals to the testing paradigm and minimize stress-induced analgesia.

Sciatic Injection

Rats were briefly anesthetized with isoflurane 3% in oxygen delivered in induction chamber for approximately 1 min to facilitate injections. A needle was introduced posteromedial to the greater trochanter, pointing in an anteromedial direction. Once bone (ischium) was contacted, 0.3 ml of solution was injected. The left leg was always used for blocks; the right served as a control and measure of sublethal systemic toxicity (see Systemic Toxicity with Sciatic Perineural Injection section).[7]

Neurobehavioral Testing

We used a neurobehavioral assessment battery modified from Thalhammer *et al.*, which uses measures of sensory-nocifensive and motor-proprioceptive impairments to assess the duration and intensity of blockade after sciatic perineural injection.[23] Investigators were blinded to dose and treatment assignment. Mechanical nocifensive block was assessed using Von Frey (VF) filament testing (Touch-Test Sensory Evaluator; North Coast Medical Inc., USA). After brief habituation to a wire mesh cage, VF hairs of ascending force were applied until observation of paw withdrawal. Care was taken to apply filaments only on lateral plantar surfaces receiving reliable innervation by the sciatic nerve. Filaments were applied in an escalating series until withdrawal was observed or until the maximum of 300 g was reached. Thermal nocifensive block was assessed by time to withdrawal from hotplate set at 56°C, as described in the study by Kohane *et al.*,[7] with a cutoff value of 12 s. Extensor postural thrust (EPT) is a mixed-strength proprioceptive test measured as grams exerted in hind paw push-off on an upright balance.[23]

Neurobehavioral measures were taken preinjection for a baseline reading. Postinjection values were taken at the following time points: 15 min and 1, 2, 3, 4, 6, 8, 10, and 12 h. After 12 h, measurements were made every 6 h until motor recovery was measured. At each time point, three replicate measurements of EPT force and hotplate latency were taken and averaged. Previous studies of site-1 sodium blockers using this paradigm have shown that higher doses cause transient contralateral impairments of neurobehavioral measures, reflecting systemic analgesia and/or systemic weakness.[1,7]

In previous studies, injections with 0.3 ml of bupivacaine 2.5 mg/ml resulted in complete block (based on cutoffs defined later in this paragraph) for greater than 98% of animals. In analyses, we therefore make the assumption that incomplete block with test formulations reflects true pharmacologic effects of that dose, rather than a technical injection failure. Baseline measures were assessed before injections. Measures recorded at 15 min were considered to demonstrate peak block intensity.

Cutoff values were 300 g for the VF test and 12 s for the hotplate test.[7] Two operational measures of recovery time were derived by linear interpolations for each neurobehavioral test: time to partial recovery (similar to "50% recovery" in previous publications) and time to near-complete recovery. These parameters were selected in part based on previous work[7] and in part to foster comparisons with measures used in the accompanying human phase 1 study. The time to partial recovery was defined in the VF test as the time to recovery to a threshold of 150 g, in the EPT test as the time point when values return to 50% of the individual rat's own baseline, and in the hotplate test as the time to reach a withdrawal latency of 7 s (halfway between cutoff value of 12 s and baseline of 2 s). Near-complete recovery was defined for the VF test as time to recovery to a threshold of 60 g, for the EPT test as time for recovery to 90% of the individual rat's own baseline, and in the hotplate test as time to reach a withdrawal latency of 3 s (mean baseline latency values 2.0 ± 0.5 s).

Systemic Toxicity with Sciatic Perineural Injection

Sublethal Systemic Toxicity. Sublethal systemic toxicity was assessed by measurement of right hindlimb sensory-nocifensive and motor-proprioceptive impairments after left hindlimb sciatic injections as described in the Neurobehavioral Testing section. These transient impairments could in principle reflect a combination of systemic analgesic, sedative, and motor effects.

LD50. At higher doses of NeoSTX, alone or in combination with bupivacaine, increasing numbers of animals developed apnea or gasping respiration. To minimize distress in this paradigm involving awake animals, any animal developing apnea or gasping was immediately euthanized with

