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# A Re-examination of Tetrodotoxin for Prolonged Duration Local Anesthesia

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***Background*: Highly potent toxins such as tetrodotoxin that block sodium channels with great specificity have been studied for many years and can provide prolonged blockade when coadministered with vasoconstrictors or conventional local anesthetics. Their utility has been constrained, however, by systemic toxicity. The authors examined the efficacy of tetrodotoxin with and without epinephrine or bupivacaine for producing prolonged-duration sciatic nerve blockade in the rat, and they assessed the degree of concomitant toxicity.**

***Methods*: Rats received percutaneous sciatic nerve blockade using tetrodotoxin with and without epinephrine or bupivacaine. A subset received subcutaneous injections at the nuchal midline. Nociceptive, proprioceptive, and motor blockade were quantified using contralateral leg responses as controls for systemic effects.**

***Results*: Tetrodotoxin without epinephrine produced sciatic nerve blockade, but with considerable toxicity at most effective doses. Epinephrine reduced the median effective concentration of tetrodotoxin for nociception from 37.6 to 11.5 $\mu$M and prolonged its duration, such that reversible blocks lasting >13 h were achieved. Epinephrine reduced measures of systemic distribution and increased the median lethal dose of tetrodotoxin from 40 to 53.6 nmole/kg, thus more than quadrupling the therapeutic index. Bupivacaine increased the local anesthetic potency of tetrodotoxin, reduced its systemic toxicity, and, when coinjected subcutaneously, increased the median lethal dose from 43.7 to 47.7 nmole/kg. The addition of epinephrine did not further improve the effectiveness of the bupivacaine–tetrodotoxin combination.**

***Conclusion*: Combinations of epinephrine or bupivacaine with tetrodotoxin or with other high-potency toxins active on sodium channels should be examined for the potential to provide clinically useful, prolonged nerve blockade. (Key words: Bupivacaine; epinephrine; toxicity.)**

TETRODOTOXIN is a naturally occurring toxin found in several organisms, most notoriously the puffer fish (*Fugu*). The local anesthetic properties of tetrodotoxin have long been known.[1–4] The mechanism of action is a unimolecular blockade of a sodium channel, at a site and by an action that differs from that of lidocaine (see the review by Ritchie and Rogart[5]). Adams *et al.*[4] applied tetrodotoxin percutaneously for nerve blocks in rats and found that it provides prolonged blockade when injected in combination with vasoconstrictors or conventional local anesthetics.

Despite these desirable characteristics, tetrodotoxin has not achieved clinical use as a local anesthetic, apparently because of the considerable systemic toxicity. Tetrodotoxin toxicity causes neural blockade and muscular weakness resulting in diaphragmatic paralysis leading to respiratory arrest and death.[6] Hypotension is also a prominent feature.[7] The addition of a vasoconstrictor decreases the risk of death from tetrodotoxin.[4] It was recently reported that tetrodotoxin does not cause local neurotoxicity.[8] This, and its comparative lack of central nervous or cardiac sequelae,[7] has lead us to re-examine the potential clinical utility of this compound as a local anesthetic.

Our interest in tetrodotoxin and similar high-potency

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drugs also stems from their potential use in the creation of anesthetic formulations that confer nerve block durations that exceed those of currently available local anesthetics. Although Adams *et al.*[4] showed that blockade from tetrodotoxin was prolonged by vasoconstrictors and conventional local anesthetics, the upper limit of that duration was not demonstrated, and synergism (rather than additivity) in the tetrodotoxin-local anesthetic interaction was not established. Whether it was possible to produce markedly prolonged blockade given the limitations imposed by the toxicity of tetrodotoxin also was not addressed. Importantly, the issue of whether tetrodotoxin and conventional local anesthetics have cumulative systemic toxicities was not investigated. Therefore, the possibility of achieving both an effective and safe long-duration blockade with a mixture of tetrodotoxin and local anesthetics or vasoconstrictors, or both, remains untested.

We investigated the potential for tetrodotoxin, alone or combined with a second drug (a vasoconstrictor or local anesthetic), to provide safe prolonged-duration local anesthesia. We quantified the toxicity of tetrodotoxin using two principal measures: (1) the degree of impairment of the contralateral leg (as a control for sublethal systemic toxicity), and (2) the median lethal dose ($LD_{50}$). We also describe the relative degrees to which tetrodotoxin affects different functional modalities using a detailed neurobehavioral model.[9]

## Materials and Methods

### *Stock Tetrodotoxin Solutions*

Tetrodotoxin stock solutions were made by dissolving 1 mg tetrodotoxin (Sigma Chemical Co., St. Louis, MO) in 10 ml of 20 mM citrate buffer (Na citrate:citrate ratio, 55:45), *p*H 4.45. Bupivacaine hydrochloride (Sigma Chemical) was formulated in 10 mM morpholinopropane-sulfonic acid (MOPS; Sigma Chemical) titrated to a *p*H of 6.55 with NaOH. This stock solution was most commonly diluted with tetrodotoxin stock and saline (Baxter Healthcare Corp., Deerfield, IL) toward a target concentration of 15.4 mM (≈0.5%). Epinephrine from a commercial 1:1,000 (1 μg/μl) solution (American Regent Laboratories, Shirley, NY) was diluted to the desired concentration. A fresh vial of epinephrine was used each day.

### *Animal Care*

Animals were cared for in compliance with protocols approved by the Children's Hospital Animal Care and Use Committee. Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). They were housed in groups and kept in a 6 A.M.–6 P.M., light–dark cycle. Young adult male Sprague-Dawley rats weighing 310–420 g were used. Rats were handled repeatedly by the investigators to diminish effects resulting from stress-induced analgesia. Rats that became flaccid as a result of tetrodotoxin injection were anesthetized with halothane (with bag-and-mask ventilation) and then killed with carbon dioxide.

### *Sciatic Blockade Technique*

Before nerve block injections, rats were anesthetized briefly with halothane (2–4% inspired concentration in 100% oxygen) by face mask. Previous research[10] indicated that this reduced aversive behaviors with repeated procedures and made injection more precise. That study also confirmed that a brief halothane anesthetic had no effect on measures of blockade after the rats emerged from anesthesia, and that block durations were also unaffected. The duration of anesthesia was usually <2 min. Halothane was withheld from one control group.

The block was initiated by introducing a 23-gauge needle posteromedially to the greater trochanter pointed in an anteromedial direction.[9] Once bone was touched, the needle was withdrawn 1 mm and the drug was injected. The final volume of injectate was 0.3 ml test solution, except in one set of experiments, in which it was 0.1 ml. The left leg was always used for blocks, the right leg served as a control.

In most cases, injected doses are reported by concentration (molarity). Because the volume of injectate is 0.3 ml (except when stated otherwise), 10 μM tetrodotoxin corresponds to approximately 1 μg tetrodotoxin (actually 0.96 μg), 20 μM corresponds to 2 μg, and so on. Similarly, 15.4 mM bupivacaine corresponds to 0.5% bupivacaine, 7.7 mM to 0.25%, and so on, and 55 μM epinephrine corresponds to 1:100,000 epinephrine. When the $LD_{50}$ was determined, the dose in nmole/kg was considered a more relevant unit.

