Nociceptor-selective Peripheral Nerve Block Induces Delayed Mechanical Hypersensitivity and Neurotoxicity in Rats

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ABSTRACT

Background: Long-lasting, sensory-specific peripheral nerve blockade would advance perioperative analgesia. Perineural injection of a combination of transient receptor potential vanilloid 1 channel agonists and lidocaine or its hydrophilic derivative, QX-314, produces prolonged sensory or nociceptor-selective nerve block in rodents. In this study, the authors tested the efficacy of these combinations in peripheral nerve block after incisional surgery in rats.

Methods: The authors administered perisciatic lidocaine (2%), QX-314 (0.2%) followed by dilute capsaicin (0.05%, 10 min later), or vehicle in rats and the duration of motor and sensory block to thermal and mechanical stimuli assessed in normal animals and those after incisional surgery to the hind paw. Other animals receiving these injections were evaluated 7 weeks later by behavior and histology for potential neurotoxicity.

Results: Perineural injection of the combination not only attenuated mechanical hypersensitivity for 72 h after incision but also resulted in delayed onset mechanical hypersensitivity several weeks later, accompanied by degeneration of central terminals of isolectin B4 (nonpeptidergic) and calcitonin gene–related peptide–containing (peptidergic) afferents in the ipsilateral spinal cord. Dorsal root ganglia ipsilateral to injection of the combination showed increased expression of activating transcription factor-3 and satellite cell activation.

Conclusions: Combined administration of local anesthetics with the transient receptor potential vanilloid 1 agonist capsaicin induced a near complete blockade of incision-induced hypersensitivity for several days. However, the same combination induced delayed mechanical hypersensitivity and neurotoxicity in naïve rats. Combination of these drugs in these concentrations is likely to result in neurotoxicity, and the safety of other concentrations warrants further study. (ANESTHESIOLOGY 2014; 120:976-86)
hydrophilic lidocaine derivative QX-314 (N-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide), which gains intracellular entry, and hence produces neural blockade, to a very limited extent itself, followed 10 min later by the agonist capsaicin to open the TRPV1 channels on nociceptors. This procedure in mice results in increased thermal and mechanical withdrawal thresholds lasting several hours with minimal effects on motor responses. Interestingly, lidocaine itself at clinically used concentrations acts as a TRPV1 antagonist as well as a transient receptor potential ankyrin 1 (TRPA1) channel agonist, and coadministration of lidocaine with QX-314 produces a similar selective block of long duration. Binshtok et al. showed that the triple application of 2% lidocaine, 0.2% QX-314, and 0.05% capsaicin in rats resulted in a maximal sensory block for 16 h. These approaches provide a potential useful strategy to provide long-term postoperative pain relief as well as a tool for investigating the contribution of specific subsets of sensory neurons to the transduction of multimodal stimuli and spinal cord plasticity.

The goal of the current study was to test the hypothesis that TRPV1-containing nociceptive afferents contribute to the mechanical hypersensitivity that develops after plantar incision in the rat. We observed that perisciatic administration of 2% lidocaine, 0.2% QX-314, and 0.05% capsaicin prevented the development of mechanical hypersensitivity for several days after incision, but unexpectedly, also resulted in delayed mechanical hypersensitivity weeks later, suggestive of neurotoxicity. Because the potential for neurotoxicity has not been thoroughly examined using these combinations, we then examined long-term effects of combining local anesthetics and large pore ion channel activators on thermal and mechanical withdrawal thresholds in naïve rats and on immunohistology of the dorsal root ganglia (DRG) and spinal cord.

Materials and Methods

Animal Procedures

A total of 56 male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN), weighing 250 to 300 g, were used for experiments. All studies conformed to the Wake Forest University Guidelines on the ethical use of animals, and studies were performed under Animal Care and Use Committee (Winston-Salem, NC) approval. Animals were housed under a 12-h light–dark cycle, with food and water ad libitum.