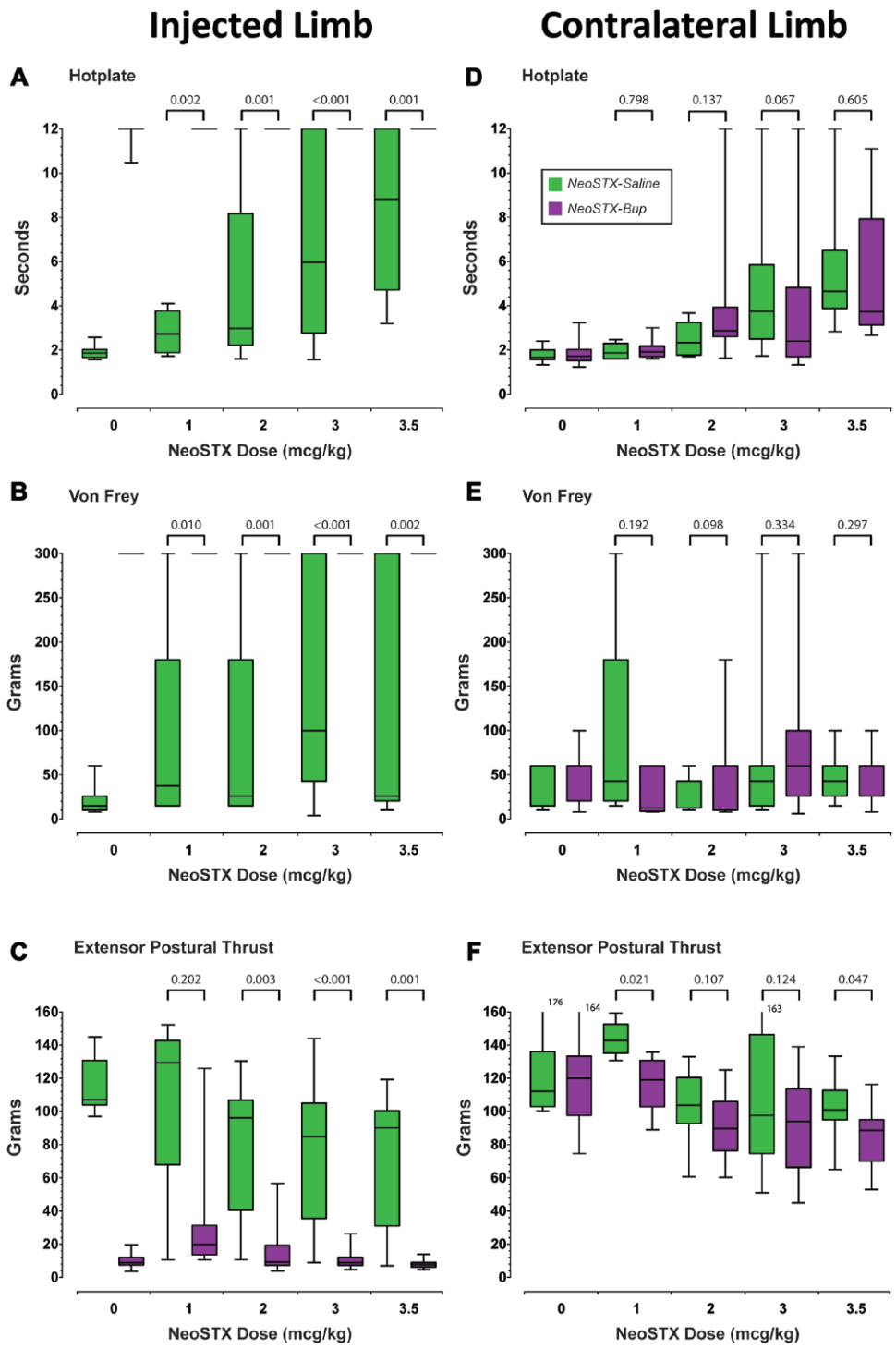

Fig. 1. Intensity of blockade at 15-min postinjection for hotplate, von Frey testing, and extensor postural thrust in the injected limb (*A–C*) and the contralateral limb (*D–F*). *Horizontal lines* represent median value, *boxes* represent interquartile range, and *whiskers* represent minimum–maximum values. For the injected limb, Kruskal–Wallis tests showed a significant difference in intensity between doses for Neosaxitoxin-saline (*NeoSTX-Saline*) (von Frey $P < 0.001$; hotplate $P < 0.001$; and extensor postural thrust $P < 0.001$). For Neosaxitoxin-Bupivacaine (*NeoSTX-Bup*), only extensor postural thrust test was significant ($P = 0.002$). For the contralateral limb, Kruskal–Wallis tests showed a significant difference in intensity between doses for *NeoSTX-Saline* (hotplate $P < 0.001$) and for *NeoSTX-Bup* (hotplate $P < 0.001$ and extensor postural thrust $P < 0.001$) tests. Comparisons have been presented for *NeoSTX-Saline versus NeoSTX-Bup* using Mann–Whitney test at each Neosaxitoxin (NeoSTX) dose. All bupivacaine-containing formulations produced dense block in all animals at 15 min.

Table 1. Time (Hours) to Partial Recovery of Injected Limb by Hotplate, Extensor Postural Thrust, and Von Frey Testing

	Neurobehavioral Test					
Treatment Groups	Hotplate	*P* Value	Extensor Postural Thrust	*P* Value	Von Frey	*P* Value
Bup (n = 20)	1.5 (1.5–2.1)		2.0 (1.5–2.4)		1.6 (1.5–1.9)	
1 μg *NeoSTX-Saline* (n = 4)	0.0 (0.0–0.0)	0.007	0.0 (0.0–1.0)	0.226	0.0 (0.0–0.9)	0.026
1 μg *NeoSTX-Bup* (n = 8)	1.6 (1.5–2.7)		1.7 (0.8–1.9)		2.5 (1.7–2.8)	
2 μg *NeoSTX-Saline* (n = 8)	0.0 (0.0–0.7)	<0.001	0.0 (0.0–1.5)	<0.001	0.0 (0.0–0.8)	<0.001
2 μg *NeoSTX-Bup* (n = 11)	3.8 (2.6–10.1)		9.1 (3.8–17.8)		3.6 (2.5–8.5)	
3 μg *NeoSTX-Saline* (n = 20)	0.2 (0.0–1.6)	<0.001	0.0 (0.80–0.4)	<0.001	0.0 (0.0–1.1)	<0.001
3 μg *NeoSTX-Bup* (n = 27)	3.5 (2.8–4.6)		17.9 (12.1–22.5)		4.5 (3.5–9.0)	
3.5 μg *NeoSTX-Saline* (n = 12)	0.9 (0.0–1.9)	<0.001	0.0 (0.0–5.0)	<0.001	0.0 (0.0–1.5)	<0.001
3.5 μg *NeoSTX-Bup* (n = 13)	10.3 (4.0–11.2)		20.6 (19.2–23.6)		6.1 (3.5–11.8)	

Values are median (interquartile range). Kruskal–Wallis tests showed significant differences between the time to partial recovery and doses for the NeoSTX-Saline (hotplate $P < 0.001$; extensor postural thrust $P = 0.002$; and Von Frey $P = 0.001$) and for NeoSTX-Bup (hotplate $P < 0.001$; extensor postural thrust $P < 0.001$; and Von Frey $P < 0.001$). *P* value compared NeoSTX-Bup with NeoSTX-Saline for the same dose using Mann–Whitney test.

Bup = bupivacaine; NeoSTX-Bup = Neosaxitoxin-Bupivacaine; NeoSTX-Saline = Neosaxitoxin-Saline.

intraperitoneal pentobarbital (100 mg/kg), and this was taken as a lethal event. LD50 calculation is described in the Statistical Procedures section.

Systemic Toxicity with IV Infusion. To model an accidental IV injection, lightly isoflurane-anesthetized, spontaneously breathing rats received infusions *via* tail vein cannula of drug-containing solution until the endpoint of asystole. A light plane of anesthesia was chosen for this model to permit the study of multiple physiologic endpoints without subjecting awake animals to the distress of an awake-paralyzed situation. Twenty-six rats were randomly assigned to four groups: *NeoSTX-Saline* (n = 6); *Bup* (n = 7); full-concentration *NeoSTX-Bup* combination (n = 7); and half-concentration *NeoSTX-Bup* combination (n = 6), using the drug concentrations and infusion rates detailed in the section entitled Drugs. Anesthesia was induced by inhalation of isoflurane 3 to 5% in oxygen *via* induction chamber. The tail vein catheter was flushed with 2 ml of 0.9% saline and connected to a Medfusion syringe pump (Smiths Medical, USA). Vital signs were monitored and physiologic data acquired continuously using PowerLab equipment and LabChart software (AD Instruments, Australia). Baseline measurements were taken (subsequent offline analysis) once all monitoring equipment was calibrated and connected (electrocardiogram, temperature, pulse oximeter, Bain circuit pressure transducer, and tail vein plethysmograph), tail vein accessed, and the rat was maintained in a stable plane of anesthesia at 1% inspired isoflurane in oxygen for at least 5 min. Infusions (as described in the paragraph entitled Drugs) were initiated immediately after a short period of baseline recording and continued until asystole was reached. Primary endpoints for analysis were as follows: (1) apnea (undetectable pressure changes in the Bain circuit) and (2) asystole. Secondary endpoints were bradycardia (heart rate <270 beats/min), deleterious change in electrocardiographic waveform (including either heart block, wide QRS complex, ectopic atrial or ventricular beats, or prolonged QTc interval), and loss of caudal artery pulsatility by plethysmography.

Histological Procedures

Seven days after sciatic injection, rats were given an overdose of pentobarbital (150 mg/kg) and fixed by transcardiac perfusion in two stages: 100 ml of 0.9% saline was infused, followed by 200 ml of a modified Karnovsky fixative containing 2.5% glutaraldehyde and 1.25% paraformaldehyde in 0.1 M phosphate buffer. The left and right sciatic nerves were dissected and stored in dilute fixative at 4°C. Sciatic tissue was plastic embedded using standard osmium tetroxide electron microscopy protocol, cut to semithin sections, and stained with toluidine blue. Sections were analyzed by an experienced neuroscientist (G.C.), using the scoring system of Estebe–Myers[24]; this neuroscientist remained blinded to group assignments throughout. Estebe–Myers scoring system follows a 0- to 5-point ordinal Likert scale: 0 = no pathology in any portion of the field, 1 = very few myelinated axons with any mild abnormality, 2 = slightly more myelinated axons with any abnormality than 1, but less than 10% abnormal, 3 = 10 to 20% abnormal myelinated axons, 4 = signs of moderate axonal degeneration, and 5 = clear signs of axonal degeneration.[24]

