

Coapplication of Lidocaine and the Permanently Charged Sodium Channel Blocker QX-314 Produces a Long-lasting Nociceptive Blockade in Rodents

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Background: **Nociceptive-selective local anesthesia is produced by entry of the permanently charged lidocaine-derivative QX-314 into nociceptors when coadministered with capsaicin, a transient receptor potential vanilloid 1 (TRPV1) channel agonist. However, the pain evoked by capsaicin before establishment of the QX-314–mediated block would limit clinical utility. Because TRPV1 channels are also activated by lidocaine, the authors tested whether lidocaine can substitute for capsaicin to introduce QX-314 into nociceptors through TRPV1 channels and produce selective analgesia.**

Methods: **Lidocaine (0.5% [17.5 mM], 1% [35 mM], and 2% [70 mM]) alone, QX-314 (0.2% [5.8 mM]) alone, and a combination of the two were injected subcutaneously and adjacent to the sciatic nerve in rats and mice. Mechanical and thermal responsiveness were measured, as was motor block.**

Results: **Coapplication of 0.2% QX-314 with lidocaine prolonged the nociceptive block relative to lidocaine alone, an effect attenuated in TRPV1 knockout mice. The 0.2% QX-314 alone had no effect when injected intraplantary or perineurally, and it produced only weak short-lasting inhibition of the cutaneous trunci muscle reflex. Perisciatic nerve injection of lidocaine with QX-314 produced a differential nociceptive block much longer than the transient motor block, lasting 2 h (for 1% lidocaine) to 9 h (2% lidocaine). Triple application of lidocaine, QX-314, and capsaicin further increased the duration of the differential block.**

Conclusions: **Coapplication of lidocaine and its quaternary derivative QX-314 produces a long-lasting, predominantly nociceptor-selective block, likely by facilitating QX-314 entry through TRPV1 channels. Delivery of QX-314 into nociceptors by using lidocaine instead of capsaicin produces sustained regional analgesia without nocifensive behavior.**

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BY blocking voltage-gated sodium channels in axons, local anesthetics disrupt action potential generation and prevent transmission of nociceptive information to the central nervous system; hence their effectiveness in producing regional anesthesia. However, local anesthetics in their uncharged hydrophobic form penetrate through the membranes of all sensory and motor axons; therefore, they also produce loss of innocuous sensation, motor paralysis, and autonomic block. Approaches that can produce selective blockade only of nociceptor (pain) fibers may have clinical utility.

We recently reported that a charged sodium-channel blocker can be targeted selectively into nociceptors through activation of transient receptor potential vanilloid 1 (TRPV1) channels, producing a nociceptive-selective local analgesia.[1] TRPV1 is a noxious thermosensitive transducer channel localized exclusively on high-threshold nociceptors. Using capsaicin, the pungent ingredient in chili peppers to activate TRPV1, we were able to selectively introduce QX-314 (*N*-ethyl-lidocaine), a permanently charged, membrane-impermeant lidocaine derivative, into nociceptors and thereby block their electrical activity. Nonnociceptive neurons that do not express TRPV1 were not blocked by the combination of QX-314 and capsaicin. *In vivo* injection of 5.8–58 mM of QX-314 and capsaicin *in vivo* together, but not the administration of each alone, abolished responses to noxious mechanical and thermal stimuli without motor or tactile deficits.

Targeting membrane-impermeant polar local anesthetics specifically into pain fibers by activation of nociceptive-specific transducer channels could be used clinically to produce a long-lasting nociceptive-selective block while preserving motor and autonomic function—a regional analgesia. However, use of capsaicin as the activator of TRPV1 channels would likely lead to an intense,

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if short-lasting, pain before the impermeant sodium channel blocker entered the nociceptors in sufficient quantities to interrupt conduction, limiting clinical application. Nonirritative activators of the TRPV1 channel are needed to adapt this drug delivery approach to clinical use. Surprisingly, lidocaine is a potential candidate because, besides its sodium channel blocking effects, it also activates TRPV1 channels with a much lower potency than capsaicin but nevertheless at clinically relevant concentrations.[2]

We therefore hypothesized that the activation of TRPV1 channels by lidocaine may be sufficient to allow selective entry of QX-314 into nociceptors and thereby produce a long-lasting nociceptive-specific regional blockade beyond the short-lived nonselective effects of lidocaine alone. To test this, we examined in rats the magnitude and duration of the effects of lidocaine alone, QX-314 alone, and the combination of lidocaine and QX-314 together on evoked nocifensive responses and motor behavior *in vivo*. We also used TRPV1 knockout mice to examine the specific contribution of TRPV1 channels in generating the nociceptive selective blockade and in mediating lidocaine-evoked calcium entry in dorsal root ganglion (DRG) neurons *in vitro*.

Materials and Methods

Animal procedures were approved by the Harvard Medical Area Standing Committee on Animals and by the Committee on Research Animal Care of the Massachusetts General Hospital, Boston, MA. Male Sprague-Dawley rats were purchased from Charles River Laboratories, Inc., Wilmington, Massachusetts. Rats were habituated to handling. At the time of injection, animals weighed approximately 250–290 g. Tests were performed with the experimenter blind to the treatment.

Capsaicin was freshly prepared with a solvent of 10% ethanol, 10% Tween 80, and 80% normal saline (pH of the final solution was 6.6). All other drug solutions were prepared freshly in 0.9% NaCl saline. The pH of tested solutions ranged from 5.0 to 6.3 and was not adjusted because it is probably buffered quickly by the pH of the tissue fluid. The osmolarities were: saline, 300 mOsm/l; 0.5% lidocaine, 335 mOsm/l; 1% lidocaine, 370 mOsm/l; 2% lidocaine, 440 mOsm/l; 0.5% lidocaine + 0.2% QX-314 (bromide salt), 349 mOsm/l; 1% lidocaine + 0.2% QX-314 (bromide salt), 382 mOsm/l; 2% lidocaine + 0.2% QX-314 (bromide salt), 452 mOsm/l.

Intraplantar Injections

Intraplantar injections of QX-314 (lidocaine *N*-ethyl bromide) (0.2%, 10 μl, 1.6 mg/kg; Sigma, St. Louis, MO) and/or lidocaine-HCl (1%, 10 μl, 8 mg/kg; Sigma) into the left hind paw (n = 6 for each group) were made; mechanical and thermal sensitivities were determined by using von Frey hairs,[3] and radiant heat of 54°C[4] focused on an 8 × 8-mm plantar skin area, respectively, at the times indicated.

Intracutaneous Injections

Rats were briefly anesthetized by inhalation of 1–2% sevoflurane (Abbott Laboratories, North Chicago, IL). Drug solutions were injected subcutaneously *via* the shaved dorsal thoracolumbar region. The injection with 0.3 ml volume resulted in a circular wheal, which was then marked with ink. Groups of eight rats were injected with each test solution: lidocaine (1%), QX-314 (0.2%), and lidocaine mixed with QX-314.