Drugs

Capsaicin (0.05% or 0.5 mg/ml) was prepared fresh in a solvent of 10% ethanol, 10% Tween-80, and 80% sterile saline. Lidocaine HCl injection (1 to 2% or 35 to 70 mM, 20 mg/ml; Hospira, Inc., Lake Forest, IL) and QX-314 (lidocaine N-ethyl bromide, 0.2% or 5.8 mM; Sigma, St. Louis, MO) was diluted or dissolved in sterile saline solution.

Perisciatic Injection of Local Anesthetic Combinations

Rats were lightly anesthetized by inhalation of 1 to 2% isoflurane. The local anesthetic drug solutions were injected into the sciatic notch of the left hindlimb between the greater trochanter and the ischial tuberosity with 27-guage needle connected to a tuberculin syringe. When the needle encountered the body of the ischium, it was withdrawn slightly and the drug solution injected over several seconds. Groups of rats were injected with 0.2 ml of drug solutions including coinjected lidocaine (2 or 1%) + QX-314 (0.2%) in saline followed by injection of 0.05% capsaicin (10 min apart). The final volume of ethanol and Tween-80 injected per rats was 20 µl each.

Plantar Incision Model

Plantar incision was performed as previously described. In brief, animals were anesthetized with inhalational isoflurane (2%) in oxygen, and the plantar aspect of the left hind paw was prepared in a sterile manner with a 10% povidone-iodine solution. A midline incision (1 cm) was made using a No. 11 blade on the left hind paw starting 0.5 cm from the heel. The plantaris muscle was elevated and incised longitudinally. The wound was closed with two 5.0-nylon mattress sutures. For sham procedures, animals were anesthetized with inhalational isoflurane (2%) in oxygen, and the plantar aspects of the left hind paw were prepared in a sterile manner with 10% povidone-iodine solution.

Behavioral Assessment of Nerve Block

Motor Function. Motor function was assessed by measuring the extensor postural thrust of the hindlimbs similar to previous methods. Rats are held upright with the hindlimb extended with the body weight supported by the distal metatarsus and toes. The extensor postural thrust is the gram force applied to a digital platform balance that resists contact of the platform by the heel of the paw. Preincision control values are 269.9 ± 10 g (n = 6). For each time point, extensor postural thrust was assessed three times and reported as an average of the three assessments. Results were reported as mean ± SD.

Nocifensive Reaction Mechanical and Thermal Stimuli

Nocifensive reactions to mechanical stimuli were assessed by the presence of vocalization and/or withdrawal reflex to pinch of a skin fold over the lateral metatarsus. This reaction was scored based on the degree of withdrawal reflex, escape behavior, and vocalization on a scale of 0 to 3 with 0 = baseline or normal rapid withdrawal reflex, escape behavior, and strong vocalization; 1 = mildly impaired; 2 = moderately impaired; and 3 = totally impaired reaction. For each time point, the nocifensive reaction was assessed three times and reported as an average of the three assessments. Withdrawal latency to radiant heat was also determined as previously described. Thermal testing was performed after the rat had been placed in a clear plastic box on a glass surface maintained at 30°C. A calibrated radiant heat source was
focused on the hind paw, and the latency to withdrawal was recorded, using a 30-s maximum exposure to avoid tissue injury. Withdrawal latency was measured two times in the ipsilateral and contralateral foot in the middle of the footpad. These two observations were averaged for each animal. Thermal withdrawal latencies and nociceptive responses were determined several time points after perisciatric nerve injection (0, 0.5, 1, 2, 4, 6, 12, and 24 h after drug administration). Withdrawal latency to thermal stimulation is reported as mean ± SD.

Assessment of Mechanical Hypersensitivity
Paw withdrawal thresholds to mechanical stimuli were determined using application of von Frey filaments as previously described. In brief, rats were placed in individual clear acrylic chambers with a plastic mesh floor and allowed to acclimate to the test apparatus at least 30 min before testing. Filaments were applied to the bending point for 6 s, and a brisk paw withdrawal was considered a positive response. Withdrawal threshold was determined using an up–down statistical method. Individuals conducting behavioral assays were blinded to the treatment.