Statistical Procedures

All measurements are summarized as medians with interquartile ranges (IQRs) or mean ± SD. No data have been lost or missing. Sample sizes with a minimum of eight rats per treatment group were based on our previous experience with 19 rat sciatic block local anesthetic studies over the past 20 yr. Kruskal–Wallis tests following by Dunn correction test were used to compare block intensity at 15 min and time to partial and time to near-complete recovery across doses for *NeoSTX-Saline* and *NeoSTX-Bup* groups, whereas Mann–Whitney U tests were used to make comparisons between *NeoSTX-Bup* and *NeoSTX-Saline* groups over the range of NeoSTX doses. Two-factor ANOVAs with Bonferroni correction for multiple comparisons were used to conduct an exploratory analysis of the effect of NeoSTX

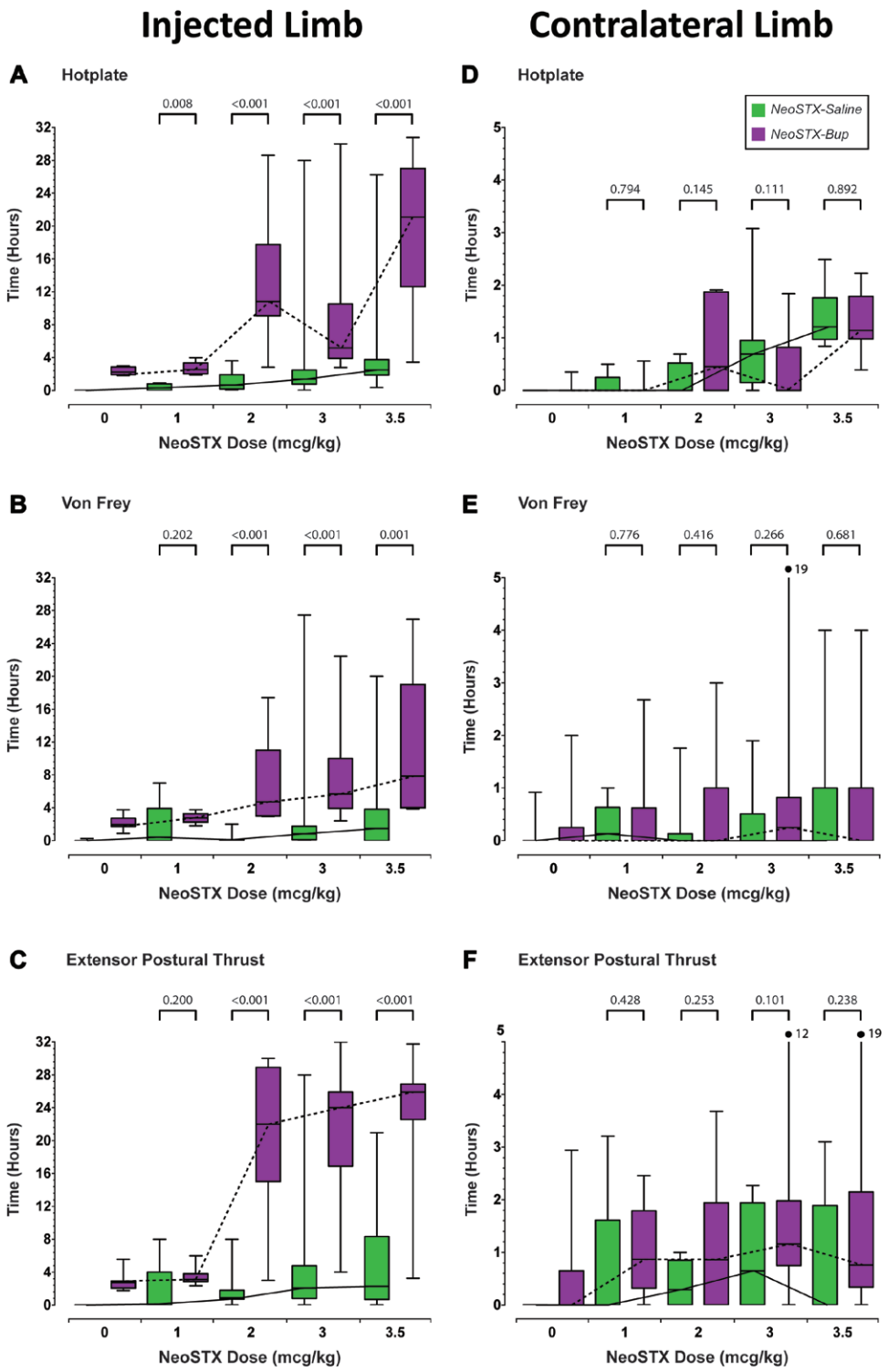

Fig. 2. Time to near-complete recovery across doses for hotplate, Von Frey testing, and extensor postural thrust in the injected limb (*A–C*) and the contralateral limb (*D–F*). *Horizontal lines* represent median value, *boxes* represent interquartile range, and *whiskers* represent minimum–maximum values. For the injected limb, Kruskal–Wallis tests showed significant differences between doses on time to near-complete recovery for both the Neosaxitoxin-saline (*NeoSTX-Saline*) (hotplate $P < 0.001$, Von Frey $P = 0.019$, extensor postural thrust $P < 0.001$) and Neosaxitoxin-Bupivacaine (*NeoSTX-Bup*) (hotplate $P < 0.001$, Von Frey $P < 0.001$, extensor postural thrust $P < 0.001$). For the contralateral limb, Kruskal–Wallis tests showed a significant difference between doses for *NeoSTX-Saline* (hotplate test $P < 0.001$) and *NeoSTX-Bup* (hotplate $P < 0.001$ and extensor postural thrust $P < 0.001$). Comparisons have been presented for *NeoSTX-Saline versus NeoSTX-Bup* using Mann–Whitney tests at each Neosaxitoxin (NeoSTX) dose.

dose and treatment combinations on the time to partial and near-complete recovery for each one of the neurobehavioral test. Spearman rank-order correlation was used to examine the association between NeoSTX dose and recovery time for *NeoSTX-Saline* and *NeoSTX-Bup* treatments.

In the IV overdose model, time-to-event data were summarized using Kaplan–Meier curves. Overall log-rank tests, followed by multiple pair-wise comparisons of survival curves to Bupivacaine alone, were conducted for various endpoints, and *P* values less than 0.017 (Bonferroni corrected for multiple comparisons) were considered statistically significant. Baseline vital signs were compared with ANOVAs. Probit analysis using maximum likelihood was applied to calculate the LD50 for each drug treatment with likelihood ratio 95% CIs obtained by the profile log-likelihood method.[25,26] Nerve histology was analyzed with a Kruskal–Wallis model. Statistical analyses were performed using the SPSS statistical package (version 19.0; SPSS Inc./IBM, USA) and SAS (version 9.3; SAS Institute, USA).

Results

Sciatic Block Neurobehavioral Measures

Block Intensity. Figure 1 shows the dependence of block intensity at 15 min on the dose of NeoSTX administered in the presence and absence of bupivacaine. All *NeoSTX-Bup* formulations show complete blockade by all three behavioral measures at that time point. *NeoSTX-Saline* formulations using NeoSTX doses less than 3 μg/kg produced incomplete block for a majority of animals.