### *Subcutaneous Injection Technique*

The nuchal area was shaved and the skin was lifted away from underlying structures. A 23-gauge needle was inserted subcutaneously and then advanced anteriorly parallel to the axis of the body to a distance of approximately 1 cm (to avoid back leakage of drug through the skin puncture site). The volume injected varied depending on the dose delivered, the concentra-

tion of the solution used, and the weight of the rat; volumes were 0.25 to 0.3 ml per 300 g weight.

*Neurobehavioral Assessment of Nerve Blockade*

The effectiveness of block was measured at various time points, applying modifications of the methods of Thalhammer *et al.*,[9] as will be described. In all experiments, the investigator testing the rats was blinded to what drug was injected into any given rat.

The following modalities/functions were measured.

- Blockade of thermal nociception (TN) was assessed using a modified hot plate test.[11] Hind paws were exposed in sequence (left then right) to a hot plate at 56°C (model 39D hot plate analgesia meter; IITC Inc., Woodland Hills, CA), and the time (thermal latency) that the animal left its paw there was measured with a stopwatch. After 12 s, the paw was removed by the experimenter to avoid injury to the animal or the development of hyperalgesia. This test was repeated three times (with a 10-s pause between tests) for each rat at every time point. It is important to emphasize that although the sensation of the lateral foot is mediated by the sciatic nerve, the hip and knee flexion necessary to remove the foot from the hot plate is mediated by the femoral nerve, which we did not block. Therefore this test was specific for nociceptive block.
- The positional placing response (PPR) tests proprioception primarily. Ordinarily, a prone rat will respond to having its hindpaw pulled back (with the dorsum in contact with the table surface) by returning it to a position alongside its flank, with the claws splayed (score = 1). Blockade results in the limb trailing behind the rat with the claws clubbed (score = 4). If the foot is returned fully to the flank but the digits are clubbed, the score is 2. Any other outcome (*e.g.*, the foot is left out at an angle) yielded a score of 3.
- Hopping is a complex integrative test of sensory, proprioceptive and motor function. When suspended above a horizontal surface in the hands of an experimenter so that only one foot touches that surface, a rat will hop when its body is slowly moved laterally. It will not do so if there is sensory or motor block. This was scored (1 or 0) according to whether the animal could hop.
- To test the extensor postural thrust (EPT), the rat was held with its posterior placed above a digital balance on which it could bear weight with one hindpaw at a time. The maximum weight that the rat could bear without its ankle touching the balance was measured.

*Data Processing*

The effects of the various drug combinations are primarily reported in terms of the duration of effective block (DEB). The DEB for TN (DEB-TN) is the time required for thermal latency to return to a value of 7 s (which is 50% of maximal block when a baseline thermal latency of approximately 2 s is considered). The DEB for PPR (DEB-PPR) is the time that it took for function to return to a score of 2 (4 being a complete block). The DEB for hopping (DEB-Hop) was defined as the midpoint between the last recorded time point at which the animal could not hop and the first time point when this ability had returned. The DEB for EPT (DEB-EPT) data was defined as the time for weight bearing to return halfway to normal from the maximal block. The halfway point for each rat was determined by the following equation: midpoint = [(highest weight borne by either leg) − (lowest weight borne by blocked leg)] divided by 2 + lowest weight borne by blocked leg. This method of analysis measures the dynamic component of the weight/force exerted by the rat, as it subtracts the weight of the flaccid paralyzed foot from the total force exerted.

Animals that did not survive the acute block were not included in the DEB calculation. However, we must emphasize that the DEBs of all other animals were included in the calculations of average DEBs. The DEB for the appropriate modality was considered 0 (zero) for all "unsuccessful" blocks, defined as injections that did not result in a thermal latency of at least 7 s, a PPR score of 2 or more, a hopping score of 0, or an EPT suppression of at least 50%. Thus "missed" blocks were not excluded from analysis. Pilot studies show that injection of 0.3 ml 0.5% bupivacaine results in a "missed block" rate by these investigators of 0% (n = 150). Therefore, causes of failure to achieve block with some solutions used herein are probably not a result of needle placement but reflect pharmacologically significant factors such as drug potency, concentration, volume, spread through tissues, partitioning between aqueous and lipophilic compartments, and so forth.

*Statistical Analysis*

Values are usually reported as means with standard deviations. Unless stated otherwise, statistical inferences (probability values) were made using the Student's *t* test (paired in comparisons between injected and contralateral legs, unpaired in all other cases) or

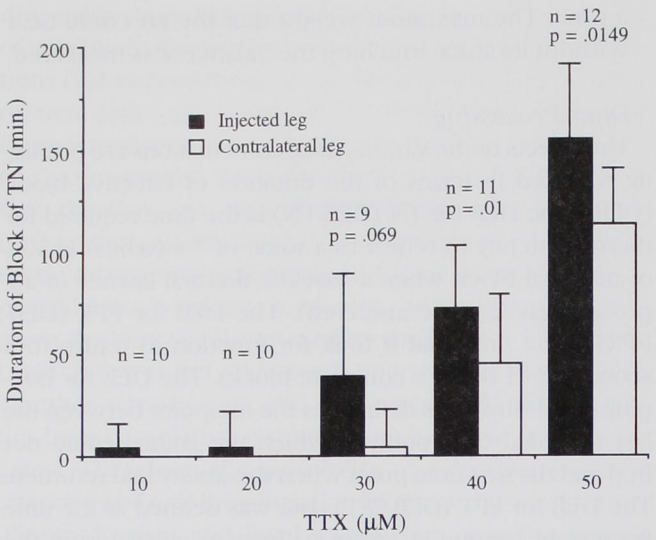


**Fig. 1. Durations of thermal nociception (DEB-TN) block in the injected and contralateral legs at various concentrations of tetrodotoxin (mean ± SD). The probability values comparing DEB-TN in both legs were calculated using a paired *t* test. Injection of high concentrations of tetrodotoxin was associated with pronounced blockade of the contralateral leg.**

analysis of variance. A subset of our data might have non-normal distributions because of the inclusion of zero-duration blocks, as described in the preceding paragraph. Having verified that the nature of the statistical test did not affect the validity of statistical assertions, we decided not to introduce a second set of (nonparametric) tests into this article to address those instances.

In most circumstances, we considered probability values of 0.05 to be significant. When many comparisons are made, we used the Bonferroni correction factor to determine the probability value. Thus the "significant" probability value is 0.05 divided by the number of comparisons. For example, if three comparisons are made, the probability value required would be 0.05 divided by three, or 0.017.

Logit (logistic regression) analyses were used to derive and compare the $LD_{50}$ and the concentration required to achieve maximal block (*i.e.*, thermal latency of 12 s) in 50% of rats ($EC_{50}$). These data analyses were conducted using Stata statistical software (Stata Corp., College Station, TX).

## Results

### *Local Anesthetic Properties and Systemic Toxicity of Tetrodotoxin*

Groups of rats received sciatic nerve injections with 10, 20, 30, 40, and 50 μM tetrodotoxin in 0.3 ml saline (corresponding to 1–5 μg, respectively). The duration of nociceptive blockade increased with increasing concentrations beyond 20 μM tetrodotoxin (fig. 1, table 1). As a control, six rats were injected with 0.3 ml 0.9% saline. None of them developed any deficits of the modalities measured.