Tissue Preparation for Immunohistochemistry
Rats collected for assessment of long-term pathological effects of nerve block or vehicle injections were anesthetized with sodium pentobarbital (intraperitoneal injection; 100 mg/kg), the thorax was opened, and 0.1 M phosphate-buffered saline (pH 7.4) followed by fixative (4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4) was perfused through the left ventricle with a peristaltic pump (20 ml/min). The spinal cord and L4-6 DRG were removed immersed in fixative for 12 h at 4°C followed by immersion in 30% sucrose at 4°C for cryoprotection until ready to be sectioned. Spinal cord cross-sections (40 μm) were cut on a cryostat, and every fourth section was processed free floating and incubated over night at 4°C with primary antibody. Sections were washed thoroughly in phosphate-buffered saline, mounted on plus-sheets, air-dried, dehydrated in ethanol, cleared in xylene, and cover slipped with DPX mounting media (Sigma-Aldrich, St. Louis, MO) at room temperature.

Image Analysis and Quantification
Tissue sections were examined with fluorescent microscopy, and images of ipsilateral and contralateral L4-5 dorsal spinal cord were captured with a charged coupled device digital camera attached to the microscope using a 10X objective at a resolution of 1,600 × 1,200 pixels. For semi-quantitative analysis of immunofluorescence levels, a square with a fixed area (250 × 250 μm²) covering the region of laminae I-II was positioned in the lateral and middle one third of the mediolateral extent of the spinal cord dorsal horn. The number of pixels occupied by immunoreactive cells within a defined threshold was measured using image analysis software (Image J; NIH Image, National Institutes of Health, Bethesda, MD). Immunofluorescent measurements were obtained from a minimum of five spinal cord sections/rat and averaged. The percentage of sensory neurons in the DRG that expressed ATF3 and TRPV1 were quantified as previously described.

Statistical Analysis
We used generalized estimating equations to test hypotheses regarding the effect of local anesthetic combinations on longitudinal behavioral endpoints (response scores to mechanical pinch, motor extensor postural thrust values, thermal withdrawal latencies, and mechanical withdrawal threshold) after nerve block on naïve and incised rats. Group/condition was specified as a between-subjects effect with time as a repeated measures effect. The outcome variables for thermal latencies and mechanical withdrawal thresholds were modeled using a normal distribution with identity link when the data were parametric. For the long-term study assessing mechanical withdrawal thresholds in normal rats administered nociceptor-selective block, the data were modeled using a normal distribution with log link due to its skewed nature. Because of the nonparametric form of the response score to mechanical pinch, a rank transformation was performed and then the ranks were modeled using a generalized estimating equations with normal distribution and identity link. In all cases, an independent covariance structure (i.e., within-animal repeated measurements were modeled as uncorrelated with each other given more complex working correlation structures failed to improve the model) was specified for the repeated measurements. Full factorial models were specified.
Results

Verification of Sensory and Motor Block with Lidocaine, QX-314, and Capsaicin in Rats without Surgery

Perisciatic injection of triple combination 2% lidocaine, 0.2% QX-314 followed by dilute 0.05% capsaicin produced a long-lasting (24 h) thermal hyposensitivity (fig. 1A). Consistent with previous results, perisciatic administration of 0.2% QX-314 alone followed by capsaicin increased thermal withdrawal latencies for 4 h (fig. 1A). We observed a significant main effect of group, time, and group × time interaction for ipsilateral thermal withdrawal latencies in treated rats (Time: \( P < 0.001 \); Group: \( P < 0.001 \); Time × Group: \( P < 0.001 \)). In rats administered the triple combination, thermal withdrawal latency in the contralateral paw was significantly different only at 2 h after administration (fig. 1B). We observed a significant main effect of time and group × time interaction but no group effect in contralateral thermal withdrawal latencies (Time: \( P < 0.001 \); Group: \( P = 0.28 \); Time × Group: \( P = 0.01 \)). Rats receiving triple combination had reduced responses to noxious mechanical pinch for greater than 24 h (fig. 1C). The QX-314 alone and capsaicin treatment group had reduced responses to noxious mechanical pinch for 4 h, similar to