Duration of Block. Time to partial recovery in hours for all tests and doses is presented in table 1. *NeoSTX-Bup* formulations produced significantly longer times to partial recovery from thermal and mechanical sensory-nocifensive and motor-proprioceptive blockade compared with *NeoSTX-Saline* at all doses greater than 1 μg/kg. Time to near-complete recovery in hours for all tests and doses is presented in figure 2. *NeoSTX-Bup* formulations produced substantially longer times to near-complete recovery from thermal and mechanical sensory-nocifensive and motor-proprioceptive blockade compared with *NeoSTX-Saline* formulations at all doses 1 μg/kg or greater. Median time to near-complete recovery after injection of *Bup* was 2.2 h (IQR = 1.9 to 2.9 h) for modified hotplate, 2.7 h (IQR = 2.1 to 2.9 h) for EPT, and 2.0 h (IQR = 1.8 to 2.7 h) for VF testing. Compared with *Bup*, median time to near-complete recovery after *NeoSTX-Bup* injection with a NeoSTX dose of 2 μg/kg was substantially longer for modified hotplate (10.8 h; IQR = 9.1 to 17.8 h; $P < 0.001$), EPT response (22.0 h; IQR = 15.0 to 28.9 h; $P < 0.001$), and VF response (4.7 h; IQR = 3.0 to 11.0 h; $P < 0.001$).

An exploratory analysis of the effect of NeoSTX dose and treatment combinations on time to partial and near-complete recovery was conducted using two-factor ANOVAs. All parameters evaluated showed an overall significant

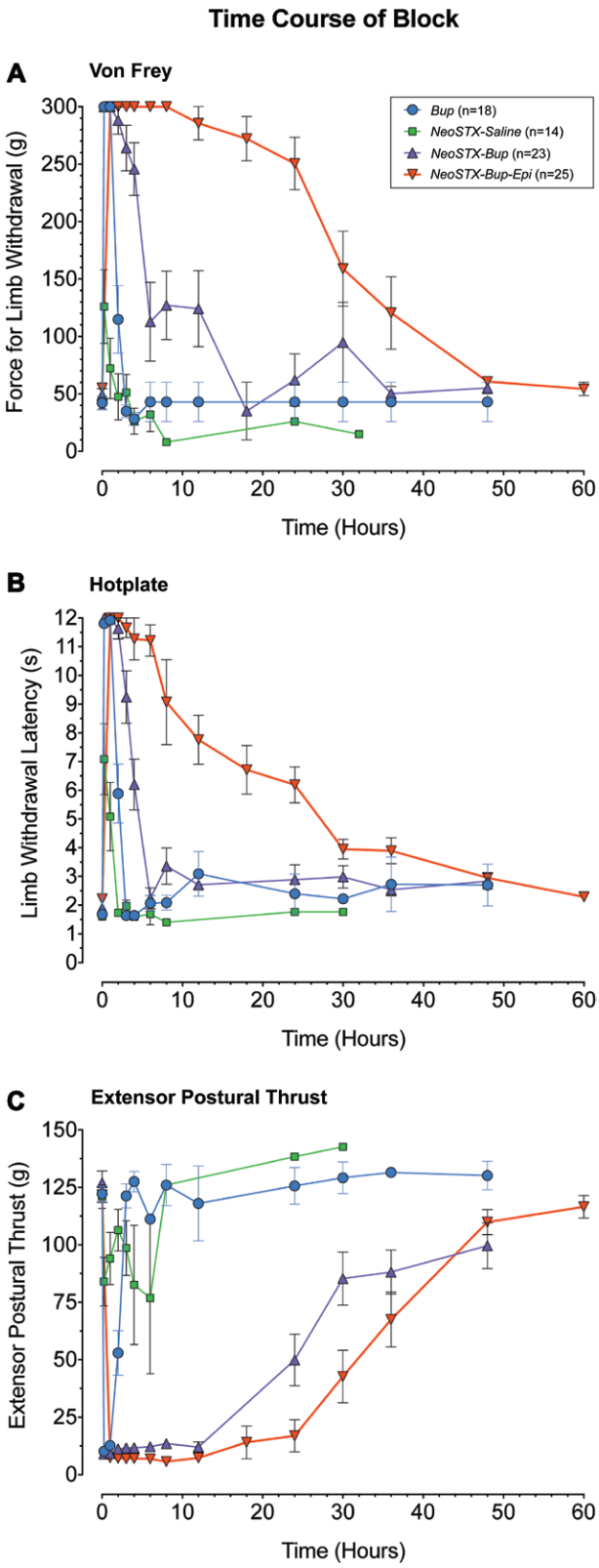

Fig. 3. Time course of blockade across treatments at constant Neosaxitoxin (NeoSTX) of 3 μg/kg with (*A*) Von Frey, (*B*) hotplate, and (*C*) extensor postural thrust. *Bup* = Bupivacaine; *NeoSTX-Bup* = Neosaxitoxin-Bupivacaine; *NeoSTX-Bup-Epi* = Neosaxitoxin-Bupivacaine-Epinephrine; *NeoSTX-Saline* = Neosaxitoxin-saline.

