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Resiniferatoxin (RTX) Causes a Uniquely Protracted Musculoskeletal Hyperalgesia in Mice by Activation of TRPV1 Receptors

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Abstract: **Inactivation of transient receptor potential vanilloid-1 (TRPV1) receptors is one approach to analgesic drug development. However, TRPV1 receptors exert different effects on each modality of pain. Because muscle pain is clinically important, we compared the effect of TRPV1 ligands on musculoskeletal nociception to that on thermal and tactile nociception. Injected parenterally, capsaicin had no effect on von Frey fiber responses (tactile) but induced a transient hypothermia and hyperalgesia in both the tail flick (thermal) and grip force (musculoskeletal) assays, presumably by its agonistic action at TRPV1 sites. In contrast, resiniferatoxin (RTX) produced a chronic (>58 days) thermal antinociception, consistent with its reported ability to desensitize TRPV1 sites. In the same mice, RTX produced a transient hypothermia (7 hours) and a protracted (28-day) musculoskeletal hyperalgesia in spite of a 35.5% reduction in TRPV1 receptor immunoreactivity in muscle afferents. Once musculoskeletal hyperalgesia subsided, mice were tolerant to the hyperalgesic effects of either capsaicin or RTX whereas tolerance to hypothermia did not develop until after 3 injections. Musculoskeletal hyperalgesia was prevented but not reversed by SB-366791, a TRPV1 antagonist, indicating that TRPV1 receptors initiate but do not maintain hyperalgesia. Injected intrathecally, RTX produced only a brief musculoskeletal hyperalgesia (2 days), after which mice were tolerant to this effect.**

Perspective: ***The effect of TRPV1 receptors varies depending on modality and tissue type, such that RTX causes thermal antinociception, musculoskeletal hyperalgesia, and no effect on tactile nociception in healthy mice. Spinal TRPV1 receptors are a potential target for pain relief as they induce only a short musculoskeletal hyperalgesia followed by desensitization.***

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Key words: *Capsaicin, pain, musculoskeletal hyperalgesia, desensitization, nociception.*

The transient receptor potential vanilloid-1 (TRPV1) receptor, a nonselective cation channel, was first cloned by Caterina et al and described as a heat sensor and transducer of thermal nociception.[7] TRPV1 receptors are expressed all over the body, including the brain, spinal cord, and peripheral nervous system,[43,52,61] where they exert their nociceptive function through the activation of primary afferent C-fibers[22] and Aδ-fibers[13] innervating the skin and muscles.[22,23,40,60] TRPV1 is crucial to the development of many human conditions and animal models of hyperalgesia.[12,16,53] In fact, TRPV1 receptor expression is increased in painful diseases like fibromyalgia,[41] irritable bowel syndrome,[8] vulvodynia,[64] mastalgia,[20] and fibrosarcoma.[36]

Information about musculoskeletal pain is important because of its prevalence in the global population.[5,69,71] Approximately 39% of men and 45% of women report chronic musculoskeletal pain,[70] describing it as dull and aching rather than epicritic.[44] A variety of modulators of primary afferent C-fiber activity (eg, lactate and ATP) are poised to serve as endogenous mediators of musculoskeletal pain.[40] Muscle pain is modulated by the same type of TRPV1 receptor–expressing C- and Aδ-primary afferent fibers that transmit thermal and mechanical nociceptive signals to the muscle as well as to the

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skin.[13,30,31] In support of this, capsaicin injected into the masseter muscle of rats induces a tactile mechanical hyperalgesia (measured using von Frey fibers) that is prevented by the TRPV1 receptor antagonists capsazepine and AMG9810.[51] Thus, although TRPV1 receptors play a crucial role in thermal hyperalgesia,[28,29,45,49] their role in tactile mechanical hyperalgesia has been disputed, with claims of no effect,[3,4] an antinociceptive effect,[9,24] and additional claims of a hyperalgesic effect.[28,63,67] With regard to muscle pain in particular, capsazepine also abolishes mechanical hyperalgesia produced by electrically induced eccentric exercise of the gastrocnemius muscle of rats measured using the Randall-Selitto apparatus,[16] suggesting an association with muscle fatigue.

Although informative, previous studies concerning TRPV1 receptors and muscle pain only measured muscle sensitivity to pressure (tactile sensitivity) applied to the muscle. They have not addressed the deep dull muscle pain that accompanies muscle use. To address this, we hypothesized that TRPV1 receptor activity modulates musculoskeletal nociception in mice, as measured using the grip force assay. In this assay, the force generated when animals grip a wire grid is measured; decreases in their ability to hold on to the grid reflect either muscle pain or weakness. We examined the ability of the TRPV1 receptor agonist capsaicin,[7] the receptor desensitizer resiniferatoxin (RTX),[62] and the TRPV1 receptor antagonist SB-366791(*N*-(3-methoxyphenyl)-4-chlorocinnamide)[21,50,65] to influence grip force responses in mice. We differentiated pain from weakness by the ability of morphine to reverse decreases in grip force. On the basis of the presence of TRPV1 receptor–expressing interneurons in the spinal cord,[26,38] we also examined their possible role in the transmission of nociception from muscles by assessing the effect of intrathecally (i.t.) injected RTX on musculoskeletal nociception.