The cutaneous trunci muscle reflex (CTMR) was elicited by a noxious pinprick. A von Frey filament (26.0 g) was affixed to an 18-gauge needle and used to standardize the stimulus intensity. After observing the animal's normal reaction to six pinpricks applied on the contralateral control side, six pinpricks were then applied inside the wheal. No CTMR response after six pinpricks was defined as complete blockade (*i.e.*, 100% of maximum possible effect); three responses defined 50% maximum possible effect; and six responses after six pinpricks defined 0% maximum possible effect.

Sciatic Nerve Injections

Rats were lightly anesthetized by inhalation of sevoflurane, and the landmarks (greater trochanter and ischial tuberosity) of the left hind limb were localized. Groups of eight rats were injected with 0.2 ml of each test solution: lidocaine (0.5%, 4 mg/kg; 1%, 8 mg/kg; 2%, 16 mg/kg), QX-314 (0.2%), and lidocaine mixed with QX-314 (0.5% lidocaine + 0.2% QX-314, 1% lidocaine + 0.2% QX-314, 2% lidocaine + 0.2% QX-314). The drug was injected in immediate proximity to the sciatic nerve with a 27-gauge hypodermic needle attached to a tuberculin syringe as described,[5] and the rat was observed for the development of sciatic nerve block, indicated by complete paralysis of the hind limb. For some experiments, coinjection of lidocaine (1% and 2%) and 0.2% QX-314 was followed by injection of 0.05% of capsaicin (10 min apart). The right hind limb was used as a control. Motor function and nocifensive reactions were evaluated immediately before inhalation of sevoflurane at, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min, and then at 4, 5, 6, 9, 12, 18, and 24 h or until complete recovery.

Motor Function. Motor function was evaluated by measuring the "extensor postural thrust" of the hind limbs. The rat was held upright with the hind limb extended so that the body's weight was supported by the distal metatarsus and toes. The extensor thrust was measured as the gram force applied to a digital platform balance (Ohaus Lopro; Fisher Scientific, Florham Park, NJ), the force that resists contact of the platform by the heel. The preinjection control value was 115.8 ± 2 g (n = 24). The reduction in this force, representing

reduced extensor muscle contraction resulting from motor blockade, was calculated as a percentage of the control force. The percentage value was assigned a score: 0 = no block or baseline; 1 = minimal block, force between 100% and 50% of preinjection control value; 2 = moderate block, force between 50% of the preinjection control value and 20 g (approximately 20 g is the weight of the flaccid limb); 3 = complete block, force equal to or less than 20 g.

Nocifensive Reaction. Nocifensive reaction was evaluated by the withdrawal reflex and/or vocalization to pinch of a skinfold over the lateral metatarsus (cutaneous pain or superficial nociceptive block). This nocifensive reaction was graded in the following manner on a scale of 0–3 and based on withdrawal reflex, escape behavior, and vocalization: 0 = baseline or normal, brisk withdrawal reflex, normal escape behavior and strong vocalization; 1 = mildly impaired; 2 = moderately impaired; and 3 = totally impaired nocifensive reaction (See also Hung *et al.*[6]).

Trpv1 Knockouts

TRPV1 knockout mice on a C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME), and male wild-type (WT) C57BL/6 mice (Orient Bio Inc., Sungnam, South Korea) were housed at a temperature of 23 ± 2°C with a 12-h light-dark cycle (light on 08:00 to 20:00), and fed food and water *ad libitum*. The animals were allowed to habituate to the housing facilities for 1 week before the experiments. Lidocaine and QX-314 were purchased from Sigma-Aldrich (Milwaukee, WI).

Intraplantar Injections and Behavior Testing. Mechanical threshold was assessed by measuring foot withdrawal thresholds in response to mechanical stimuli to the right hind paw. The 50% withdrawal threshold was determined using the up-down method[7] with a set of von Frey filaments (0.02–6 g; Stoelting, Wood Dale, IL). Mice were placed in a plastic cage with a wire mesh bottom and were allowed at least 30 min for behavioral accommodation. After pretest thresholds were determined by two sets of experiments, 10 μl of 5% lidocaine, 5% lidocaine with 0.2% QX-314, 0.2% QX-314, or normal saline was injected into the plantar aspect of the right hindpaw plantar. Then, thresholds were measured at 10, 20, 30, 60, 90, 120, 180, 240, and 300 min or until complete recovery. All tests were performed blinded.

DRG Culture Preparation and Calcium Imaging Experiments

Cell Culture. Primary DRG neuron cultures were prepared from 6- to 8-week-old Sprague-Dawley male rats. DRGs were removed and placed into Dulbecco's Modified Eagle's Medium (Sigma) and 1% penicillin–streptomycin (Sigma) and then digested in 5 mg/ml collagenase, 1 mg/ml Dispase II (Roche, Indianapolis, IN), and 0.25% trypsin, followed by addition of 2.5% trypsin inhibitor. Cells were triturated in the presence of Dnase I inhibitor (50 U) and centrifuged through 15% bovine serum albumin (Sigma). The cell pellet was resuspended in 1 ml of Neurobasal (Sigma) containing B27 supplement (Invitrogen, Carlsbad, CA), penicillin and streptomycin (Sigma), 10 μM AraC, 100 ng/ml 2.5S Nerve Growth Factor (Promega, Madison, WI), and Human Glial Cell-Line Derived Neurotropic Factor (2 ng/ml, Promega). Cells were plated onto polylysine (500 μg/ml)–coated and laminin (5 mg/ml)–coated 35-mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) at 8000–9000 per well, at 37°C, 5% carbon dioxide.

Ratiometric Calcium Imaging. Cultured adult DRG neurons were loaded for 1 h with 1 μM Fura-2 acetoxymethyl ester (stock in dimethy sulfoxide in a bath solution composed of 145 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride, 10 mM Glucose, and 10 mM HEPES and then rinsed for 1 h for de-esterification of intracellular AM esters. Neurons were perfused continuously at 2 ml/min and examined with an inverted microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) equipped with Epi-Fl attachment (Nikon) and CoolSpan ES monochromator (Roper Scientific, Tucson, AZ) by using imaging processing software (Photomatrix, HDRsoft, Montpellier, France). Intracellular $[Ca^{2+}]_i$ was measured fluorometrically as absorbance ratio at 340 nm and 380 nm (ΔF340/380) (510 nm for emission). Images were taken every 8 s, monitored online, and analyzed offline using IPLab 3.7 software (Scanalytics, Rockville, MD). Lidocaine (30 mM) and capsaicin (1 μM) were briefly applied (20 s) by using a multibarrel fast drug delivery system positioned approximately 200 to 300 μm from the recording area. In all responding neurons (n = 130) ΔF after application of lidocaine and capsaicin were larger than 0.1ΔF and easily distinguishable from optic noise, which was less than 0.025ΔF.