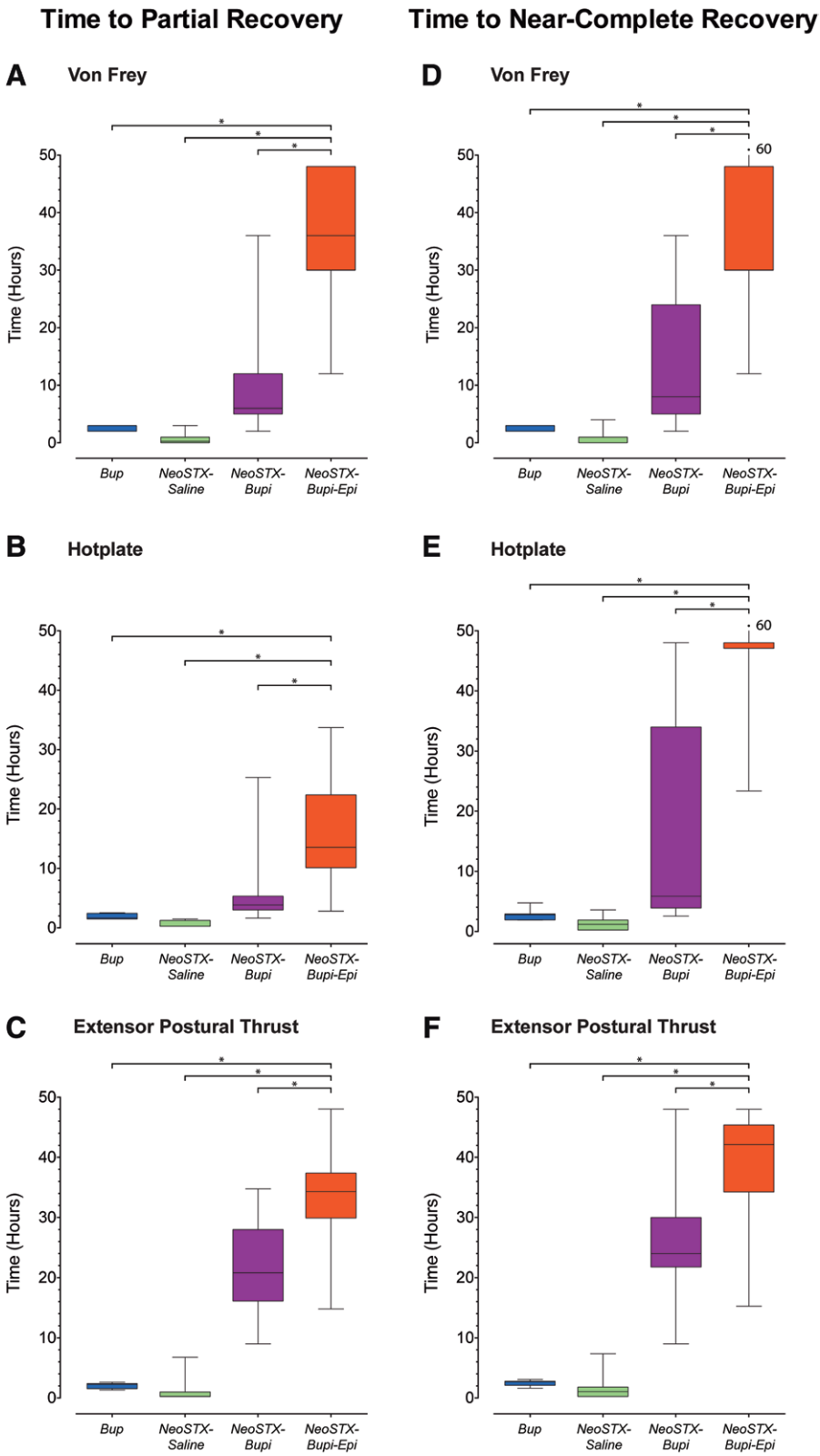

Fig. 4. Times to partial recovery (*A–C*) and near-complete recovery (*D–F*) across treatments at constant Neosaxitoxin (NeoSTX) dose of 3 μg/kg for all neurobehavioral tests. *Bup* = Bupivacaine (n = 18); *NeoSTX-Bup* = Neosaxitoxin-Bupivacaine (n = 23); *NeoSTX-Bup-Epi* = Neosaxitoxin-Bupivacaine-Epinephrine (n = 25); *NeoSTX-Saline* = Neosaxitoxin-saline (n = 16). *Horizontal lines* represent median value, *boxes* represent interquartile range, and *whiskers* represent minimum–maximum values. For both, time to partial recovery and time to near-complete recovery, Kruskal–Wallis tests showed a significant effect of treatment for each neurobehavioral test ($P < 0.001$). *$P < 0.001$ when compared with the *NeoSTX-Bup-Epi* using Dunn test.

independent effect of dose ($P < 0.001$ for all) and treatment ($P < 0.001$ for all). The interaction of dose and treatment combination also had a significant effect on the time to partial recovery and near-complete for modified hotplate (partial recovery $P = 0.008$, near-complete recovery $P = 0.050$) and EPT only (partial recovery $P = 0.002$, near-complete recovery $P = 0.001$). Spearman rank-order correlation demonstrated a significant, moderately strong, positive association between NeoSTX dose and time to partial (EPT: $R = 0.60$, $P < 0.001$; hotplate: $R = 0.47$, $P < 0.001$; and VF: $R = 0.45$, $P < 0.001$) and near-complete recovery for the *NeoSTX-Bup* group across all neurobehavioral tests (EPT: $R = 0.48$, $P < 0.001$; hotplate: $R = 0.44$, $P < 0.001$; and VF: $R = 0.49$, $P < 0.001$). The *NeoSTX-Saline* group only showed a significant association between NeoSTX dose and time to partial and near-complete recovery for the hotplate test only (partial recovery: $R = 0.35$, $P < 0.021$; near-complete recovery: $R = 0.48$, $P = 0.001$). This correlation was generally stronger for the *NeoSTX-Bup* compared with *NeoSTX-Saline*.

At a constant NeoSTX dose of 3 μg/kg, addition of epinephrine (*NeoSTX-Bup-Epi*) showed a significantly longer time to partial and near-complete recovery than *NeoSTX-Saline*, *NeoSTX-Bup*, and *Bup* groups for all parameters evaluated ($P < 0.001$ for all parameters) (figs. 3 and 4). For *NeoSTX-Bup-Epi*, the median time to partial (36 h; IQR = 30 to 48 h) and near-complete recovery (48 h; IQR = 30 to 48 h) for VF was at least six-fold longer than that observed for the *NeoSTX-Bup*, *NeoSTX-Saline*, and *Bup* groups, at least three-fold longer for hotplate and at least 1.5-fold longer for EPT (fig. 4).

Systemic Toxicity

Transient Contralateral Impairments. As a marker for systemic drug distribution after sciatic injection, neurobehavioral measures were obtained from the uninjected right limb. *NeoSTX-Bup* and *NeoSTX-Saline* combinations only at doses greater than 3 μg/kg produced transient right limb impairments, at 15-min postinjection (fig. 1, D–F). Median duration of right limb impairments lasted less than 2 h across all *NeoSTX-Bup* and *NeoSTX-Saline* doses (fig. 2, D–F).

LD50 Testing with Sciatic Perineural Injection. As *NeoSTX-Saline* and *NeoSTX-Bup* doses increased, more animals developed apnea or gasping respiration and thus were subsequently euthanized. LD50 for *NeoSTX-Saline* was 4.9 μg/kg (95% CI, 4.2 to 6.2) and for *NeoSTX-Bup* was 5.7 μg/kg (95% CI, 4.9 to 7.9) (fig. 5). *NeoSTX-Saline* and *NeoSTX-Bup* LD50 values were not significantly different.

IV Overdose Model. There were no significant differences in rat vital signs before testing (table 2). Compared with bupivacaine infusion, *NeoSTX-Saline* infusion had significantly longer times to apnea, first arrhythmia, and asystole endpoints. High-dose *NeoSTX-Bup* caused asystole and first arrhythmia more rapidly than *Bup*; however, low-dose *NeoSTX-Bup* was not significantly different than *Bup* for any endpoint (fig. 6 and table 3).

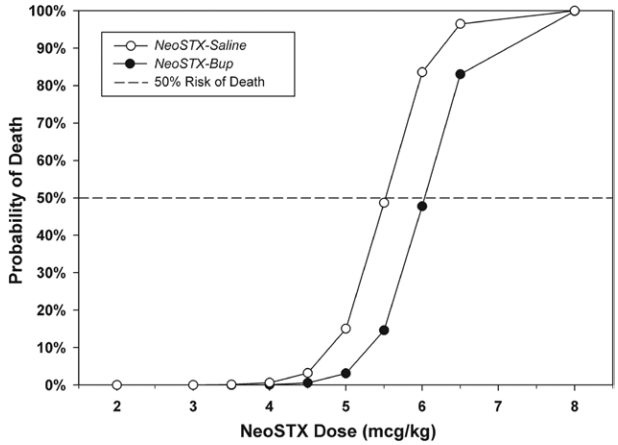

Fig. 5. Median lethal dose curve of Neosaxitoxin (NeoSTX#5) formulation with and without bupivacaine. Remarkably, coinjection of bupivacaine in an extravascular (sciatic perineural) location does not worsen the systemic toxicity of NeoSTX in this model. *NeoSTX-Bup* = Neosaxitoxin-Bupivacaine; *NeoSTX-Saline* = Neosaxitoxin-saline.

Nerve Histology

Estebe–Myers scoring of nerve injury revealed a very benign histologic profile after sciatic injection. For all treatments, the median Estebe–Myers nerve injury score was 0 (IQR = 0 to 0), with no individual scores greater than 2. Numbers of nerves receiving each treatment were as follows: vehicle: 19, bupivacaine 0.25% plain: 19, *NeoSTX-Saline* 1 μg/kg: 4, *NeoSTX-Bup* 1 μg/kg: 8, *NeoSTX-Saline* 2 μg/kg: 4, *NeoSTX-Bup* 2 μg/kg: 7, *NeoSTX-Saline* 3 μg/kg: 19, *NeoSTX-Bup* 3 μg/kg: 27, *NeoSTX-Saline* 3.5 μg/kg: 12, *NeoSTX-Bup* 3.5 μg/kg: 13, *NeoSTX-Saline* 4 μg/kg: 1, N*eoSTX-Bup* 4 μg/kg: 6, and uninjected (right side control): 16. There were no statistical differences between any treatment group and noninjected control (right) sciatic nerves. As a validation of the blinded histologist's readings, slides were obtained from sections of positive control nerves taken from animals who had received deliberate nerve injury (loose ligation model), processed under the same protocol. These positive control nerves all received high injury ratings, with Estebe–Myers scores of 3 or 4. Representative micrographs are shown in figure 7.