Methods

Animals

Adult female Swiss Webster mice (Harlan Sprague Dawley Inc, Indianapolis, IN) weighing 20 to 25 g were housed 5 per cage and allowed to acclimate for at least 1 week prior to use. Mice were allowed free access to food and water and were housed in a room with a constant temperature of 23°C on a 12-hour light–dark cycle. Females were used to explore movement-evoked painful disorders and to reflect the higher incidence of musculoskeletal pain in most anatomic sites in females than in males when studied in humans.[70] Because fluctuations in sex hormones were not the focus of our study, the estrus cycle of each mouse was not examined. All experimental procedures and measurements were done blinded to the treatment and performed according to the guidelines of the International Association for the Study of Pain, the University of Minnesota Animal Care and Use Committee, and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication NIH 78-23, revised 1995).

Drugs and Chemicals

Capsaicin, a TRPV1 ligand that activates TRPV1 sites at low doses and desensitizes them at higher doses, was purchased from Sigma Chemical Company (St. Louis, MO). Capsaicin was dissolved in sesame oil prior to each use and delivered subcutaneously (s.c.) at a dose of 2 mg/kg.

RTX, a TRPV1 ligand that is reported to rapidly desensitize TRPV1 receptors with little or no agonistic activity, was obtained from LC Laboratories, Inc (Woburn, MA) and delivered s.c. at doses of .1 mg/kg or .02 mg/kg in sesame oil or i.t. at a dose of .125 μg/animal dissolved in 5% Tween and 5% dimethyl sulfoxide (DMSO). SB-366791, a TRPV1 antagonist, was purchased from Enzo Life Sciences, Inc (Farmingdale, NY) and injected intraperitoneally (i.p.) at a dose of .5 mg/kg. This dose was based on a dose previously found to be effective by its ability to inhibit the number of eye wipe responses produced by application of capsaicin to the eye and to block capsaicin-induced hypothermia in rats.[65] Because of its low solubility, SB-366791 was dissolved in 40% DMSO and injected 20 minutes before capsaicin or RTX. Injections made i.t. in mice were delivered at approximately the L5-L6 intravertebral space using a 30-gauge, .5-inch disposable needle on a 50-μL Luer tip Hamilton syringe (Becton Dickinson and Company, Franklin Lakes, NJ) in lightly restrained, unanesthetized mice.[25] Injections made s.c. were delivered in the medial lower back of the animal to avoid injection into muscle responsible for either grip force or von Frey fiber responses. Morphine sulfate, a μ-opioid receptor agonist, was purchased from Mallinckrodt (St. Louis, MO). Morphine sulfate was dissolved in saline (pH 5.0) and injected i.p. at doses from 10 to 60 mg/kg, a range of doses that induce a potent antinociception in the tail flick assay in mice. For each drug treatment, control mice were injected with the same amount of vehicle.

Grip Force Assay

Forelimb grip force was measured using a grip force apparatus as described in rats[33] and mice.[34,39,66] The grip force apparatus consisted of a force transducer that is connected to a wire mesh grid (12 × 7 cm^2 outer diameter with a .5-cm^2 wire grid) and positioned on top of an aluminum frame approximately 30 cm above the bench top. During testing, each mouse was held by its tail and gently passed in a horizontal direction over the wire grid until it grasped the grid with its forepaws. The peak force that is exerted by the forelimbs of each mouse when pulling on the grid was recorded by the force transducer, to which the grid is attached. Two grip force measures were obtained at each time point; the average of these measurements was used to represent each animal's forelimb grip force at that particular time. Animals were familiarized with the grip force apparatus for 3 days by grip force testing prior to initiation of the experiment. On the third day, grip force measurements were obtained prior to each intervention to

establish baseline values for each animal. Then the drug was administered and grip force measured at the times indicated. Grip force data are represented as raw data and expressed in terms of grams (g).

Tail Flick Assay

Animals were manually restrained and the tail submerged to a distance of 1 cm from the base of the tail in a water bath maintained at 49 to 51°C. The withdrawal latency was defined as the time for the animal to withdraw its tail from the water. To avoid tissue damage, cut-off times of 10 to 15 seconds were used.