![Fig. 1. Effects of nociceptor-selective block on sensory and motor function in na"ive rats. Rats were administered perisciatic injections of 2% lidocaine and 0.2% QX-314 in combination followed by 0.05% capsaicin (red symbols) or administered 0.2% QX-314 alone followed by capsaicin (blue symbols) and assessed duration of elevation of ipsilateral (A) and contralateral (B) hind paw to radiant heat stimuli. Responses to noxious mechanical pinch of a skin fold over the lateral metatarsal (C) were also scored and recorded. Motor block was assessed by evaluating extensor postural thrust of the ipsilateral hind paw (D). Individual data points are presented for each rat along with a longitudinal trend line based on the mean group values. n = 3 rats per group. * \( P < 0.05/7 (0.007) \) versus preinjection baseline values.](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/930983/ on 06/19/2017)
previous observations. For responses to mechanical pinch, we observed a main effect of time, group, and a significant interaction (Time: $P < 0.001$; Group: $P < 0.001$; Time $\times$ Group: $P < 0.001$). Minimal but demonstrable motor block was present for 1 h in rats receiving the triple combination and 30 min in rats receiving QX-314 plus capsaicin (fig. 1D). We observed a main effect of time, group, and a significant interaction in motor responses (Time: $P < 0.001$; Group: $P < 0.001$; Time $\times$ Group: $P < 0.001$).

Short-term Effects of Nociceptor-selective Block on Mechanical Hypersensitivity after Incisional Surgery

On the basis of the duration of blockade achieved with the triple combination, we tested the effect of two perisciatic injections of this combination, the first 1 h before and the second 12 h after plantar incision. For acute effects of treatment on mechanical hypersensitivity, we observed a main effect of time, group, and a significant time $\times$ group interaction (Time: $P < 0.001$; Group: $P < 0.001$; Time $\times$ Group: $P < 0.001$). Incisional surgery resulted in hypersensitivity to mechanical stimuli in the ipsilateral hind paw from 6 to 30 h after incision in perisciatic vehicle-treated animals (fig. 2A). In contrast, in rats receiving the triple combination, mechanical hypersensitivity was significantly attenuated compared with vehicle-treated incised rats from 6 to 30 h after incision as rats with triple combination did not have significant mechanical hypersensitivity at most time points after incision compared with preincision values (fig. 2A). Neither triple combination nor vehicle solutions altered withdrawal threshold to von Frey filament testing in sham-operated animals at any time point examined (fig. 2A) although we did not assess response to noxious pinch as in naïve animals. Perisciatic administration of capsaicin (0.05%) solution alone or lidocaine + QX-314 without capsaicin developed mechanical hypersensitivity postoperatively similar to rats administered vehicle (fig. 2A).

Long-term Effects of Nociceptor-selective Block on Mechanical Hypersensitivity after Incisional Surgery