Discussion

In this study, rat sciatic blockade was used to assess the intensity and duration of local anesthetic effect with NeoSTX alone and in combination with bupivacaine, with and without epinephrine. *NeoSTX-Saline* gave inconsistent and short-duration block at lower doses, and when doses were escalated to greater than 3 μg/kg, there was increasing evidence of systemic as well as local action, as evidenced by transient impairments in contralateral (right) hindlimb neurobehavioral assessments (fig. 1). *NeoSTX-Bup* produced dense block at all NeoSTX doses, as assessed by three behavioral tests. Increasing NeoSTX doses in *NeoSTX-Bup* combinations produced longer block durations (fig. 2). In

Table 2. Vital Signs at Baseline for Intravenous Overdose Model across Four Treatment Groups (n = 26)

	Treatment Groups				
	Bupivacaine (n = 7)	NeoSTX (n = 6)	Half-dose Combination (n = 6)	Full-dose Combination (n = 7)	*P* Value
Weight (kg)	0.34 ± 0.03	0.35 ± 0.02	0.36 ± 0.02	0.34 ± 0.03	0.19
Temperature (°C)	37.7 ± 0.3	37.6 ± 0.4	37.4 ± 0.4	37.4 ± 0.5	0.59
Heart rate (beats/min)	354 ± 33	377 ± 35	359 ± 32	357 ± 26	0.58
Respiratory rate (rpm)	71 ± 12	82 ± 11	77 ± 12	78 ± 11	0.41
QT interval (ms)	0.10 ± 0.04	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.03	0.19
PR interval (ms)	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.09
QRS interval (ms)	0.015 ± 0.003	0.013 ± 0.003	0.014 ± 0.003	0.015 ± 0.003	0.61

Values are Mean ± SD, overall ANOVA test *P* values presented.

NeoSTX = Neosaxitoxin; PR interval = duration the impulse takes to reach the ventricles from the sinus node; QRS interval = duration of ventricular depolarization; QT interval = duration from the depolarization to the repolarization of the ventricles; rpm = respirations per minute.

an add-on study using constant NeoSTX dosing of 3 μg/kg, addition of epinephrine (*NeoSTX-Bup-Epi*) gave further prolongation of blocks compared with *NeoSTX-Bup* (fig. 4).

Two models for study of systemic toxicity were used. An intramuscular (sciatic perineural) injection model was used to simulate systemic toxicity in an extravascular site, as

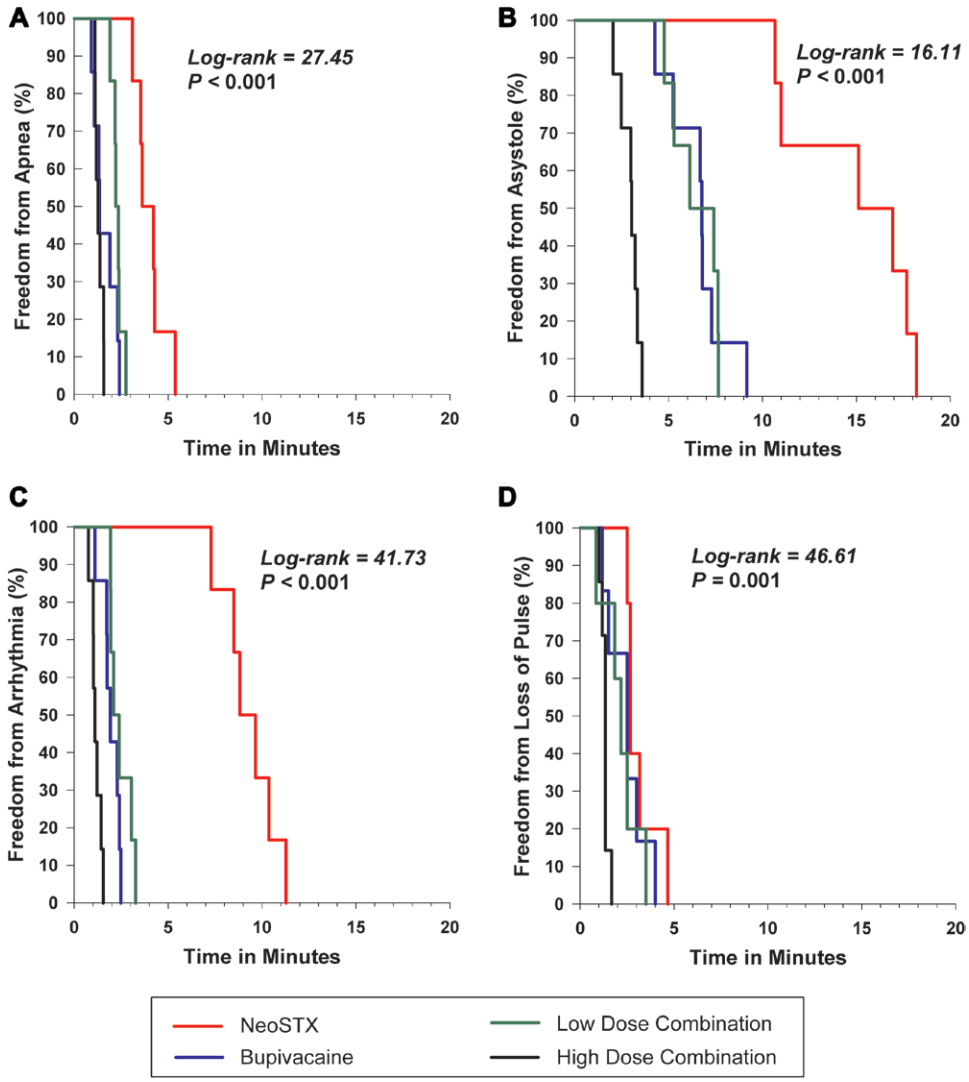

Fig. 6. Kaplan–Meier Survival curves of time-to-event data in intravenous overdose model showing times to apnea (*A*), asystole (*B*), first arrhythmia (*C*), and loss of caudal artery pulsatility (*D*). *Bup* = Bupivacaine; *NeoSTX-Bup* = Neosaxitoxin-Bupivacaine; *NeoSTX-Saline* = Neosaxitoxin-saline.

Table 3. Time (Minutes) to Cardiac Events between Treatment Groups Using the Kaplan–Meier Product-limit Method

	Treatment Groups			
Cardiac Event	Bupivacaine (n = 7)	NeoSTX (n = 6)	Half-dose Combination (n = 6)	Full-dose Combination (n = 7)
Apnea	1.3 (1.2–1.4)	3.6 (2.8–4.5)*	1.3 (1.0–1.5)	2.2 (2.0–2.4)
	Ref.	$P < 0.001$	$P = 0.23$	$P = 0.14$
Loss of pulse	2.5 (1.3–3.7)	2.7 (2.4–3.9)	1.3 (1.2–1.5)	2.2 (1.5–2.9)
	Ref.	$P = 0.29$	$P < 0.001$	$P = 0.53$
First arrhythmia	1.9 (1.5–2.3)	8.8 (7.4–10.2)*	1.1 (0.9–1.3)*	2.1 (1.6–2.6)
	Ref.	$P < 0.001$	$P < 0.001$	$P = 0.17$
Asystole	6.8 (5.2–7.3)	16.0 (11.0–17.7)*	3.0 (2.5–3.3)*	6.8 (5.3–7.6)
	Ref.	$P < 0.001$	$P < 0.001$	$P = 0.92$
Heart rate <270 beats/min	1.3 (0.9–1.8)	5.2 (4.0–6.4)	2.0 (1.4–2.6)	1.2 (0.7–1.6)
	Ref.	$P < 0.001$	$P = 0.59$	$P = 0.76$

Data represent the median time to event in minutes (95% CI). *P* values are based on the log-rank test, using Bupivacaine treatment group as the reference group.