Statistical Analysis

Data are presented in the text as mean ± SEM, and graphical presentation of the score data was also done as mean ± SEM to visually capture the time effects of the drugs on nerve blockade. However, to assess statistical significance, we used nonparametric analysis. Analysis for intraplantar and intracutaneous injections was done with either one -way analysis of variance (ANOVA) followed by Dunnett's test (compared to baseline values) or two-way ANOVA followed by Bonferroni (comparison between different treatments). To evaluate the significance of differences in the area under the curve (AUC) among all approaches for noncategorical data, ANOVA with *post hoc* Dunnett's test was performed. Independent two-tailed *t* test was also performed to compare the means of AUCs between different phenotypes (knockout and WT). For the block scores, due to the ordinal categorical nature of the data, the overall *P* value was calcu-

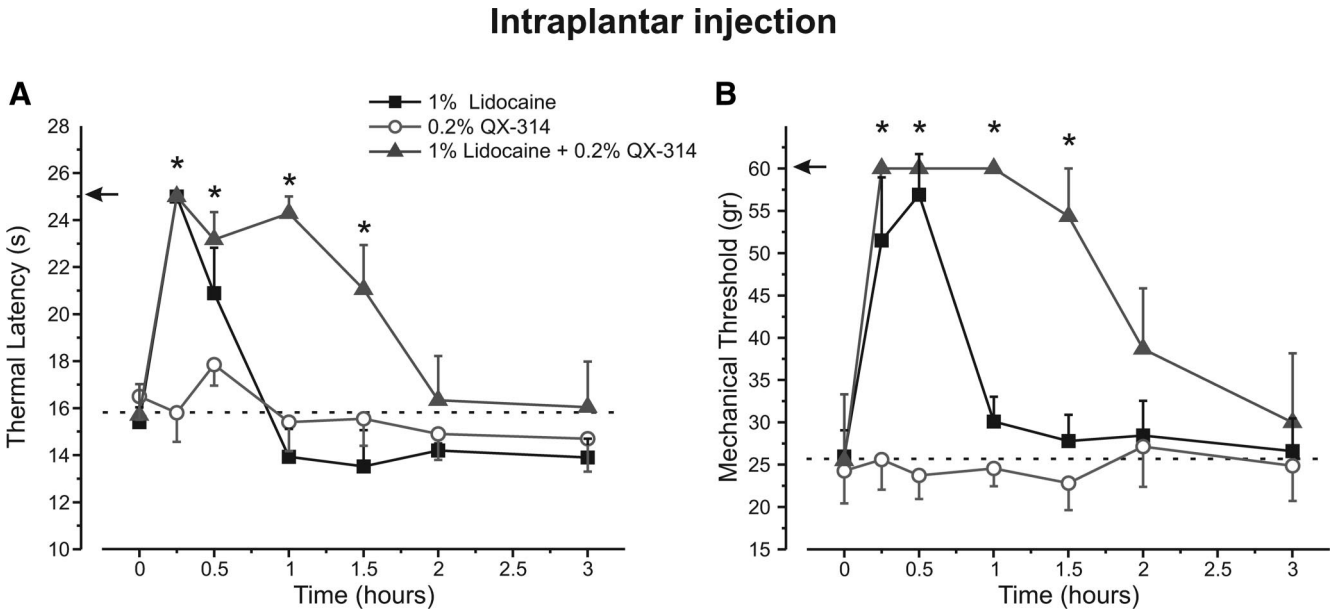

Fig. 1. The duration of the elevation in thermal (radiant heat) response latency (*A*) and mechanical threshold (von Frey) (*B*) produced by intraplantar injection of a combination of QX-314 together with lidocaine (0.2% QX-314, 1% lidocaine, 10 μl) exceeds that produced by lidocaine alone (1%, 10 μl). QX-314 alone did not alter mechanical or thermal responsiveness (0.2%, 10 μl). *Arrows* indicate the cutoffs, and the *dotted line* indicates baseline level (mean ± SEM; * $P < 0.05$, ANOVA followed by Dunnett's test; n = 6 for each group).

lated *via* generalized estimating equations using PROC GENMOD (SAS 9.1; Cary, NC).[8] A cumulative logistic ordinal model was fit with a linear and quadratic trend in time and time by group interaction. To calculate differential block, the differences between the motor and nociceptive block scores for each group were first analyzed by generalized estimating equations. Using the Mann-Whitney test, we determined the time of recovery for motor and nociceptive function by establishing the time at which the difference with baseline was no longer statistically significant ($P > 0.05$). The time from the beginning of the experiment until the last test immediately preceding recovery was defined as the effective block time. Differential block is the difference in the effective block times for motor function and nociception.

Results

In our previous investigation into nociceptive-selective blockade[1] and in further pilot experiments for this study, we found that intraplantar or perisciatic injections of up to 60 mM (approximately 2%) QX-314 alone never produced any sensory block at all. However, a study by another group has reported a slow-onset nociceptive and motor block in response to high concentrations of QX-314 alone, with a threshold of 30 mM (approximately 1%).[9] To avoid any possible independent action of QX-314, we used a low concentration of QX-314 (0.2%, approximately 6 mM), where there is agreement that it is without any blocking effect.[9] Intraplantar injection of QX-314 (0.2%) alone did not affect the latency of response to a noxious thermal stimulus, measured as time to withdrawal from a standardized radiant noxious heat stimulus applied to the plantar surface of the hind paw ($AUC_{QX\text{-}314} = 1.4 \pm 0.6$, n = 6; fig. 1A). Injection of 1% (approximately 35 mM) lidocaine into the subcutaneous tissue in the hind paw led to a substantial but brief increase in the thermal latency (fig. 1A). At 30 min postinjection, 50% of the animals were unresponsive to the radiant noxious heat stimulus applied for 25 s (cutoff) and the mean latency increased from 15.4 ± 1 to 20.8 ± 1 s ($P < 0.05$, n = 6; fig. 1A). This effect reversed completely 1 h after injection. The injection of 1% lidocaine did not produce any pain-like behavior; no flinching was observed at all during the first 5 min after injection.

A combination of 1% lidocaine and 0.2% QX-314 injected together into the plantar surface of the hind paw produced an elevation in thermal latency significantly longer than lidocaine alone ($AUC_{lidocaine + QX\text{-}314} = 16.8 \pm 3$ *vs.* $AUC_{lidocaine} = 4.7 \pm 1$, n = 6, $P < 0.001$, n = 6; fig. 1A). The increase in response latency peaked 1 h after the injection (25.1 ± 1 g *vs.* 15.7 ± 1, $P < 0.01$, n = 6). Unlike 1% lidocaine alone, the effects of which fully reversed by 60 min, the block produced by injection of 1% lidocaine and QX-314 only reversed fully at 2 h (fig. 1A). Similar relative effects were found on the mechanical threshold for eliciting a flexion reflex, measured by monofilament von Frey hairs applied to the plantar skin (fig. 1B). The effect of 1% lidocaine increased from 30 min (when given alone) to 1.5 h when combined with the 0.2% of

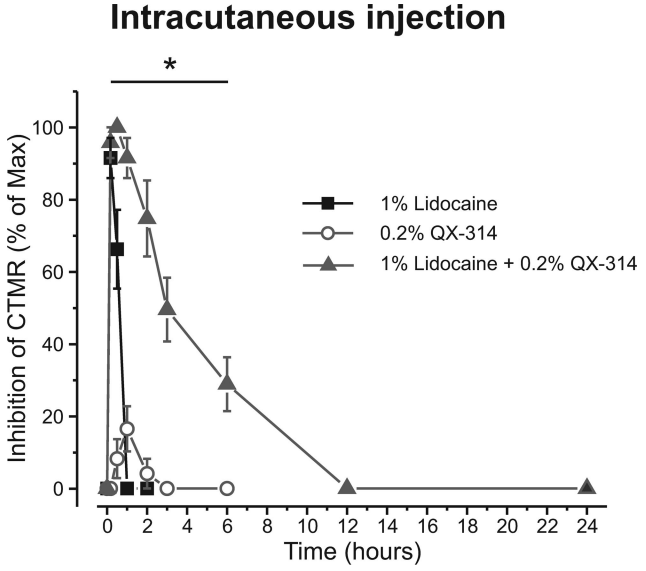

Fig. 2. Inhibition of the pinprick-evoked cutaneous trunci muscle reflex (CTMR) produced by intracutaneous injection of a combination of 1% lidocaine and 0.2% QX-314, lidocaine alone (1%), and QX-314 alone (0.2%) (100% = no response to the pinprick) expressed as percentage of maximal block (* $P < 0.05$, analysis of variance [ANOVA] followed by Dunnett's test, n = 8 for each group).