In a subset of rats, we examined mechanical withdrawal thresholds for several days to determine whether nociceptor-selective block had a lasting effect on resolution of hypersensitivity. For long-term effects of treatment on mechanical hypersensitivity, we observed a main effect of time, group, and a significant group $\times$ time interaction (Time: $P < 0.001$; Group: $P < 0.001$; Time $\times$ Group: $P < 0.001$). Rats administered vehicle had hypersensitivity to mechanical stimuli after surgery which was nearly resolved within 7 days (fig. 2B). Unexpectedly, rats administered the triple combination exhibited a delayed reduction in mechanical withdrawal thresholds 17 days after incision suggesting that this approach may be toxic to peripheral nerves (fig. 2B). To examine the functional integrity of TRPV1-containing afferents in these same rats, we assessed the ipsilateral and contralateral response to noxious thermal stimuli at this time, and we observed prolonged latency (hyposensitivity) ipsilateral to injection in rats receiving the triple combination ($24.4 \pm 7.2$ s) compared with those receiving vehicle ($9.7 \pm 1.6$ s; $P = 0.026$). However, their withdrawal latency did not differ contralateral to injection ($10.1 \pm 1.0$ s vs. $9.5 \pm 0.4$ s in triple combination and vehicle groups, respectively; $P = 0.33$). We did not observe overt signs of ongoing pain including spontaneous guarding or flinching in the rats that received nociceptor-selective block at the time point when mechanical hypersensitivity developed.
Long-term Behavioral Assessment of Neurotoxicity in Normal Rats Administered Nociceptor-selective Block

Behavior was assessed after two dosing regimens—the same dosing as in the incisional study (two perisciatic injections of 2% lidocaine/0.2% QX-314/0.05% capsaicin separated by 12 h) or a single injection of the triple combination with a reduced concentration of lidocaine (1%). Two vehicle injections served as control. We observed a main effect of time, group, and a significant interaction for ipsilateral thermal withdrawal latencies (Time: \( P < 0.001 \); Group: \( P < 0.001 \); Time \( \times \) Group: \( P < 0.001 \)). Rats with two perisciatic injection of 2% lidocaine/0.2% QX-314/cap exhibited increased paw withdrawal latencies to noxious heat in the ipsilateral paw throughout the 35 days of testing, whereas single injection of 1% lidocaine/0.2% QX-314/cap produced a transient increase in paw withdrawal latency only at 12 h after administration (fig. 3A). There was no main effect of treatment on contralateral thermal response latencies for any treatment group (fig. 3B; \( P = 0.095 \)). In contrast to this early onset hyposensitivity to thermal stimuli, there was a delayed onset hypersensitivity to mechanical stimuli beginning 14 days after the two injections of the triple combination with 2% lidocaine, lasting through 35 days of testing (fig. 3C). This was not observed with the single injection of the combination containing 1% lidocaine (fig. 3C). We observed a main effect of time, group, and a significant time \( \times \) group interaction (Time: \( P < 0.001 \); Group: \( P = 0.009 \); Time \( \times \) Group: \( P < 0.001 \)) for ipsilateral mechanical withdrawal thresholds. Withdrawal threshold contralaterally varied inconsistently although it was reduced in the two injection, 2% lidocaine group on day 35 after injections (fig. 3D). We observed no main effect of group for contralateral mechanical withdrawal thresholds, but we did observe a significant effect of time and time \( \times \) group interaction (Time: \( P < 0.001 \); Group: \( P = 0.448 \); Time \( \times \) Group: \( P < 0.001 \)) for contralateral mechanical withdrawal thresholds.

Long-term Immunohistochemistry Analysis of Toxicity in Spinal Cord and DRG of Normal Rats Receiving Nociceptor-selective Block

ATF3, a marker of neuronal injury,\(^{1,3}\) was increased 35 days after the two injections of the 2% lidocaine containing