The standard deviations of the DEBs were large relative to the mean values, especially at the lower concentrations. The coefficients of variation for DEB with 10, 20, 30, 40, and 50 μM tetrodotoxin are 425%, 332%, 128%, 41%, and 23%, respectively. For comparison, the coefficient of variation for block with 15.4 mM (0.5%) bupivacaine, which gives a DEB-TN approximately equal to that of 50 μM tetrodotoxin, was 26%. The large coefficients of variation for tetrodotoxin resulted from the fact that in calculating the mean DEB we included unsuccessful blocks (as defined in Materials and Methods), with a defined DEB of zero. We included all failed blocks in our calculations because (1) there was no *a priori* way to distinguish between blocks that failed because a given drug was too weak and the injection was misplaced, and (2) our rate of successful block with 15.4 mM bupivacaine was 100%, suggesting that the drug was being deposited at an effective location. Un-

**Table 1. Duration of Thermal Nociceptive Block (DEB-TN) from TTX Alone or with 55 μM Epinephrine**

| TTX (μM) | TTX Alone | n | DEB-TN (min) with Epinephrine | n | *P* Value |
|---|---|---|---|---|---|
| 10 | 4 ± 17 | 10 | 266 ± 250 | 10 | 0.009 |
| 20 | 5 ± 17 | 10 | 446 ± 283 | 10 | 0.0003 |
| 30 | 39 ± 50 | 9 | 656 ± 123 | 10 | $5.2 \times 10^{-9}$ |
| 40 | 72 ± 30 | 11 | 655 ± 186 | 10 | $4.2 \times 10^{-6}$ |
| 50 | 154 ± 36 | 12 | 795 ± 230 | 11 | $3.6 \times 10^{-6}$ |
| 100 | — | 6/6 dead | 979 ± 218 | 2 (4/6 dead) | — |

Values for DEB-TN are mean ± SD. *P* values comparing DEB-TN of TTX with and without epinephrine were determined by Student's *t* test.

successful blocks were common at the lower concentrations of tetrodotoxin. When the zero DEBs are assumed to be missed blocks and are discarded, the standard deviations become much smaller. For example, the coefficient of variation for the DEB-TN of 30 μM tetrodotoxin decreases from 128% to 38%, and for 40 μM tetrodotoxin it decreases from 41% to 24%.

At low concentrations of tetrodotoxin, successful TN block was infrequent but was always unilateral in the injected leg. As the dosage of tetrodotoxin was increased, the number of successful TN blocks increased, but the fraction of successful blocks that were associated with sensory and motor deficits in the contralateral (uninjected) limbs also increased markedly (table 2). At high concentrations, TN blockade was uniformly "successful," but the contralateral leg was also strongly affected, presumably by sublethal systemically distributed toxin (fig. 1). The deficits were significantly greater in the injected than in the uninjected limbs. None of the rats showed deficits in the contralateral leg only. These observations imply that deficits in behavior from tetrodotoxin alone resulted from a combination of local blockade and systemic effects.

Control experiments reconfirmed our previous experience[10] that the contralateral deficits were unrelated to the presence or absence of a brief halothane general anesthetic; impairment was similar in animals having an injection when awake (n = 4) or when anesthetized (data not shown). Furthermore, animals given halothane without sciatic nerve block (n = 3) had normal latencies in the contralateral leg when they awoke.

Another indication that the observed deficits in both the injected and contralateral legs were at least partly due to systemic toxin was the finding that subcutaneous injection of 40 μM tetrodotoxin at the nuchal midline produced increased thermal latency in both legs. Thermal nociception in the left leg was affected, with a DEB-TN of 100 ± 32 min, comparable to the 72 ± 30 min for the same concentration of tetrodotoxin injected at that leg's sciatic nerve.

**Table 2. Frequency of Successful Thermal Nociceptive Blocks in the Injected and Contralateral Leg**

| TTX (μM) | % Successful Blocks Injected Leg | % Successful Blocks with Contralateral Block |
|---|---|---|
| 10 | 10 (1/10) | 0 (0/1) |
| 20 | 10 (1/10) | 0 (0/1) |
| 30 | 44 (4/9) | 25 (1/4) |
| 40 | 91 (10/11) | 80 (8/10) |
| 50 | 100 (12/12) | 100 (12/12) |

For each concentration of TTX in the first column, the second column shows the percentage of injections that resulted in a successful block (defined as resulting in a thermal latency of at least 7 s). The third column shows the percentage of those successful blocks that were associated with blockade in the contralateral limb.

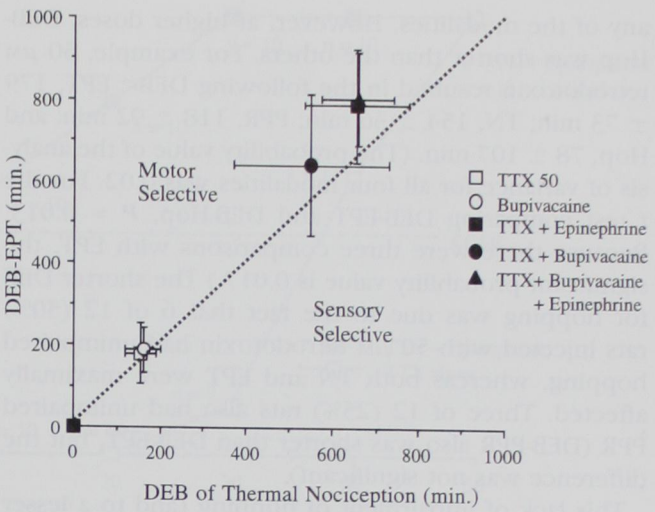


**Fig. 2. Duration of block for thermal nociception (sensory function) and extensor postural thrust (motor function) for 50 μM (5 μg) tetrodotoxin alone, 15.4 mM (0.5%) bupivacaine alone, and 30 μM tetrodotoxin (3 μg) combined with bupivacaine, 55 μM (1:100,000) epinephrine, or both. The dotted line is a line of identity between nociceptive and motor blockade.**

Deficits in the contralateral leg were accompanied by a range of symptoms that varied from none to death, depending on the tetrodotoxin concentration. Some rats developed lower extremity impairment of TN without appearing grossly sick or weak (although fine testing, such as EPT, would reveal marked weakness). There was overt toxicity at the higher concentrations. One of 12 animals in the 40-μM group and 3 of 15 in the 50-μM group died, and many others appeared lethargic or flaccid or had difficulty breathing. All of the six animals given 100 μM tetrodotoxin injections (≈20 μg/kg or 62 nmole/kg) died within 30 min. The $LD_{50}$ from percutaneous injection of tetrodotoxin alone was 40 nmole/kg (12.9 μg/kg; 95% CI: 34.8 to 45.2 nmole/kg).