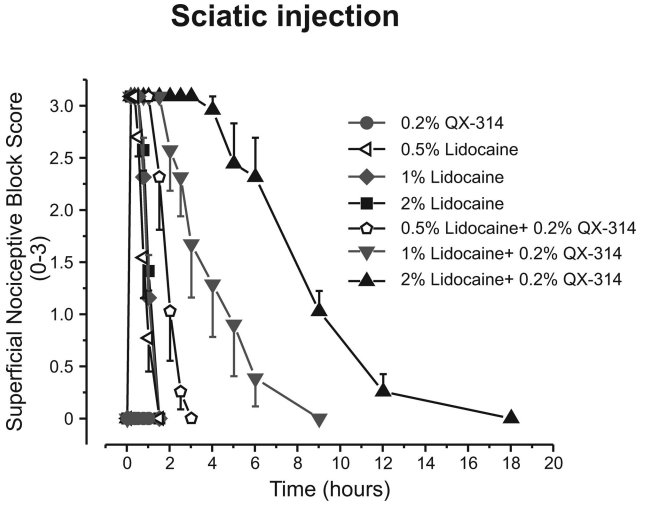

Fig. 3. Blockade of the pinch-induced withdrawal response after perisciatic nerve injection of a combination of lidocaine (0.5%, 1%, and 2%) and 0.2% QX-314; lidocaine alone (0.5%, 1%, and 2%) and QX-314 (0.2%) alone. Note that application of QX-314 alone (0.2%) did not produce detectable changes in the response to the pinch. Grading was as follows: 3 = complete block, no withdrawal, no vocalization; 2 = partial block, vocalization accompanied with slow withdrawal and flexion of the leg; 1 = minimal block, rapid flexion of the leg, or other escape response with loud vocalization; 0 = baseline. n = 8 for each group.

QX-314 ($AUC_{lidocaine + QX-314} = 64.9 \pm 19$ *vs.* $AUC_{lidocaine} = 16.3 \pm 4$, n = 6; $AUC_{QX-314} = 2.7 \pm 0.3$, $P < 0.001$, n = 6; fig. 1B).

We then examined the effect of lidocaine alone, QX-314 alone, and their combination when administered subcutaneously in another model of cutaneous pain sensitivity, the pinprick-evoked CTMR (fig. 2). QX-314 (0.3 ml of 0.2%) alone produced a small effect (less than 20% of the maximal possible block) with full recovery at 2 h. Injection of 1% lidocaine caused an almost complete blockade (approximately 95%, n = 8) of the CTMR 30 min after the injection, with full recovery at 1 h. The combination of 0.2% QX-314 and 1% lidocaine produced a long-lasting complete block with a peak at 2 h (100% inhibition, n = 8) and an effect still detectable at 6 h (fig. 2, table 1). The difference in block duration between the intraplantar and CTMR experiments likely reflects the different mechanical stimuli used: a punctate stimulus at threshold for the hind paw *versus* a suprathreshold tissue-damaging pinprick stimulus on the back. In addition, the animals were anesthetized before the intracutaneous injection but not before the intraplantar application; therefore, the difference in duration of the blockade may also be influenced by entry of QX-314 through sevoflurane-mediated activation of TRPV1 channels[10] as well as endogenous activation of TRP channels by tissue-injuring stimuli.

Table 1. Statistical Analysis of the Inhibition of the CTMR Followed Intracutaneous Injection of Lidocaine Alone, QX-314 Alone, and a Combination of Lidocaine and QX-314

		ANOVA, *post hoc*	
Drug	AUC (% × h)	1% Lidocaine	0.2% QX-314
1% lidocaine	50.4 ± 4.7		
0.2% QX-314	19.9 ± 9.1	0.825	
1% lidocaine + 0.2% QX-314	437.9 ± 62.0	<0.001*	<0.001*

P values obtained by one-way analysis of variance (ANOVA) and *post hoc* Dunnett's Test; n = 8.

* $P < 0.001$.

AUC = area under curve; CTMR = cutaneous trunci muscle reflex.

To test whether a lidocaine/QX-314 combination can induce a long-lasting nociceptor-specific block when applied close to a major nerve, we tested the response to pinch of the skin on the hind paw at the lateral metatarsus using standard forceps after injection of the combined drugs adjacent to the sciatic nerve. Motor function was measured as the plantar thrust force exerted by the hind limb when placed on a digital balance (see Materials and Methods). Perisciatic injection of 0.2 ml of 0.2% QX-314 alone did not produce any detectable motor or sensory deficit in all examined animals (fig. 3; tables 2 and 3, n = 8). A complete but short-lived (30 min) nociceptive and motor block was elicited by 0.2 ml of 1% lidocaine (figs. 3 and 4, tables 3 and 4, n = 8). The combination of both drugs resulted in a complete nociceptive block for approximately 3 h, with recovery at approximately 6 h (fig. 3). The duration of the nociceptive blockade was dependent on the concentration of lidocaine used, and it varied between 1.5 h when 0.5% was used to approximately 10 h when 2% of lidocaine was used (fig. 3 and 4A). Application of lidocaine alone (0.5–2%) produced no differential block because the

Table 2 Stastical Analysis, Using Generalized Estimating Equations (GEE) of Nociceptive Block Followed by Sciatic Injections of Lidocaine Alone, QX-314 Alone, and the Combination of Lidocaine and QX-314

	Lidocaine (dose)				Lidocaine (dose) + 0.2% QX-314		
	(0.5%)	(1%)	(2%)	0.2% QX-314	(0.5%)	(1%)	(2%)
0.5% Lidocaine		0.3192	0.2806	<0.001*	<0.001*		
1% Lidocaine	0.3192		0.649	<0.001*		<0.001*	
2% Lidocaine	0.2806	0.649		<0.0001			<0.001*
0.2% QX-314	<0.001*	<0.001*	<0.0001		<0.001*	<0.0001	<0.0001
0.5% Lidocaine + 0.2% QX-314	<0.001*			<0.001*		0.020‡	
1% Lidocaine + 0.2% QX-314		<0.001*		<0.0001	0.020‡		0.003‡
2% Lidocaine + 0.2% QX-314			<0.001*	<0.0001	<0.001*	0.003‡	<0.001*

P values obtained by generalized estimating equations (GEE), n = 8.