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**Fig. 3.** Long-term effects of nociceptor-selective block on mechanical and thermal sensitivity in naive rats. Time course of paw withdrawal latency to noxious radiant heat stimulus (A and B) and paw withdrawal thresholds to mechanical stimuli (C and D) in rats that received two perisciatic injections of 2% lidocaine/0.2% QX-314 followed by 0.05% capsaicin (12 h apart), a single perisciatic injection of 1% lidocaine/0.2% QX-314 followed by 0.05% capsaicin or vehicle solutions. Data represent mean ± SD. \( n = 4–6 \) rats per group. * \( P < 0.05/7 (0.007) \) compared with preinjection baseline values or # \( P < 0.05 \) compared with vehicle-injected rats. BL = baseline; cap = capsaicin; lid = lidocaine.
combination in the ipsilateral L4 and L5 DRGs as measured by percentage of ATF3-immunoreactivity (IR) sensory neurons compared with vehicle injection ($P < 0.05$) or the contralateral DRG ($P < 0.05$) within the same treatment group (fig. 4, A and B and table 1). The percentage of ATF3-IR neurons was also greater in ipsilateral compared with contralateral DRGs after single injection of the 1% lidocaine combination (table 1; $P = 0.029$) although in this case the percentage did not differ compared with vehicle controls (table 1). ATF3-IR was not present in motor neurons within the spinal cord (data not shown). The percentage of neurons expressing TRPV1-IR was less 35 days after the two injections of the 2% lidocaine combination compared with vehicle (fig. 4, A and B, and table 1; $P < 0.05$) or contralaterally (table 1; $P = 0.002$), whereas this was not the case after the single injection of the 1% lidocaine combination (table 1). GFAP-IR was barely detectable around sensory neuron cell bodies within ipsilateral or contralateral DRG of rats 35 days after administration of vehicle solutions, but was dramatically increased approximately threefold in the ipsilateral DRG of rats that received two injections of the 2% lidocaine combination (fig. 4, C and D; $P < 0.05$). In contrast, GFAP-IR was not significantly increased in the ipsilateral DRG of rats receiving the single injection of the 1% lidocaine combination (table 1).

In the spinal cord, both IB4-IR and CGRP-IR in the mediolateral aspects ipsilateral to injection were reduced similarly 35 days after two injections of the 2% lidocaine combination and the single 1% lidocaine combination injection compared with control or contralaterally (fig. 5). Overall, GFAP-IR did not differ among groups although some rats in the two injection 2% lidocaine combination group exhibited clear hypertrophy of astrocytes in the superficial aspects of the ipsilateral spinal cord (fig. 5I; data not shown).

Discussion

Pure blockade of pain without sympathetic or motor block is not possible with perineural injection of local anesthetics on a mixed peripheral nerve, because the local anesthetic will block sodium channels in all fibers to which it has access. Therefore, there has been considerable excitement regarding the concept that local anesthetic entry can be selectively enhanced in sensory neurons by temporarily opening pores into these fibers with agonists to large pore ion channels. This pore opening can even allow entry of highly hydrophilic local anesthetic derivatives such as QX-314 which normally fail to traverse the cell membrane. The current study extends knowledge in this exciting concept in two manners—by applying it to the setting of surgery and by uncovering a potentially dangerous neurotoxicity of this approach.