* Corrected Bonferroni significance levels $P < 0.017$ when compared with bupivacaine.

NeoSTX = Neosaxitoxin.

intended for nerve blockade or infiltration in clinical use. In this model, coinjection of bupivacaine along with NeoSTX does not increase the systemic toxicity compared with animals receiving the same dose of NeoSTX in saline (figs. 1, D–F, and 5). In this model, the bupivacaine dose was constant and always below the range that produces cardiotoxicity. The transient contralateral effects at the 3 and 3.5 μg/kg doses did not significantly differ between the *NeoSTX-Saline* and *NeoSTX-Bup* groups, except for a borderline significant difference in the EPT test at 3.5 μg/kg ($P = 0.047$). In this model, the presumed mode of death is from respiratory muscle weakness. Because *NeoSTX-Bup* produces more reliable and longer-lasting block compared with *NeoSTX-Saline*, whereas not increasing the systemic toxicity of NeoSTX, *NeoSTX-Bup* provides a significant improvement in therapeutic index compared with *NeoSTX-Saline*.

The second systemic toxicity model used deliberate IV rapid infusion to simulate the effects of accidental intravascular injection (fig. 6). NeoSTX and bupivacaine were each infused either alone, in full-concentration combination, or in combinations with half-concentrations of each of the two components, and time-to-event endpoints (apnea, electrocardiogram deterioration, asystole, and loss of peripheral perfusion) were used as measures of toxicity. In animals receiving IV NeoSTX alone, apnea occurred long before deterioration of the cardiac rhythm, whereas these two events occurred much closer in time with IV bupivacaine alone (table 3). With full concentrations of both drugs, the toxicity was slightly greater (time to event was shorter) than either drug alone. However, with half-concentrations of each drug, the toxicity was not greater than with bupivacaine alone. Interpretations regarding the clinical risks of intravascular injection of the combinations should be cautious. Clinical toxicity of NeoSTX-bupivacaine combinations will probably depend on the injected doses, rates of injection, baseline physiologic status of the patient, and on the speed of resuscitative interventions, including respiratory support, vasoactive medications, medications to terminate convulsions, and treatment of rhythm disturbances.[11–14]

Previous research has demonstrated that amino-amide local anesthetics produce local myotoxicity[10] and neurotoxicity[8] that increases with local anesthetic concentration and duration of exposure. The local toxicities of traditional local anesthetics appear not to arise from sodium channel blockade *per se*, but rather from actions on several other cellular targets.[9,16] Several approaches to prolonged local anesthesia involving controlled release of bupivacaine from microparticles or liposomes found local inflammation[27,28] as well as myotoxicity and neurotoxicity. Although the clinical significance of these tissue effects for different formulations is open to debate, in our view avoidance of inflammation, neurotoxicity, and myotoxicity is a desirable feature of any proposed approach to prolonged local anesthesia. In contrast to these observations with prolonged delivery of amino amides, previous histologic studies of several site-1 sodium channel blockers have shown minimal injury or inflammatory effects on muscle or nerve.[9,16] Even when the site-1 blocker is delivered *via* liposomes over periods of weeks, the tissue response is extremely mild.[15] In the current study, sciatic nerves were assessed by a blinded neuroscientist using an established nerve preparation protocol and scoring system. Overall, the results from these histologic studies gave very low injury scores, providing preliminary support for anticipating benign local tissue effects from NeoSTX with bupivacaine combinations. Additional studies are planned using different staining procedures and different time courses.

Conclusions

A formulation of NeoSTX developed for clinical trials provides prolonged longer-duration sciatic nerve blockade when administered in combination with bupivacaine, with or

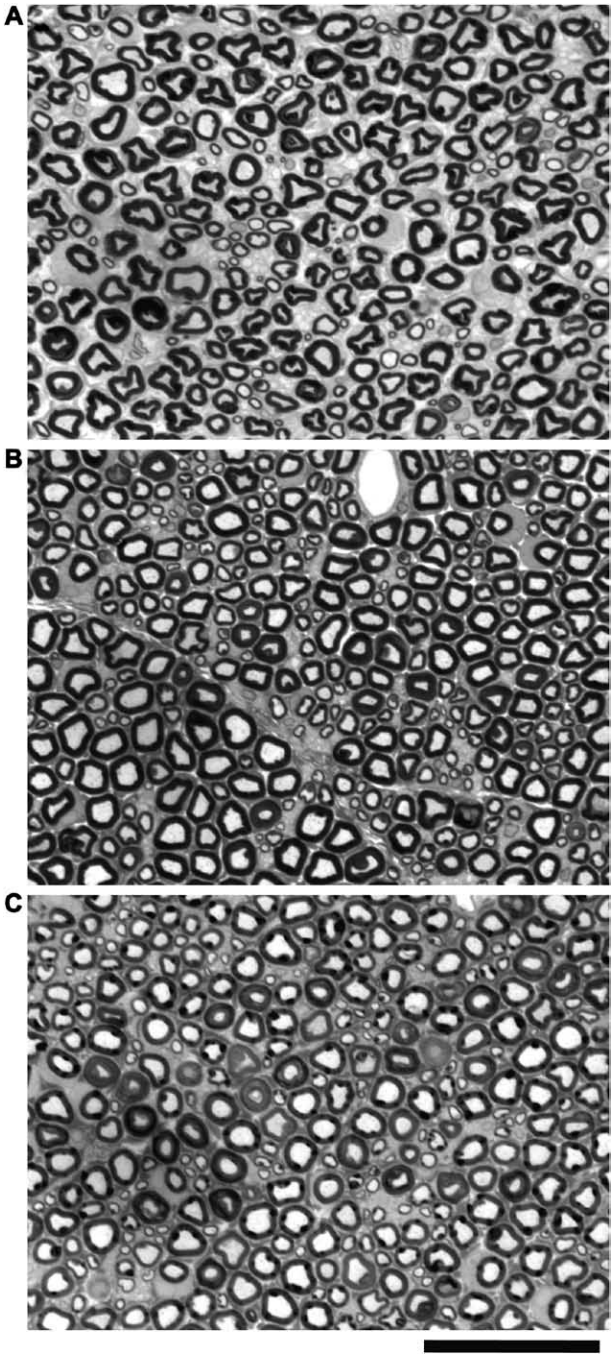

Fig. 7. Photomicrographs of sciatic nerve sections from treatments with Bupivacaine (*A*), Neosaxitoxin-saline (*NeoSTX-Saline*) (*B*), and Neosaxitoxin-Bupivacaine (*NeoSTX-Bup*) (*C*). *Scale bar* = 50 μm. The Neosaxitoxin concentration was 3 μg/kg for both *NeoSTX-Saline* and *NeoSTX-Bup*.

without epinephrine. Coinjection with bupivacaine improves the reliability and duration of blockade compared with NeoSTX alone, and addition of epinephrine gives further prolongation of block durations. Sciatic nerve histology is reassuring. These data provide further support for proceeding with clinical trials of NeoSTX-bupivacaine combinations, with or without epinephrine, for prolonged local anesthesia.