* $P < 0.001$; † $P < 0.01$; ‡ $P < 0.05$

duration of motor block was similar to the sensory block (approximately 30 min with full recovery at 1 h) (fig. 4B, table 3, n = 8).

When 1% of lidocaine was used together with 0.2% QX-314, a complete motor block lasted for 30 min, with full recovery at 2 h, which was significantly shorter than the nociceptive block (fig. 4A, table 3), providing approximately 2.5 h of nociceptive-specific block that persisted after recovery from the nonselective motor and sensory block (fig. 4C). Application of 2% of lidocaine together with 0.2% of QX-314 prolonged the differential block to approximately 9 h (fig. 4, A and C), whereas reduction of lidocaine to 0.5% resulted in a loss of the differential block (fig. 4, A and C).

Since lidocaine potentiates the capsaicin-mediated activation of TRPV1 channels,[2] we examined whether a triple combination of lidocaine, capsaicin, and QX-314 would further prolong the dual capsaicin-QX-314 or of lidocaine-QX-314–mediated block and whether this triple application would prevent the initial irritant effects of capsaicin. We injected lidocaine and QX-314 first, followed 10 min later by capsaicin, according to the same protocol we used for QX-314 and capsaicin.[1] Complete nociceptive blockade after the triple injection (1% lidocaine, 0.2% of QX-314, and 0.05% capsaicin) lasted 5 h, fully recovered only after 12 h, and was significantly longer that that produced by either a capsaicin 0.1%/QX-314 0.2% combination[1] or lidocaine 1%/QX-314 0.2% (table 4, $P < 0.01$, n = 8; fig. 5A). The motor block produced by the triple application was similar to that produced by lidocaine/QX-314 (table 4) and lasted for 2 h. The differential block (sensory beyond motor) achieved by triple application of 1% lidocaine, 0.2% of QX-314, and 0.05% capsaicin lasted 8 h (fig. 5A); an increase in lidocaine to 2% significantly further prolonged the nociceptive selective blockade, with a complete block of 10 h and recovery only at 48 h (fig. 5B), and the differential block lasted about 16 h (fig. 5, C and D) with the motor block no different in duration from triple injection with 1% lidocaine (table 4).

To assess the relative irritancy of the compounds alone and in combination, we measured the number of flinches 5 min after intraplantar injection of 10 μl of 0.05% capsaicin alone, 0.05% capsaicin + 0.2% QX-314, 1% lidocaine + 0.2% QX-314, and 0.05% capsaicin + 1% lidocaine + 0.2% QX-314. Application of QX-314 together with capsaicin failed to prevent the initial irritative response produced by capsaicin alone. Application of lidocaine together with capsaicin and QX-314 totally abolished the irritant properties of capsaicin (fig. 6).

To examine the specific contribution of TRPV1 channels to the prolongation of the sensory-blocking effects of lidocaine when used in combination with QX-314, we exploited TRPV1 knockout mice. Application of 1% or 2% lidocaine alone (10 μM) into hind paw of either WT or TRPV1 knockout mice failed to produce any change in response parameters to noxious mechanical stimuli. Injection of 5% of lidocaine produced a short-lasting complete block for 10 min with full recovery after 20 min (fig. 7A). Coinjection of 5% lidocaine and 0.2% QX-314 induced an increase in mechanical threshold that was greater than lidocaine alone, and this was significantly shorter in TRPV1 knockout mice than in WT mice ($AUC_{TRPV1\ knockout\ (lidocaine\ +\ QX\text{-}314)} = 3.5 \pm 1$ *vs.* $AUC_{WT\ (lidocaine\ +\ QX\text{-}314)} = 8.5 \pm 1$, $P < 0.05$, n = 4) with recovery after 1 h *versus* 3 h in WT animals (fig. 7A). However, the effect of the combination of lidocaine and QX-314 on mechanical threshold in the TRPV1-null mice was still significantly longer than the effect of lidocaine alone ($AUC_{TRPV1\ knockout\ (lidocaine)} = 1.6 \pm 0.2$, $P < 0.05$; fig. 7A). This suggests that lidocaine activates some additional channel beyond TRPV1 that enables delivery of QX-314 into nociceptor fibers. To examine this possibility *in vitro*, we assayed changes in intracellular calcium concentrations evoked by lidocaine in cultured adult rat dorsal root ganglion neurons. Application of lidocaine (30 mM) produced an increase in intracellular calcium concentration in TRPV1-expressing (capsaicin-sensitive) neurons (141 of 200 cells), as previously reported.[2] We also found that lidocaine evoked an increase in intracellular calcium in a subpopulation (66 out of 207 cells) of capsaicin-insensitive small (less than 25 μm) DRG neurons (fig. 7B). In the majority (approximately 60%) of the capsaicin-insensitive neurons, removal of extracellular