Nociceptor-selective block approaches using combined administration of TRPV1 activators and QX-314 to silence sensory afferents have only recently been examined in preclinical models of acute and more persistent pain states. Notably, the ability of TRPV1 silencing to prevent mechanical hypersensitivity after plantar incision may be dependent on the therapeutic approach. In the current study, rats administered triple combination did not develop mechanical hypersensitivity for several days suggesting that TRPV1-containing afferents contribute to mechanical hypersensitivity after incision. Similar to our findings, strategies that induce degeneration of TRPV1 afferents or their terminals such as local infiltration of high concentrations of capsaicin in the hind paw (1.5% or 15 mg/ml) or perisciatic administration of the potent capsaicin analog resiniferatoxin (0.03%) completely prevented the development of mechanical hypersensitivity in rats when administered before plantar incision. However, local infiltration or perisciatic injection of more dilute capsaicin (0.05 to 1.0%) before plantar incision blocks the development of thermal hypersensitivity and spontaneous guarding but fails to prevent mechanical hypersensitivity or increased physiological responses of Aδ and C fibers to mechanical stimuli in vitro. Besides a difference in concentration, it is conceivable that we targeted a wider afferent population than TRPV1-expressing afferents. Lidocaine activates TRPA1 channels in addition to TRPV1, and sensitization of TRPA1-containing afferents has been shown to contribute to mechanical hypersensitivity after acute plantar incision. Although TRPA1 and TRPV1 are coexpressed in some sensory neurons of the rat, a
Fig. 5. Representative photomicrographs of dorsal spinal cord from rats 35 days after perisciatric administration of vehicle (A and D) or 2% lidocaine/0.2% QX-314, and 0.05% capsaicin (ipsilateral: B and E; contralateral: C and F). Rats that received nerve block had reduced immunostaining for isolectin B4 (IB4, green) and calcitonin gene–related protein (CGRP, red) in the ipsilateral spinal cord compared with vehicle-injected rats indicating degeneration of sensory nerve terminals. Quantification of immunofluorescence levels for IB4, CGRP, and glial fibrillary acidic protein (GFAP) in the dorsal spinal cord of rats administered two injections of 2% lidocaine/QX-314 and capsaicin (2% Lid/QX/cap), a single injection of 1% lidocaine/QX-314 and capsaicin (1% Lid/QX/cap) or vehicle solution (G–I). Insets in A indicate sampling area for immunofluorescence analysis. Two-way ANOVA followed by post hoc Bonferroni contrasts *P < 0.05 versus vehicle. †P < 0.05 versus contralateral. N = 4–6. Scale bar in A and D = 100 μm. cap = capsaicin; ipsi = ipsilateral; Lid = lidocaine.
significant population of TRPA1 afferents do not overlap and are functionally distinct.\textsuperscript{25,26} Previous studies examining nociceptor-selective blockade with capsaicin plus lidocaine and/or QX-314 have focused on acute analgesic or antihypersensitivity effects, and have observed a prolonged time course (6 to 24 h) of action similar to our results in naïve animals (fig. 1). The current study uniquely followed animals for several weeks after perineural injection of this combination either after incisional surgery or in naïve animals. We were surprised to observe delayed and long-term hypersensitivity to mechanical stimuli, similar to what is observed in standard models of neuropathic pain.\textsuperscript{27,28} The delayed onset of behavioral hypersensitivity in the current study is similar to that observed by Pan et al.\textsuperscript{29} who showed that systemic administration of neurolytic doses of resiniferatoxin produced persistent thermal hypersensitivity several weeks later. Assuming destruction of TRPV1-expressing afferents, locally in this study and globally in the study by Pan et al., the reason that mechanical hypersensitivity would follow after a delay of many days is unknown. But other models of neuropathic hypersensitivity demonstrate clearly that injured afferents affect their uninjured neighbors coursing through the same peripheral nerve in a manner that results in up-regulation of cytokines and growth factors contributing to peripheral sensitization.\textsuperscript{30,31} Spinal plasticity including possible sprouting of myelinated primary afferents into laminae II may also be responsible for the delayed hypersensitivity.\textsuperscript{29,32} In our study, histologic examination 5 weeks after injection was consistent with nerve injury, as evidenced by increased numbers of sensory neurons expressing the injury marker ATF3, perisomatic activation of satellite cells in the DRG, and loss of peptidergic and nonpeptidergic fiber markers in the spinal cord. The loss of CGRP- and IB4-containing terminals in the rat is consistent with destruction of TRPV1-expressing afferents targeted for temporary blockade with this combination based on the coexpression of both these markers with TRPV1 in the rat.\textsuperscript{33} We did not observe a complete loss of TRPV1-IR in the DRG probably due to dynamic changes in TRPV1 expression associated with nerve injury as decreased expression of TRPV1 on injured and increased expression in uninjured neurons have been reported in several a peripheral nerve injury models.\textsuperscript{19,34–36} Although our study was not designed to test the time of onset and mechanisms of neurotoxicity from this combination, the potential cause of this toxicity and its implication deserve further comment. It is unlikely that nerve injury from intraneural injection \textit{per se} was the cause of the toxicity, because neither abnormal behavior nor histologic evidence of toxicity was present in any vehicle-injected animals. The doses of lidocaine (1 to 2%) used in the current study are routinely used clinically\textsuperscript{37} and although generally not considered to be toxic in the rat, some studies have reported minor axonal degeneration and edema in rats 24 to 48 h after perineural injection of 2 to 4% lidocaine.\textsuperscript{38} Long-term effects several weeks after injections have not previously been reported. QX-314 when injected perineurally at the concentration (0.2% or 5.8 mM) used in the current study did not produce signs of neurotoxicity based on histological and ultrastructural analysis of nerve fibers.\textsuperscript{39} Significantly higher doses of QX-314 (25 to 100 mM) have been shown to induce signs of tissue injury around the injection site.\textsuperscript{40} Spinal administration of QX-314 at doses used in the current study (5 to 10 mM) produces severe irritation and death in mice\textsuperscript{41} and rats.\textsuperscript{18} The mechanisms responsible for the adverse spinal effects of QX-314 are not clear, however they may be due to the ability of QX-314 to block inhibitory synaptic activity \textit{via} interactions with spinal nicotinic receptors\textsuperscript{4,18} as rats administered spinal QX-314 do not show histological or immunohistochemical evidence of inflammation, neuronal degeneration, or glial activation in the spinal cord.\textsuperscript{18} It is tempting to speculate that opening the TRPV1 channel with capsaicin allowed higher intracellular concentrations of lidocaine and QX-314 enhancing the neurotoxic potential of these agents. This is consistent with the observation that toxicity required the combined effects of administering lidocaine, QX-314, and capsaicin, because administration of these compounds alone did not cause gross behavioral impairment or delayed hypersensitivity in rats.