### *Pattern of Functional Impairment from Tetrodotoxin*

Block durations after tetrodotoxin injection for TN and EPT (a measure of motor block) were approximately equal (fig. 2). At low concentrations (*e.g.*, 30 μM, table 3) there was no significant difference between

any of the modalities. However, at higher doses, DEB-Hop was shorter than the others. For example, 50 μM tetrodotoxin resulted in the following DEBs: EPT, 179 ± 73 min; TN, 154 ± 36 min; PPR, 118 ± 92 min; and Hop, 78 ± 107 min. (The probability value of the analysis of variance for all four modalities was 0.02. For the *t* test comparing DEB-EPT and DEB-Hop, $P = 0.013$. Because there were three comparisons with EPT, the significant probability value is 0.017.) The shorter DEB for hopping was due to the fact that 6 of 12 (50%) rats injected with 50 μM tetrodotoxin had unimpaired hopping, whereas both TN and EPT were maximally affected. Three of 12 (25%) rats also had unimpaired PPR (DEB-PPR also was shorter than DEB-EPT, but the difference was not significant).

This lack of impairment of hopping (and to a lesser extent, PPR) was not seen with 15.4 mM (0.5%) bupivacaine, where all four modalities were impaired in all rats (table 3). It also had not been observed in previous studies of local anesthetic blockade using similar measures.[9,11,12] However, it occurred in 100% of rats injected with 40 μM tetrodotoxin subcutaneously in the neck (n = 12).

### *Effect of a Second Drug on Duration and Effectiveness*

**Epinephrine.** Groups of rats were injected with 10–50 μM tetrodotoxin made up in 55 μM (1:100,000) epinephrine. The vasoconstrictor greatly increased the duration of blockade of all concentrations of tetrodotoxin (fig. 3, table 1). Concentrations of tetrodotoxin that had little effect alone produced strong anesthesia when coinjected with epinephrine, whereas the higher concentrations had their DEBs prolonged by several times.

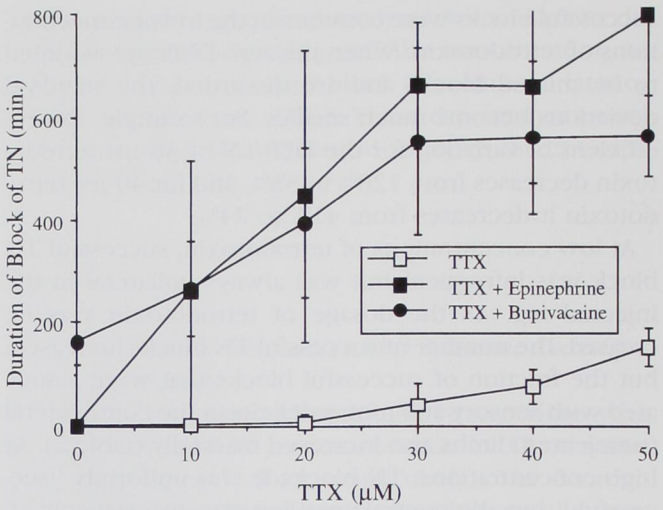


**Fig. 3. Duration of effective block of thermal nociception for various concentrations of tetrodotoxin alone (*open squares*) or in combination with 55 μM (1:100,000) epinephrine (*dark squares*) or 15.4 mM (0.5%) bupivacaine (*dark circles*).**

We also investigated the effect of the addition of epinephrine on the frequency with which tetrodotoxin achieved maximal thermal nociceptive block (*i.e.*, thermal latency = 12 s; fig. 4). Groups of rats were given sciatic nerve blocks with various concentrations of tetrodotoxin with or without epinephrine. The fraction developing a maximal block was plotted against the concentration delivered, and the $EC_{50}$ was derived for each group. The $EC_{50}$ was decreased by more than three times by the addition of epinephrine, from 37.6 μM (95% CI, 34.2 to 41 μM) to 11.5 μM (95% CI, 8 to 15 μM; $P < 0.0001$).

The increase in DEB-TN due to epinephrine was concentration dependent (fig. 5). Very low concentrations

**Table 3. Duration of Effective Block for Each of the Functional Modalities**

| Drug or Combination | Duration of Effective Block (in minutes.) TN | PPR | Hop | EPT | n | ANOVA |
|---|---|---|---|---|---|---|
| TTX alone | 39 ± 50 | 25 ± 52 | 27 ± 67 | 55 ± 54 | 9 | 0.65 |
| Bupivacaine alone | 161 ± 42 | 162 ± 44 | 143 ± 48 | 190 ± 56 | 18 | 0.04 |
| TTX + epinephrine | 656 ± 123 | 846 ± 126 | 787 ± 137 | 860 ± 109 | 11 | 0.003 |
| TTX + bupivacaine | 550 ± 181 | 629 ± 182 | 587 ± 190 | 642 ± 174 | 10 | 0.63 |
| TTX + bupivacaine + epinephrine | 659 ± 83 | 766 ± 98 | 731 ± 125 | 804 ± 115 | 10 | 0.03 |

Values are mean ± SD. TTX = 30 μM (3 μg), epinephrine = 55 μM (1:100,000), bupivacaine = 15.4 mM (0.5%). ANOVA lists the *P* value of the comparison of the four modalities for each drug.

TN = thermal nociception; PPR = positional placing response (proprioception); Hop = hopping; EPT = extensor postural thrust (motor function).

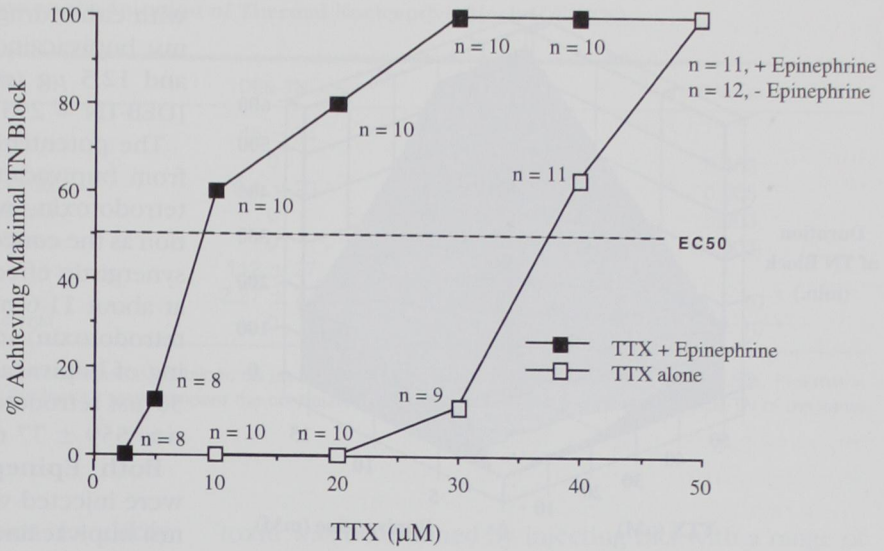


**Fig. 4. Determination of the median effective concentration ($EC_{50}$; to achieve a maximal thermal nociceptive block; *i.e.*, a thermal latency of 12 s) for tetrodotoxin, alone or with 55 μM (1:100,000) epinephrine, in the injected leg. The $EC_{50}$s for tetrodotoxin with epinephrine and tetrodotoxin alone were 11.5 μM and 37.6 μM, respectively ($P < 0.0001$).**

of epinephrine were still able to yield very prolonged blockade. For example, 30 μM tetrodotoxin with 1.1 μM epinephrine (1:5,000,000, one twenty-fifth of the concentration traditionally used with local anesthetics) had no signs of systemic toxicity and had a DEB-TN of 408 ± 243 min, a 10-fold prolongation over the DEB-TN of 30 μM tetrodotoxin alone (39 ± 50 min; $P$ = 0.002). Although the DEB-TN for 30 μM tetrodotoxin with 0.6 μM epinephrine (131 ± 132 min) was considerably increased over that of tetrodotoxin alone, the difference was not significant ($P$ = 0.06).