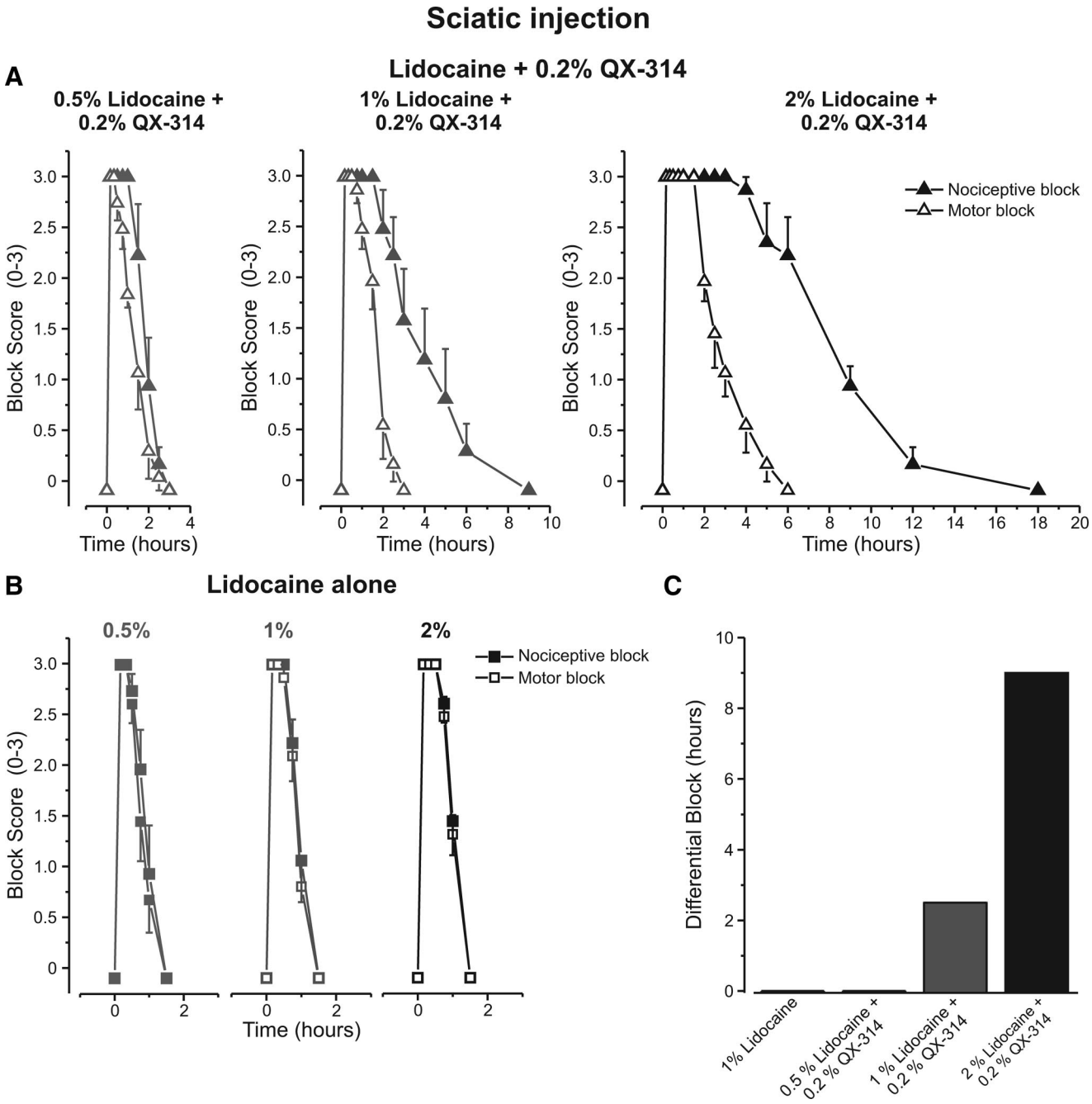

Fig. 4. Injection of lidocaine and QX-314 close to the sciatic nerve produces a short complete sensory and motor block followed by a prolonged nociceptive-selective block. (*A*) Coinjection of lidocaine (0.5%, 1%, and 2%) and QX-314 (0.2%) produced a differential block since the block to a noxious pinch exceeds the short-duration motor deficit. (*B*) Lidocaine (0.5%, 1%, and 2%) injected alone produces a short-duration nonselective local anesthesia where nociceptive (*closed squares*) and motor (*open squares*) block have exactly the same duration. Grading for the nociceptive block is as in Fig. 3. Grading for motor block: 0 = baseline (115.8 g ± 2, n = 24), 1 = minimal block (50–100% of baseline); 2 = moderate block (less than 50% of baseline to 20 g); 3 = complete block, force less than 20 g (see Materials and Methods). (*C*) Summary of differential block produced after sciatic injection of 1% lidocaine alone and lidocaine coinjected with 0.2% QX-314.

calcium totally abolished lidocaine-mediated changes in Fura-2 fluorescence, suggesting that lidocaine produced a calcium influx in these neurons, but not through TRPV1. In the remaining approximately 40% of the capsaicin-insensitive cells, although the response to lidocaine decreased by 75 ± 5%, it was not abolished by absence of extracellular calcium, implying some lidocaine-mediated release of calcium from internal stores, as previously demonstrated.[11]

Discussion

We find that a combination of the commonly used local anesthetic, lidocaine, and the membrane-imperme-

Table 3. Comparison of Motor and Nociceptive Block after Perisciatic Injection of Different Concentrations of Lidocaine Alone, 0.2% QX-314 Alone, and a Combination of Lidocaine/QX-314

Lidocaine Alone	0.5% Lidocaine	1% Lidocaine	2% Lidocaine	
P value	0.72	0.725	0.878	
0.2% QX-314	0.2% QX-314 alone	0.5% lidocaine + QX-314	1% lidocaine + QX-314	2% lidocaine + QX-314
P value	—§	0.005†	0.023*	< 0.001‡

P-value obtained by generalized estimating equations.

n = 8.

* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$; § the algorithm failed to converge due to the single level of measured values (all 0).

able sodium channel blocker, QX-314, a simple permanently charged quaternary derivative of lidocaine, produces prolonged local analgesia in contrast to the short-lived, nonselective effect of lidocaine when administered alone. The effects of the lidocaine/QX-314 combination are greatly reduced in TRPV1-null mice; therefore, it is likely mediated in part by the activation by lidocaine of TRPV1. *In vitro*, extracellularly applied QX-314 at 1–5 mM has little or no effect on sodium currents in DRG cell bodies[1,12]; however, when introduced into the cytoplasm, it completely blocks sodium channels with an IC_{50}less than 100 μM (unpublished data, June 2008, Alexander Binshtok, Ph.D., and Michelino Puopolo, Ph.D., Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts; original traces of sodium current recordings with analysis), suggesting that intracellular QX-314 blocks the channels as effectively as lidocaine in DRG neurons, consistent with results from other neuronal cells.[13–17] A slow onset and prolonged blockade of the CTMR was reported by other investigators in response to 70 mM and higher concentration injections of QX-314 alone, with lower concentrations producing less consistent and smaller effects.[9] The mechanism of this is unclear. The authors suggested that QX-314 at very high concentrations may penetrate slowly through the lipid membrane.[9] It is also possible that very high concentrations of QX-314 might block sodium channels from the outside, as it does with the cardiac sodium channel Nav1.5.[18,19] We find that 0.2% (5.8 mM) QX-314 alone did not produce any detectable block when given by the intraplantar or perisciatic route, but we did find a small (less than 20%) reduction in the pin prick–evoked CTMR after subcutaneous injection. One possible explanation for the variable blocking effects of QX-314 applied alone is that certain routes of administration and tests may result in the release of endogenous ligands for TRP channels such as endocannabinoids or protons,[20–23] which could contribute to QX-314 entry into axons *via* the channels. Furthermore, inhalational anesthetics act as agonists for TRPV1 and transient receptor potential A1 channels (TRPA1)[10,24]; in some experimental settings, this may be sufficient to contribute to a transfer of QX-314 into nociceptors. It is notable that the only model in which we saw some effect of QX-314 alone included general anesthesia, intracutaneous injection, and repeated intense (pin prick) stimuli, all of which could potentially activate TRP channels. It is also conceivable that QX-314 could enter axons to a limited extent through channels other than TRPs that have some endogenous opening activity, or by endocytosis. However, our data show that such entry is minimal compared to that mediated through TRPs activated by application of capsaicin or lidocaine.

Table 4. Comparison of Motor and Nociceptive Blockade after Application of Lidocaine/QX-314 and Lidocaine/Capsaicin/QX-314

	1% Lidocaine + 0.2% QX-314 + 0.05% Capsaicin	2% Lidocaine + 0.2% QX-314 + 0.05% Capsaicin
Nociceptive block		
1% lidocaine + 0.2% QX-314	0.007†	
2% lidocaine + 0.2% QX-314		0.004†
2% lidocaine + 0.2% QX-314 + 0.05% capsaicin	0.05*	
Motor block		
1% lidocaine + 0.2% QX-314	0.071	
2% lidocaine + 0.2% QX-314		0.071
2% lidocaine + 0.2% QX-314 + 0.05% capsaicin	0.08	

P value obtained by generalized estimating equations (GEE), n = 8.

* $P < 0.05$; † $P < 0.01$.