Acknowledgments

Support was received from the Boston Children's Hospital's Technology and Innovation Development Office and the Sara Page Mayo Endowment for Pain Research, Boston, Massachusetts (to Dr. Berde).

Competing Interests

Dr. Berde and his collaborators (Drs. Kohane, Strichartz, and Langer) hold issued patents on site-1 blockers, including Neosaxitoxin, for prolonged-duration local anesthesia. Dr. Berde is Investigational New Drug holder for Neosaxitoxin. Boston Children's Hospital has a collaboration agreement with Proteus SA (Santiago, Chile) for commercial development of Neosaxitoxin. In the event of future commercial development, Dr. Berde, his coinventors, and Boston Children's Hospital could potentially receive royalties. Dr. Berde has received no research support, equity, consulting fees, or other income from Proteus SA or any other commercial partner related to this study. The other authors declare no competing interests.

Correspondence

Address correspondence to Dr. Berde: Department of Anesthesiology, Perioperative, and Pain Medicine, Boston Children's Hospital, 333 Longwood Avenue, Boston, Massachusetts 02115. charles.berde@childrens.harvard.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

1. Kohane DS, Lu NT, Gökgöl-Kline AC, Shubina M, Kuang Y, Hall S, Strichartz GR, Berde CB: The local anesthetic properties and toxicity of saxitonin homologues for rat sciatic nerve block *in vivo*. Reg Anesth Pain Med 2000; 25:52–9
2. Rodriguez-Navarro AJ, Lagos N, Lagos M, Braghetto I, Csendes A, Hamilton J, Figueroa C, Truan D, Garcia C, Rojas A, Iglesias V, Brunet L, Alvarez F: Neosaxitoxin as a local anesthetic: Preliminary observations from a first human trial. Anesthesiology 2007; 106:339–45
3. Rodríguez-Navarro AJ, Berde CB, Wiedmaier G, Mercado A, Garcia C, Iglesias V, Zurakowski D: Comparison of neosaxitoxin *versus* bupivacaine *via* port infiltration for postoperative analgesia following laparoscopic cholecystectomy: A randomized, double-blind trial. Reg Anesth Pain Med 2011; 36:103–9
4. Rodriguez-Navarro AJ, Lagos M, Figueroa C, Garcia C, Recabal P, Silva P, Iglesias V, Lagos N: Potentiation of local anesthetic activity of neosaxitoxin with bupivacaine or epinephrine: Development of a long-acting pain blocker. Neurotox Res 2009; 16:408–15
5. Catterall WA: Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu Rev Pharmacol Toxicol 1980; 20:15–43
6. Penzotti JL, Lipkind G, Fozzard HA, Dudley SC Jr: Specific neosaxitoxin interactions with the Na^+ channel outer vestibule determined by mutant cycle analysis. Biophys J 2001; 80:698–706
7. Kohane DS, Yieh J, Lu NT, Langer R, Strichartz GR, Berde CB: A re-examination of tetrodotoxin for prolonged duration local anesthesia. Anesthesiology 1998; 89:119–31
8. Lambert LA, Lambert DH, Strichartz GR: Irreversible conduction block in isolated nerve by high concentrations of local anesthetics. Anesthesiology 1994; 80:1082–93

9. Drasner K: Local anesthetic neurotoxicity: Clinical injury and strategies that may minimize risk. Reg Anesth Pain Med 2002; 27:576–80
10. Padera R, Bellas E, Tse JY, Hao D, Kohane DS: Local myotoxicity from sustained release of bupivacaine from microparticles. Anesthesiology 2008; 108:921–8
11. Butterworth JFt: Models and mechanisms of local anesthetic cardiac toxicity: A review. Reg Anesth Pain Med 2010; 35:167–76
12. Di Gregorio G, Neal JM, Rosenquist RW, Weinberg GL: Clinical presentation of local anesthetic systemic toxicity: A review of published cases, 1979 to 2009. Reg Anesth Pain Med 2010; 35:181–7
13. Wolfe JW, Butterworth JF: Local anesthetic systemic toxicity: Update on mechanisms and treatment. Curr Opin Anaesthesiol 2011; 24:561–6
14. Neal JM, Bernards CM, Butterworth JF IV, Di Gregorio G, Drasner K, Hejtmanek MR, Mulroy MF, Rosenquist RW, Weinberg GL: ASRA practice advisory on local anesthetic systemic toxicity. Reg Anesth Pain Med 2010; 35:152–61
15. Epstein-Barash H, Shichor I, Kwon AH, Hall S, Lawlor MW, Langer R, Kohane DS: Prolonged duration local anesthesia with minimal toxicity. Proc Natl Acad Sci U S A 2009; 106:7125–30
16. Sakura S, Bollen AW, Ciriales R, Drasner K: Local anesthetic neurotoxicity does not result from blockade of voltage-gated sodium channels. Anesth Analg 1995; 81:338–46
17. Wylie MC, Johnson VM, Carpino E, Mullen K, Hauser K, Nedder A, Kheir JN, Rodriguez-Navarro AJ, Zurakowski D, Berde CB: Respiratory, neuromuscular, and cardiovascular effects of neosaxitoxin in isoflurane-anesthetized sheep. Reg Anesth Pain Med 2012; 37:152–8
18. Zhang H, Ji H, Liu Z, Ji Y, You X, Ding G, Cheng Z: Voltage-dependent blockade by bupivacaine of cardiac sodium channels expressed in *Xenopus* oocytes. Neurosci Bull 2014; 30:697–710
19. González T, Arias C, Caballero R, Moreno I, Delpón E, Tamargo J, Valenzuela C: Effects of levobupivacaine, ropivacaine and bupivacaine on HERG channels: stereoselective bupivacaine block. Br J Pharmacol 2002; 137:1269–79
20. Shin HW, Soh JS, Kim HZ, Hong J, Woo DH, Heo JY, Hwang EM, Park JY, Lee CJ: The inhibitory effects of bupivacaine, levobupivacaine, and ropivacaine on K2P (two-pore domain potassium) channel TREK-1. J Anesth 2014; 28:81–6
21. Butterworth J, James RL, Grimes J: Structure-affinity relationships and stereospecificity of several homologous series of local anesthetics for the β2-adrenergic receptor. Anesth Analg 1997; 85:336–42
22. Zimmer T: Effects of tetrodotoxin on the mammalian cardiovascular system. Mar Drugs 2010; 8:741–62
23. Thalhammer JG, Vladimirova M, Bershadsky B, Strichartz GR: Neurologic evaluation of the rat during sciatic nerve block with lidocaine. Anesthesiology 1995; 82:1013–25
24. Estebe JP, Myers RR: Amitriptyline neurotoxicity: Dose-related pathology after topical application to rat sciatic nerve. Anesthesiology 2004; 100:1519–25
25. Finney DJ: The median lethal dose and its estimation. Arch Toxicol 1985; 56:215–8
26. Faraggi D, Izikson P, Reiser B: Confidence intervals for the 50 percent response dose. Stat Med 2003; 22:1977–88
27. Curley J, Castillo J, Hotz J, Uezono M, Hernandez S, Lim JO, Tigner J, Chasin M, Langer R, Berde C: Prolonged regional nerve blockade. Injectable biodegradable bupivacaine/polyester microspheres. Anesthesiology 1996; 84:1401–10
28. Castillo J, Curley J, Hotz J, Uezono M, Tigner J, Chasin M, Wilder R, Langer R, Berde C: Glucocorticoids prolong rat sciatic nerve blockade *in vivo* from bupivacaine microspheres. Anesthesiology 1996; 85:1157–66