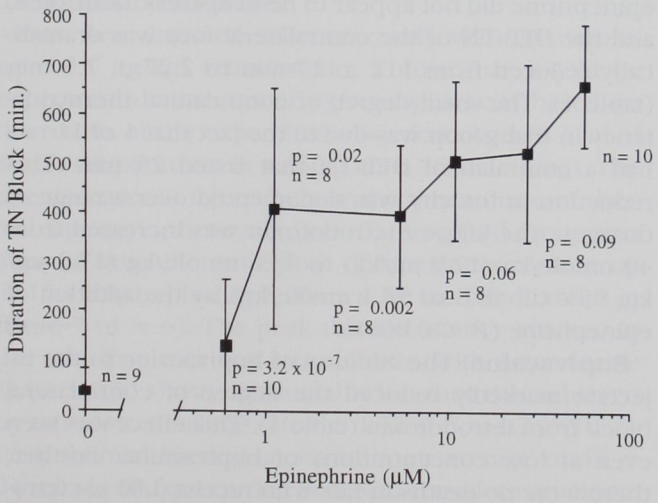


**Fig. 5. Effect of epinephrine concentration on the duration of effective block for thermal nociception (DEB-TN) from 3 μg tetrodotoxin. 55 μM = 1:100,000 epinephrine. Values of DEB-TN are mean ± SD. The probability values result from *t* tests comparing the DEB-TN of tetrodotoxin combined with various epinephrine concentrations with tetrodotoxin combined with 55 μM epinephrine. Epinephrine concentrations as low as 1.1 μM (1:5,000,000) prolonged the DEB-TN.**

The potential for even longer blockade was demonstrated by delivering the same dose of tetrodotoxin (in micrograms) in a 0.1-ml volume (*i.e.*, three times the concentration). When rats were injected with 3 μg tetrodotoxin with epinephrine in 0.1 ml (90 μM tetrodotoxin), the resulting DEB-TN was 948 ± 100 min (n = 6), a 45% increase in block duration over 3 μg in the more dilute formulation ($P$ = 0.00023), with no overt toxicity.

**Bupivacaine.** When the sciatic nerve was blocked with various combinations of tetrodotoxin and bupivacaine (n = 4–24), a marked prolongation of DEB was observed. For example, the DEB-TN for 30 μM tetrodotoxin was 39 ± 50 min (n = 9), that for 15.4 (0.5%) mM bupivacaine was 161 ± 42 min (n = 18), and the DEB-TN of the combination was 556 ± 147 min (n = 11, $P = 1.15 \times 10^{-6}$ *vs.* tetrodotoxin alone, $P = 2.2 \times 10^{-5}$ *vs.* bupivacaine alone). This result showed that the combination of the two drugs yielded a duration of block greater than the sum of the durations from the individual drugs.

Figure 6 shows a three-dimensional surface that describes the DEB-TN as a function of tetrodotoxin injected with bupivacaine. The end points of the curve superimposed on that surface are concentrations of bupivacaine and tetrodotoxin that separately yield equivalent DEB-TNs (154 ± 36 min for 50 μM tetrodo-

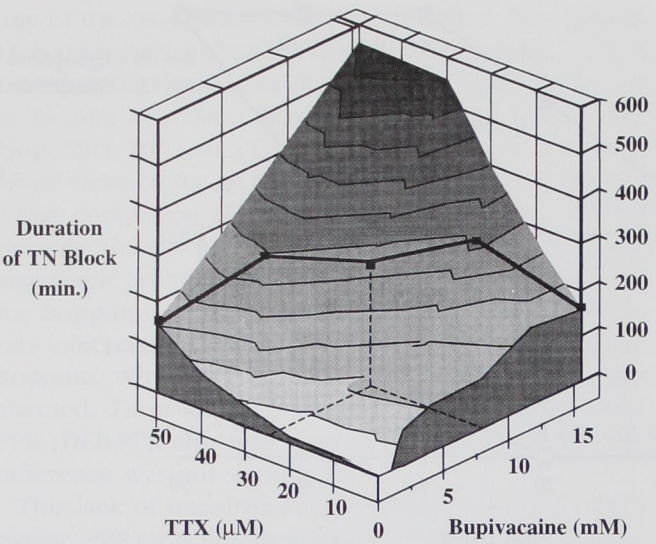


**Fig. 6. Duration of effective block of thermal nociception obtained from combinations of various concentrations of bupivacaine and tetrodotoxin, as well as either drug alone. Each gradation on the surface represents a 50-min increment. The ridge on the leftward face of the surface shows a plateau at bupivacaine doses >11.6 mM, which is not obvious in this perspective. The thick contour line connecting the DEB-TN for 50 μM tetrodotoxin and 15.4 mM (0.5%) bupivacaine intersects the DEB-TN achieved by three combinations that would yield equal DEBs (≈160 min) if tetrodotoxin and bupivacaine were merely additive. The dotted lines demarcate the DEB-TN resulting from one-half the maximal dose of either drug alone. The synergism of tetrodotoxin with bupivacaine is shown.**

toxin, 161 ± 42 min for 15.4 mM bupivacaine). The three points along that line represent the DEB-TN obtained from combinations of lower concentrations of bupivacaine and tetrodotoxin that should last as long as either drug alone if the combinations were merely additive (*i.e.*, should also last ≈160 min). The analysis of variance for the DEB-TN at those three points and for 50 μM tetrodotoxin and 15.4 mM bupivacaine yielded a probability value of 0.00067.

The dotted lines in figure 6 demarcate the point representing the combination of one half the concentration of bupivacaine and one half the concentration of tetrodotoxin, which each provide a DEB-TN of approximately 160 min (*i.e.*, 25 μM tetrodotoxin with 7.7 mM bupivacaine). The actual DEB-TN from this combination was 276 ± 149 min (n = 24), which was a significant increase over 15.4 mM bupivacaine ($P = 0.001$) and 50 μM tetrodotoxin ($P = 0.0007$). (Because there are six comparisons, the significant probability value is 0.0083.) Probability values for the other two points along the line were also highly significant compared with either drug alone (37.5 μM tetrodotoxin with 3.85 mM bupivacaine [DEB-TN = 317 ± 86 min, n = 16)] and 12.5 μg tetrodotoxin with 11.6 mM bupivacaine [DEB-TN = 294 ± 180 min, n = 16]).

The potentiation by tetrodotoxin of block durations from bupivacaine depended on the concentration of tetrodotoxin, with progressively increasing potentiation as the concentration of tetrodotoxin increased. The synergistic effects of the two drugs reached a plateau at about 11.6 mM bupivacaine (for constant dosing of tetrodotoxin) and 30 μM tetrodotoxin (for constant dosing of bupivacaine). The highest DEB-TN, achieved by 50 μM tetrodotoxin with 15.4 mM (0.5%) bupivacaine was 559 ± 77 min.