Lidocaine reduces both tetrodotoxin-sensitive (IC_{50} of approximately 50 μM) and tetrodotoxin-resistant (IC_{50} of approximately 200 μM) sodium currents[25] in a use-dependent way by binding to the channel pore on its internal or cytoplasmic face.[26,27] When injected in therapeutic concentrations (approximately 35–70 mM) into the hind paw or adjacent to the sciatic nerve, lidocaine produces a short-lasting (approximately 30 min) nonselective sensory and motor block in keeping with its nonselective sodium channel blocker action. However, lidocaine also acts as an agonist for the TRPV1 receptor.[2] Application of 30 mM (approximately 1%) lidocaine to dorsal root ganglion neurons produces a calcium influx similar to that evoked by 1 μM of capsaicin, an effect that was reported to be found only in capsaicin-sensitive DRG neurons and absent in $TRPV1^{-/-}$ mice.[2] We find, however, that lidocaine produces a calcium influx in some capsaicin-insensitive DRG neurons as well, indicating action also on some non-TRPV1 channel(s). Application of lidocaine evokes inward currents in capsaicin-

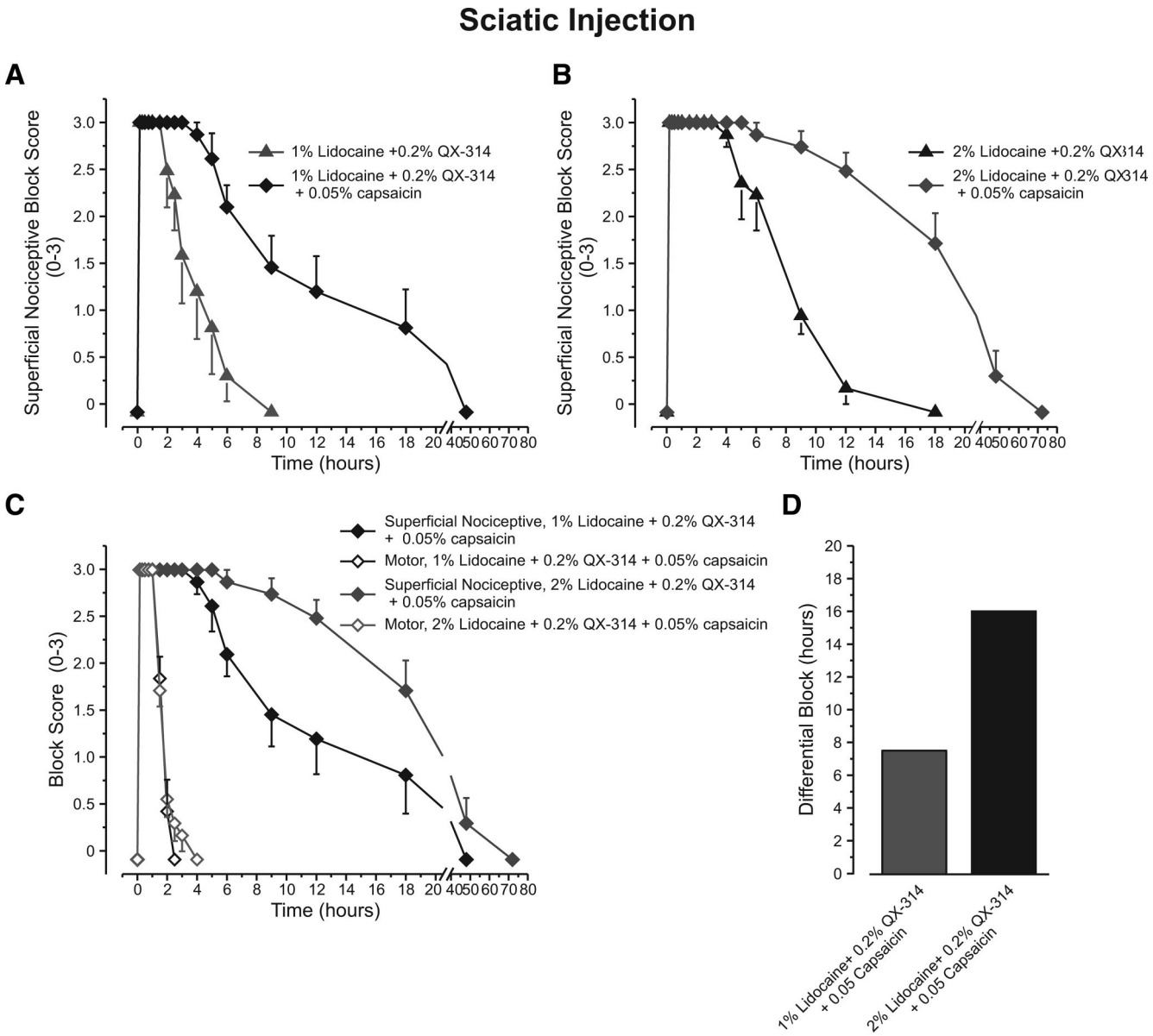

Fig. 5. Application of capsaicin (0.05%) after injection of either (*A*) 1% or (*B*) 2% of lidocaine + QX-314 (0.2%) significantly increased the nociceptive blockade produced by lidocaine coinjected only with QX-314 (0.2%). (*C*) Comparison of the duration of nociceptive (*closed diamonds*) and motor (*open diamonds*) blockade produced by triple application of 1% lidocaine/capsaicin/QX-314 (*black diamonds*) or 2% lidocaine/capsaicin/QX-314 (*gray diamonds*). Grading for the nociceptive block is as in Fig. 3. Grading for the motor block is as in Fig. 4. (*D*) Differential block produced after sciatic nerve injection of lidocaine, QX-314, and capsaicin.

sensitive DRG neurons that become smaller at a lower pH, implying that it is the uncharged, membrane-permeable form of lidocaine that activates TRPV1 channels; consistent with this, QX-314 does not activate TRPV1 channels.[2] The lidocaine-induced inward current is abolished by capsazepine, a competitive TRPV1 antagonist, suggesting that lidocaine activates TRPV1 channels at the putative capsaicin binding site on the inner face of TRPV1.[2]

Recent studies have shown that QX-314 can carry current through TRPV1 channels when it is the primary cation present,[28] offering direct support for the hypothesis that QX-314 enters nociceptors by permeating through TRPV1 channels when these are activated.[1] The EC_{50} of lidocaine's activation of TRPV1 channels is substantially higher than the IC_{50} for its blockade of sodium channels,[2,25,29] and the sodium channel blockade will prevent any sensory consequences of coincident TRPV1 activation; therefore, lidocaine is much less likely to be irritating and evoke pain than capsaicin. Indeed, lidocaine produces a transient (seconds) stinging sensation[30–32] that is much shorter and less intense than that produced by capsaicin (tens of minutes).[33,34]

We find that substitution of capsaicin by clinically-used concentrations of lidocaine (35–70 mM, 1–2%) did not produce the pain-like response found with capsaicin, but it was still able to produce a period of effective, long-