Regardless of the cause of neurotoxicity, there is some evidence that it is exposure dependent. We varied two aspects of exposure in the brief toxicity assessment, by simultaneously reducing the concentration of one component (lidocaine from 2 to 1%) and the exposure to all components by comparing two injections to one injection. The degree of loss of IB4 and CGRP-IR within sensory nerve terminals was equivalent between rats that received these two different exposures despite a lack of mechanical hypersensitivity in the lower exposure group. In addition, the percentage of ATF3-IR neurons and perisomatic satellite cell activation was greater in the ipsilateral DRG of rats that received two injections with 2% lidocaine in combination. This is consistent with exposure-dependent toxicity. This study was not designed to assess the key components and threshold concentrations for neurotoxicity from this combination. Nonetheless, it suggests that future laboratory work with these combinations should either include histologic analysis to demonstrate that reversible but not toxic block was obtained or to consider that this was an ablative strategy. And it suggests that future clinical application should follow extensive preclinical toxicity testing to define presumptively safe concentrations, including with intraneural injection.

In summary, we investigated whether silencing nociceptors with a combination of lidocaine/QX-314 and capsaicin reduces mechanical hypersensitivity in a rat model of acute incisional pain. Perisciatric administration of 2% lidocaine/QX-314 and capsaicin prevented the development of mechanical hypersensitivity for several days after incision. Unexpectedly, rats that received triple combination developed delayed mechanical hypersensitivity weeks later suggesting
that this regimen was neurotoxic. In naïve rats, administration of 2% lidocaine/QX-314 and capsaicin produced persistent thermal hypoalgesia and delayed mechanical hypersensitivity that lasted for several weeks. Signs of neurotoxicity were evident as degeneration of primary afferent terminals in the spinal cord and pathological changes indicative of nerve injury in the ipsilateral DRG weeks after administration. Importantly, the current study does not preclude the use of sensory neuron subtype selective approaches for basic science studies or exploration of nociceptor-selective block approaches for clinical development. Our results underscore the importance of conducting appropriate screening of neurotoxicity with these new combinations as safe doses of local anesthetics may be toxic in combination with TRPV1/TRPA1 ion channel activators. Future preclinical and clinical studies, investigating novel sensory-selective long-term regional anesthesia approaches should include long-term behavioral outcome measures \(^2\)\(^{2,3}\) and assessment of neurotoxicity to better assess these risks.

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Competing Interests
The authors declare no competing interests.

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