**Both Epinephrine and Bupivacaine.** Ten rats were injected with 30 μM tetrodotoxin with both 15.4 mM bupivacaine and 55 μM epinephrine. The resulting DEB-TN was 659 ± 83.3 min. This was not a significant improvement over the synergism between 30 μM tetrodotoxin and 15.4 mM bupivacaine (550 ± 81 min), or tetrodotoxin and 55 μM epinephrine (656 ± 123 min).

### *Effect of a Second Drug on Toxicity*

**Epinephrine.** We investigated the effects of a vasoconstrictor added to the injectate on the systemic actions and lethality of tetrodotoxin. Animals that received 50 μM tetrodotoxin (11–14 μg/kg) in 55 μM epinephrine did not appear to be in distress, none died, and the DEB-TN of the contralateral foot was dramatically reduced from 112 ± 27 min to 2.27 ± 7.5 min (table 4). The small degree of contralateral thermal latency in that group was due to the fact that 1 of 11 rats had a contralateral DEB-TN that lasted 25 min. This reduction in toxicity was documented over a range of dosages. The $LD_{50}$ of tetrodotoxin was increased from 40 nmole/kg (12.9 μg/kg) to 53.6 nmole/kg (17.3 μg/kg; 95% CI, 48.8 to 58.3 nmole/kg) by the addition of epinephrine ($P < 0.0001$).

**Bupivacaine.** The addition of bupivacaine to the injectate markedly reduced the degree of contralateral block from tetrodotoxin (table 4). This effect was seen even at low concentrations of bupivacaine. Further, there were no deaths in rats who received 50 μM tetrodotoxin with 15.4 mM bupivacaine (compared with a 20% mortality rate for 50 μM tetrodotoxin alone).

To elucidate the protective interaction between tetrodotoxin and bupivacaine, we performed a series of experiments in which tetrodotoxin was injected subcutaneously with or without bupivacaine. We did not use

**Table 4. Effect of Epinephrine and Bupivacaine on the Duration of Thermal Nociceptive Block (DEB-TN) in the Contralateral Leg**

| TTX (μM) | Combined with | DEB-TN | n | *P* Value |
|---|---|---|---|---|
| 40 | — | 45 ± 34 | 11 | |
| | Epinephrine | 0 | 10 | 0.003 |
| | Bupivacaine 15.4 mM | 6.4 ± 23 | 13 | 0.005 |
| | Bupivacaine 11.6 mM | 0 | 4 | 0.003 |
| | Bupivacaine 1.93 mM | 0 | 4 | 0.003 |
| 50 | — | 112 ± 27 | 12 | |
| | Epinephrine | 2.27 ± 7.5 | 11 | $5.6 \times 10^{-9}$ |
| | Bupivacaine 15.4 mM | 0 | 10 | $2 \times 10^{-8}$ |

Values of DEB-TN are mean ± SD. Epinephrine signifies that the solution contained 55 μM (1:100,000) epinephrine. Bupivacaine 15.4 mM = 0.5%, 11.6 mM = 0.375%, 1.93 mM = 0.0625%. *P* values (determined by Student's *t* test) compare the contralateral DEB-TN of TTX to the contralateral DEB-TN of the same dose of TTX in combination with a second drug.

the sciatic nerve as the site of injection to clarify whether impairments measured in the hindpaws were due to systemic toxicity or some region-specific effect (such as epidural spread of local anesthetics along the sciatic nerve to the epidural space). We also wanted to eliminate the sciatic nerve and its associated vasculature from the experiments so that any protective effect of bupivacaine could be ascribed to an effect on the surrounding tissue rather than an interaction with a large nerve or blood vessel. The skin at the nuchal midline was selected for this purpose because it is remote from the hindquarters and is easy to inject reproducibly in the subcutaneous plane.

Rats were injected subcutaneously with 35 nmole/kg (11.4 μg/kg) tetrodotoxin, a dose that would be expected to cause lower extremity deficits based on the results of the experiments we described (0.3 ml of 40 μM tetrodotoxin in a 350-g rat is 35 nmole/kg). The open squares in figure 7 show the time course of thermal latency in those rats (n = 6). A second group was injected with the same dose of tetrodotoxin made up in 15.4 mM bupivacaine, shown by the filled circles in figure 7 (n = 6). The peak thermal latency attained in the rats that received tetrodotoxin with bupivacaine (4.83 ± 0.97 s) was considerably less than that achieved by tetrodotoxin alone (10.8 ± 1.32 s; $P = 1.2 \times 10^{-5}$). Peak thermal latency occurred in an average of 44 ± 24 min in the group that received tetrodotoxin alone, compared with 75 ± 0 min for the group that received tetrodotoxin with bupivacaine ($P = 0.025$). These properties are similar to the effect that 55 μM epinephrine has when coinjected with tetrodotoxin (fig. 7, filled squares).

The effect of bupivacaine on the lethality of tetrodotoxin was determined by injecting rats with a range of doses (24.3 to 52.7 nmole/kg, or 8 to 17 μg/kg) of tetrodotoxin alone (n = 101) or tetrodotoxin with 15.4 mM bupivacaine (n = 68). The coinjection of bupivacaine increased the $LD_{50}$ of tetrodotoxin from 43.7 nmole/kg (14.1 μg/kg; 95% CI, 42.1 to 45.4 nmole/kg) to 47.7 nmole/kg (15.4 μg/kg); 95% CI, 45 to 50.4 nmole/kg; $P < 0.007$). In those rats that died from

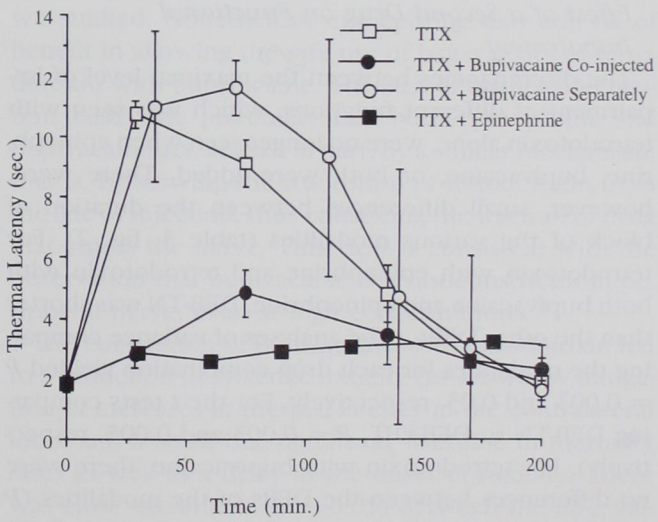


**Fig. 7. Comparison of thermal latency in the left leg resulting from subcutaneous drug injections at the nuchal midline. Thirty-five nanomoles per kilogram (11.4 μg/kg) tetrodotoxin was injected alone (*open squares*) or coinjected in 15.4 mM (0.5%) bupivacaine (*dark circles*), or with bupivacaine injected simultaneously at a separate site (*dark triangles*). Thermal latency is increased when bupivacaine is not coinjected with tetrodotoxin. Coinjection of 55 μM (1:100,000) epinephrine has a similar effect on the latency time course of tetrodotoxin (*dark squares*). Mean ± SD; n = 6 for all groups.**

tetrodotoxin toxicity, the time to death was delayed from 63.5 ± 19 min (n = 25) to 83 ± 12 min (n = 14) by the addition of bupivacaine ($P = 0.0003$). Thus the addition of bupivacaine to tetrodotoxin decreased the magnitude of the thermal latency increase from tetrodotoxin, reduced the associated mortality rate, and delayed both latency increases and death.