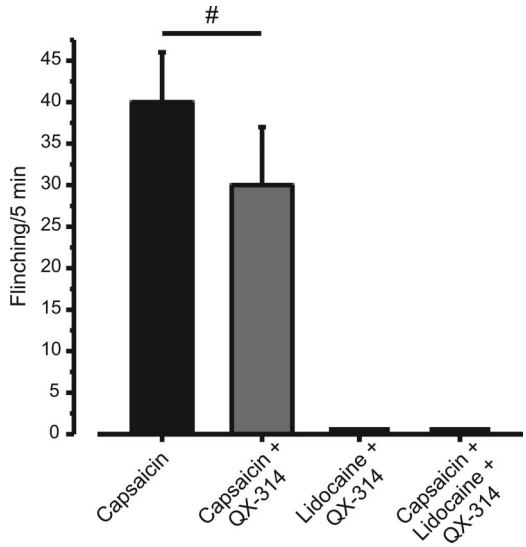

Fig. 6. Number of flinches during the first 5 min after intraplantar application of capsaicin alone (0.05%), capsaicin (0.05%) + QX-314 (0.2%), lidocaine (1%) + QX-314 (0.2%), and capsaicin (0.05%) + lidocaine (1%) + QX-314 (0.2%). # $P > 0.05$ analysis of variance (ANOVA), n = 6.

lasting, nociceptive-specific nerve block by allowing entry of QX-314 into nociceptors. Nevertheless, unlike the capsaicin–QX-314 combination, the lidocaine–QX-314 combination began with a short phase of nonselective block of the same duration as lidocaine alone, consistent with the nonselective sodium channel blocking action of lidocaine.

Moreover, we found that application of lidocaine together with capsaicin and QX-314 not only prevented the initial capsaicin-mediated irritation found with QX-314 and capsaicin coadministration, but it also significantly prolonged the nociceptive-selective blockade beyond that with either QX-314/capsaicin or QX314/lidocaine. Synergistic activation of TRPV1 channels by capsaicin and lidocaine[2] with increased entry of QX-314 may underlie this effect.

The effects of the lidocaine QX-314 combination on nociceptive-specific blockade were only partially attenuated in TRPV1 knockout mice, suggesting that lidocaine activation of TRPV1 channels is not an exclusive mechanism for lidocaine-mediated QX-314 entry into nociceptors. A lidocaine-mediated activation of transient receptor potential A1 channels[2] or other large-pore cation nonselective channels might also contribute to facilitate the action of QX-314. In mice, however, TRPV1 and transient receptor potential A1 channels are usually coexpressed in a subset of TRPV1 neurons.[35,36] Because we find that lidocaine induces an increase in intracellular calcium concentration in capsaicin-insensitive, and therefore possibly transient, receptor potential A1 channel-negative neurons, we conclude that lidocaine activates some additional calcium permeable channel(s). Further work will be required to identify these channels.

In conclusion, we show that application of lidocaine paired with QX-314 produces in rodents a long-lasting

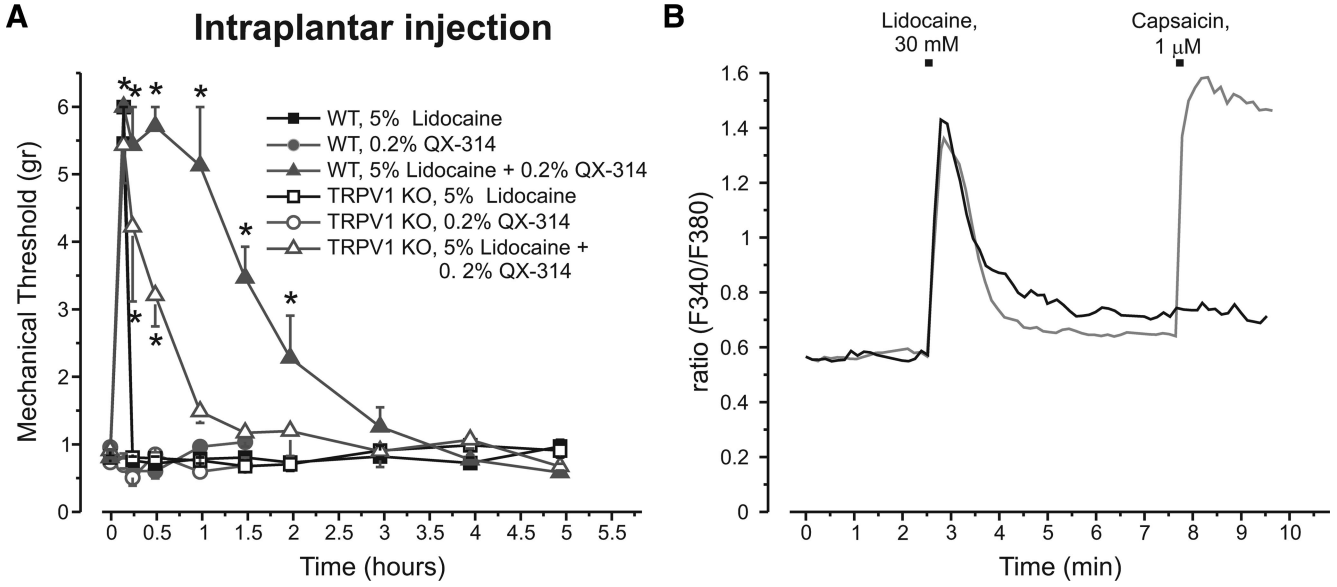

Fig. 7. The prolonged analgesic effect of the lidocaine–QX-314 combination depends largely, but not exclusively, on activation of transient receptor potential vanilloid1 (TRPV1) channels. (*A*) In TRPV1 knockout (KO) mice, the duration of the elevation of mechanical threshold assessed using von Frey hairs after combined injection of lidocaine and QX-314 (0.2% QX-314, 5% lidocaine, 10 μl, n = 6) is significantly shorter than in wild type (WT) animals ($P < 0.0001$, analysis of variance [ANOVA], n = 4), but it exceeds that produced by lidocaine alone (5%, 10 μl; $P < 0.05$, ANOVA, n = 4). Lidocaine alone (5%, 10 μl) produced similar effects in TRPV1 KO and WT animals ($P = 0.4$, n = 4, two-way ANOVA). Injection of QX-314 alone (0.2%, 10 μl) did not change mechanical threshold both in TRPV1 KO and WT mice (n = 4). * $P < 0.05$. (*B*) Representative traces from two cells (*black* and *gray*) recorded simultaneously during consecutive application of lidocaine (20 s, 30 mM) and capsaicin (20 s, 1 μM). Lidocaine induces an increase in intracellular calcium concentration (increase in fluorescent ratio [ΔF340/380]) through both TRPV1-dependent (capsaicin-sensitive) and TRPV1-independent mechanisms.

regional analgesia that is even longer-lasting when QX-314 is tripled with lidocaine and capsaicin. Although the short-lasting, nonselective effects of lidocaine itself make such an approach less nociceptive-selective than when using capsaicin as the TRPV1 agonist, it is likely clinically preferable because it avoids the transient initial but intense painful responses mediated by activation of TRPV1 channels, yet it still produces a regional analgesia that long outlasts the block in motor function. Furthermore, a lidocaine and QX-314 combination that induces a relatively short motor block followed by a much longer-lasting regional analgesia might be ideal clinically, producing initial immobilization of a surgical area, followed by sustained analgesia after motor function recovers.

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