If this protective effect were mediated at some site remote to the injection site (for example, some unforeseen effect on the nervous system or diaphragm), we would expect the degree of toxicity to be independent of whether the two drugs are injected together. Conversely, if the protective effect were mediated locally, we would expect that it would be necessary for the two drugs to be administered at the same site. To investigate this, we injected tetrodotoxin subcutaneously in the nuchal area, and we injected an equal volume of bupivacaine simultaneously in the lower back, at a distance at least 5 c. from the site of tetrodotoxin injection. As shown by the open circles in figure 7, there was no reduction ($P = 0.22$) in the peak thermal latency (11.7 ± 0.82 s) in the lower extremities when 35 nmole/kg tetrodotoxin and 0.3 ml of 15.4 mM bupivacaine were injected at separate sites (n = 6), nor was there any delay in the time to peak thermal latency ($P = 0.87$).

### *Effect of a Second Drug on Functional Impairment*

The discrepancies between the maximal level of impairment of different functions, which was seen with tetrodotoxin alone, were no longer seen when epinephrine, bupivacaine, or both were added. There were, however, small differences between the duration of block of the various modalities (table 3, fig. 2). For tetrodotoxin with epinephrine and tetrodotoxin with both bupivacaine and epinephrine, DEB-TN was shorter than the other DEBs. (The analyses of variance comparing the modalities for each drug combination yielded $P = 0.003$ and 0.03, respectively. For the $t$ tests comparing DEB-TN to DEB-EPT, $P = 0.003$ and 0.005, respectively). For tetrodotoxin with bupivacaine, there were no differences between the DEBs of the modalities ($P = 0.63$ by analysis of variance). (Because there are three comparisons for each drug combination, the significant probability value was 0.017.)

## Discussion

We have described in detail the dose-response relation for rat sciatic nerve blockade by tetrodotoxin, separating the local from the systemic effects. We also examined the interactions of a range of doses of tetrodotoxin, bupivacaine, and epinephrine and quantified the degree and duration of blockade of various neurological functions.

Apparent nerve blockade attributed to tetrodotoxin alone is due largely to a systemic analgesic effect, as evidenced by the finding that the contralateral limb experienced an increase in thermal nocifensive and withdrawal latency almost as large as the injected leg at intermediate and high doses of tetrodotoxin. Contralateral effects probably resulted from the distribution to systemic nerves and muscles rather than from an action on the central nervous system.[6,7] The prolongation of latency could be mimicked by subcutaneous injection of tetrodotoxin in the nuchal region and ablated by coinjection of vasoconstrictors. In our experience, selective (unilateral) blockade of the injected extremity was possible, but in the absence of vasoconstrictors could only be achieved at doses at which few injections were effective. Reliable blockade with tetrodotoxin alone was achieved only at doses in which a large portion of the apparent nerve blockade appeared to result from systemic toxicity.

A comparison of relative *in vivo* and *in vitro* potencies is instructive. Tetrodotoxin inhibits most neuronal sodium current ($I_{Na}$) with a $K_I = 1-2$ nM[13,14] and reduces amplitudes of action potentials in the same nerves with a median inhibitory concentration ($IC_{50}$) of 5–6 nM.[15] For bupivacaine, the corresponding values are $K_I = 25$ μM[16] and the $IC_{50}$ is 180 μM.[17] In these isolated neuronal tissues, the equilibrium potency ratio of tetrodotoxin to bupivacaine is $1.0-2.5 \times 10^4$ ($I_{Na}$) and $3.0-3.6 \times 10^4$ (action potentials). In contrast, local nocifensive blockade *in vivo* by tetrodotoxin and bupivacaine (both in 0.3 ml with 55 μM epinephrine) has $EC_{50}$s, respectively, of 12 μM and 0.86 mM (95% CI, 0.74 to 0.98; Kohane, Berde, and Strichartz unpublished observations), resulting in a potency ratio of about 70. Thus tetrodotoxin is much less potent *in vivo* than *in vitro* and requires 2,000 times the *in vitro* $EC_{50}$ for 50% functional blockade, whereas bupivacaine requires only five times the $EC_{50}$ for the same functional effect. There are two possible sources of this discrepancy. One is that the perineural sheath is a far steeper barrier to penetration of the charged and very hydrophilic toxin molecules than to the partially neutral (pKa = 8.2) and amphipathic local anesthetic. The second is that impulses in primary afferent thermal nociceptors depend in part on tetrodotoxin-insensitive

sodium channels,[18] such that much higher local concentrations of this toxin are necessary to effect nociceptive impulse blockade. However, we found comparable $EC_{50}$ values for nociceptive and motor (EPT) blockade by tetrodotoxin, yet motor fibers are not reported to use tetrodotoxin-insensitive sodium channels. From this it appears that the pharmacokinetic argument is the major explanation for the relatively low potency of tetrodotoxin *in vivo*.

Epinephrine produced a large reduction in the $EC_{50}$ for tetrodotoxin-induced nerve blockade and a large increase in the duration of the resulting blockade. Our data further extend previous descriptions[4] of the prolongation of tetrodotoxin's effect by epinephrine by showing that extremely long duration peripheral nerve blockade (13.3 h for 50 $\mu$M tetrodotoxin in 55 $\mu$M epinephrine) can be obtained from some combinations of tetrodotoxin and vasoconstrictor. This duration is five times longer than that from 15.4 mM (0.5%) bupivacaine alone (2.7 h) and markedly exceeds the previously described duration of "about 6 h" for a combination of tetrodotoxin, lidocaine, and epinephrine.[4] Even longer blocks are possible using smaller volumes of more concentrated solutions containing an even smaller dose of tetrodotoxin: 90 $\mu$M tetrodotoxin (3 $\mu$g tetrodotoxin in 0.1 ml) in 55 $\mu$M epinephrine yielded blocks lasting 16 h (six times the duration of 15.4 mM bupivacaine alone). All these blocks were achieved with no deaths or signs of respiratory distress or contralateral limb dysfunction.

The benefits of epinephrine were present at low concentrations (as low as 1.1 $\mu$M, or 1:5,000,000). Interestingly, given that epinephrine's vasoconstrictive effects[19–21] are usually invoked to explain its amplification of the local anesthetic potency of tetrodotoxin, our data show potentiation of tetrodotoxin at concentrations of epinephrine that have been shown to be weakly vasodilatory in the rat sciatic nerve: Partridge[21] documented that 1:100,000 (55 $\mu$M) epinephrine reduced nerve blood flow by 35% and 1:200,000 (27.5 $\mu$M) epinephrine reduced it by 20%, but 1:400,000 (14 mM) epinephrine actually caused a transient 20% increase, with no subsequent reduction. This is well above the concentration at which we still observed potentiation of tetrodotoxin by epinephrine. Therefore it is possible that this effect of epinephrine is not only due to vasoconstriction.

Epinephrine prevented the contralateral impairment of modalities (a measure of systemic effect) seen with tetrodotoxin and increased the $LD_{50}$ from 40 nmole/kg (12.9 $\mu$g/kg) to 53.6 nmole/kg (17.3 $\mu$g/kg). Although a 34% increase in $LD_{50}$ may not seem enormous, it allows an important margin of safety for the more prolonged blocks. The concomitant decrease in $EC_{50}$ creates a broad range of block durations, all of which are great improvements over conventional local anesthetics. The addition of epinephrine increases the therapeutic index (the $LD_{50}$:$EC_{50}$ ratio) of tetrodotoxin from 1.06 (for tetrodotoxin alone) to 4.66, which is an increase of more than four times. Lower concentrations of tetrodotoxin also yield highly prolonged blockade, with a wider margin of safety. For example, blockade by 30 $\mu$M tetrodotoxin (3 $\mu$g) with epinephrine lasts 10.9 h, which is 4.1 times the duration of 15.4 mM bupivacaine.

The durations of blockade obtained from combinations of bupivacaine and tetrodotoxin were greatly prolonged over those attained by either drug alone. This potentiation was synergistic. Concentrations of either drug alone that had relatively little anesthetic effect (*e.g.*, 25 $\mu$M tetrodotoxin and 3.85 mM [0.125%] bupivacaine) became effective in combination. Blocks lasting 11 $\pm$ 1.28 h were obtained with 50 $\mu$M tetrodotoxin combined with 15.4 mM bupivacaine.

Epinephrine had little effect on the duration of the tetrodotoxin-bupivacaine combination, and bupivacaine had little effect on the duration of the tetrodotoxin-epinephrine combination at the concentrations we studied. Nonetheless, epinephrine may still be of benefit in allowing the safe use of larger doses of tetrodotoxin with bupivacaine. The lack of further potentiation raises the possibility that both epinephrine and bupivacaine act, at least in part, by a similar mechanism; that is, by slowing the resorption of tetrodotoxin from the site of injection, thus enhancing the fraction of drug that enters the nerve. This view is consistent with the observation that bupivacaine is a vasoconstrictor in peripheral nerve, even at high concentrations.[21]

The coinjection of bupivacaine with tetrodotoxin led to a reduction in systemic toxicity (as shown by mitigation of increases in thermal latency in the contralateral limb, and a small but significant decrease in mortality rate) as well as a delay in the onset of toxicity. There was close agreement ($P > 0.05$) between the $LD_{50}$ values for tetrodotoxin alone obtained from injection at the sciatic nerve (40 nmole/kg) and subcutaneously (43.7 nmole/kg). The amelioration of systemic toxicity by bupivacaine was surprising: It had seemed likely that the toxicities of the two drugs would be cumulative, given the similarities in their cellular actions. The protective effect was locally mediated, because it depended on tetrodotoxin and bupivacaine being coinjected, and

might be due to the vasoconstrictive effects of bupivacaine. Bupivacaine has been attributed vasoconstricting[21-24] and vasodilating[25-27] properties, or both.[28,29] Aps and Reynolds[29] reported that vasoconstriction (assessed by skin color changes) occurs when bupivacaine is injected subcutaneously at concentrations of $1.25 \times 10^{-3}$ μg/ml (which is 0.125%, or 3.85 mM) and vasodilation at $>2.5 \times 10^{-3}$ μg/ml (which is 0.25%, or 7.7 mM). Johns *et al.*[24] applied bupivacaine topically for 10 min on the rat cremaster muscle and found that vasoconstriction (assessed by television microscopy) occurred at concentrations $<10^{-3}$ μg/ml (0.1%, or 3.1 mM) and had no effect on the diameter of the microvasculature at that concentration or higher. However, it is not obvious how the local tissue concentrations achieved by a 10-min pulse of topically applied bupivacaine compare with those from percutaneous injections. Partridge[21] applied bupivacaine and other local anesthetics directly on the exposed rat sciatic nerve. In this model, which seems relevant to our study, bupivacaine caused a decrease in nerve blood flow at concentrations as high as 0.75% (23.1 mM). However, unlike lidocaine, the degree of reduction of nerve blood flow was inversely related to the concentration of bupivacaine (0.75% bupivacaine reduced blood flow by 15-20%; 0.25% bupivacaine reduced it by 35%). In our study, the protective effect of bupivacaine against tetrodotoxin toxicity occurred at concentrations of bupivacaine as high as 15.4 mM (0.5%). Although some of these investigations would suggest that this concentration is outside the vasoconstrictive range of bupivacaine, the work by Partridge[21] argues otherwise. Nonetheless, those other studies raise the possibility that some other mechanism is involved.

Accidental intravascular injection of tetrodotoxin-containing solutions would probably be associated with profound systemic toxicity.[6] However, because the mechanism of death is respiratory paralysis, animals and humans can survive large doses of tetrodotoxin if they receive ventilatory support. If inadvertent intravenous injection were to occur in the presence of a vigilant anesthesiologist, most episodes could probably be managed successfully without sequelae by proper attention to ventilation. This is in contrast to commonly used local anesthetics such as bupivacaine, for which rapid intravascular injection of the doses commonly used in humans for major plexus block or epidural block can produce seizures or cardiac arrest (or both) that are resistant to resuscitative measures.[30-33] The potential lethality of inadvertent intravascular injection of bupivacaine has been documented in adults and children[30,34] at doses that are far less than the 4-5 mg/kg range derived from animal studies (which is equivalent to 280-350 mg in a 70-kg adult).

Epinephrine and bupivacaine both altered the pattern of functional selectivity of tetrodotoxin blockade. In the case of epinephrine, this change may be due to a higher local concentration of tetrodotoxin secondary to vasoconstriction, although it is also possible that it is the result of a more specific (*e.g.*, adrenergic receptor mediated) effect on nerve. With bupivacaine, the change could similarly result from either a drug-specific interaction or a vasoconstrictive mechanism. The fact that the DEB for different modalities is altered differently by bupivacaine and epinephrine suggests that there may be more than one mechanism involved.

In summary, the combinations of tetrodotoxin with bupivacaine, epinephrine, or both described in a preliminary manner by Adams *et al.*[4] can produce an effective and apparently safe peripheral nerve blockade in the rat that far exceeds the performance of currently available local anesthetics. Sakura[8] found that tetrodotoxin infused intrathecally does not produce local neurotoxicity, whereas concentrated solutions of aminoamide local anesthetics can. Combinations of tetrodotoxin with dilute solutions of epinephrine, conventional local anesthetics, or both may have the additional advantage that the primary systemic toxic side effects of tetrodotoxin overdose, respiratory arrest and hypotension, are more easily treated by supportive care. The toxin does not cause the seizures and life-threatening dysrhythmias seen with amide and ester local anesthetics.[30-33] Drug combinations such as those described here have important clinical advantages over alternative methods. Catheter-infusion techniques involve certain risks and inconveniences, including catheter dislodgement or migration, infection, foreign body reaction to the catheter, and the requirement that the patient be tethered to an infusion pump, which limits mobility. Investigational controlled-release preparations using polymer microspheres containing bupivacaine[35] leave polymer residue, require a large-bore needle for injection, and necessitate the use of a rotary vortex mixer immediately before injection. Further, the microsphere preparations require coincorporation of a glucocorticoid to produce prolonged blockade.[36] Further preclinical testing of tetrodotoxin and related biotoxins in combination with local anesthetics, vasoconstrictors, or both appears warranted for the development of these agents for clinical use as local anesthetics.

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