Von Frey Fiber Assay

Mechanical sensitivity to a von Frey fiber (Semmes-Wein standard nylon filament #4.17 equivalent to 1.4 g) was measured in mice prior to and after s.c. injection of RTX or capsaicin. The responses to the von Frey fiber prior to injection did not differ among groups. After injection, mice were placed on a wire mesh under glass 6-ounce (177 mL) custard cups that prevent escape but allow movement of all 4 limbs and head. The fiber was applied to the plantar surface of each hind paw, to the point of bending, for a total of 10 applications/paw. A positive response was defined as a brisk shaking or licking of the paw. The number of positive responses elicited from both paws out of 20 trials was recorded as the mechanical sensitivity.

Body Temperature Measurement

Body temperature measurement took place in a room with an ambient temperature of 25°C. The animal was removed from its cage and placed under a clean towel to create a barrier on 3 sides, and its colonic temperature was measured using a rectal thermometer (ETI Microtherma 2K Thermometer connected to a RET-3 rectal probe; Electronic Temperature Instruments Ltd, Worthing, West Sussex, UK).

Retrograde Tracing and Immunohistochemistry

Mice were deeply anesthetized with isoflurane and a small incision made in the calf to expose the gastrocnemius muscle. A total volume of 10 μL of 4% fluorogold (FG; Fluorochrome LLC, Denver, CO) in distilled water was injected in the medial, proximal, and distal regions of the muscle using a Hamilton syringe with a 31-gauge needle. Care was taken during these injections to prevent leakage of tracer into surrounding tissues by injecting slowly and wiping any excess tracer. The skin was then sutured and the mice were placed in a temperature-controlled cage. After recovery, mice were returned to their home cage and allowed food and water ad libitum. Some mice were injected first with FG and then 5 days later with RTX (.1 mg/kg s.c.), and finally killed 2 days after RTX. Other mice, injected first with RTX and then 2 days later with FG, were killed 5 days after the FG injection.

Mice were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.) and perfused through the heart with phosphate-buffered saline (PBS, .1 M, pH 7.4) followed by fixative (4% paraformaldehyde, .4% picric acid, pH 6.9) at room temperature. Dorsal root ganglia (DRGs) from the lumbar region (L3-L5) were extracted and stored in 10% sucrose in PBS until sectioned. Each DRG was sectioned at a plane that is parallel to its widest axis on a cryostat (15-μm thickness) and thaw-mounted on gelatin-coated slides. From each DRG, every fourth section was taken for analysis, totaling 10 to 15 sections per DRG. From the sections taken, those with the highest number of FG projections were used for the analyses, resulting in 5 sections counted for L3 and L4 DRGs and in 3 sections counted for L5 DRGs such that equal numbers of sections were used from each DRG at each level (L3-L5) in all animals. Entire sections were counted, and counting was done in a blinded fashion. Tissue was washed in blocking solution (.1 M PBS, .3% Triton X-100, 10% normal donkey serum) for 1 hour at room temperature and then incubated overnight with the primary antibody (rabbit anti-TRPV1 C-terminus [1:1,000 in blocking solution]; Neuromics, Minneapolis, MN). Following incubation with the primary antibody, the tissue was rinsed with PBS and incubated for 4 hours with the secondary antibody (Cy3-conjugated AffiniPure donkey anti-rabbit IgG [1:200 in PBS]; Jackson Immunoresearch, West Grove, PA). The tissue was then dehydrated in ascending concentrations of alcohol, cleared in xylene, and coverslipped with DPX mountant (Fluka, Milwaukee, WI). Tissue sections were visualized under a fluorescence microscope, and images were captured with a cooled CCD camera system (Dage model CCD-300T-IFG, Michigan City, IN).

Statistical Analyses

Mean values (± standard error of the mean) are presented throughout the figures. Statistical analysis of the results was performed using Student unpaired t-test between 2 groups or analysis of variance followed by post hoc Bonferroni or Newman-Keuls test for comparison between groups, as indicated. A difference was considered significant if the probability that it occurred because of chance alone was less than 5% ($P < .05$).

Results

TRPV1 Agonists Decreased Grip Force Responses

A single injection of capsaicin (2 mg/kg s.c.) at a dose in the range reported to induce hypothermia[48,51] decreased grip force responses (Fig 1A), tail flick latencies (Fig 1D), and body temperatures (Fig 1J) when compared to vehicle-injected control mice. All changes induced by capsaicin were maximal 30 minutes after injection and faded between 90 and 180 minutes later (Figs 1A, 1D, 1J). In spite of the decrease in grip force responses, there was no effect of capsaicin on von Frey fiber responses (Fig 1G), indicating that musculoskeletal nociception, measured using grip force, differs from tactile mechanical nociception, measured using von Frey fibers, in their sensitivity to TRPV1

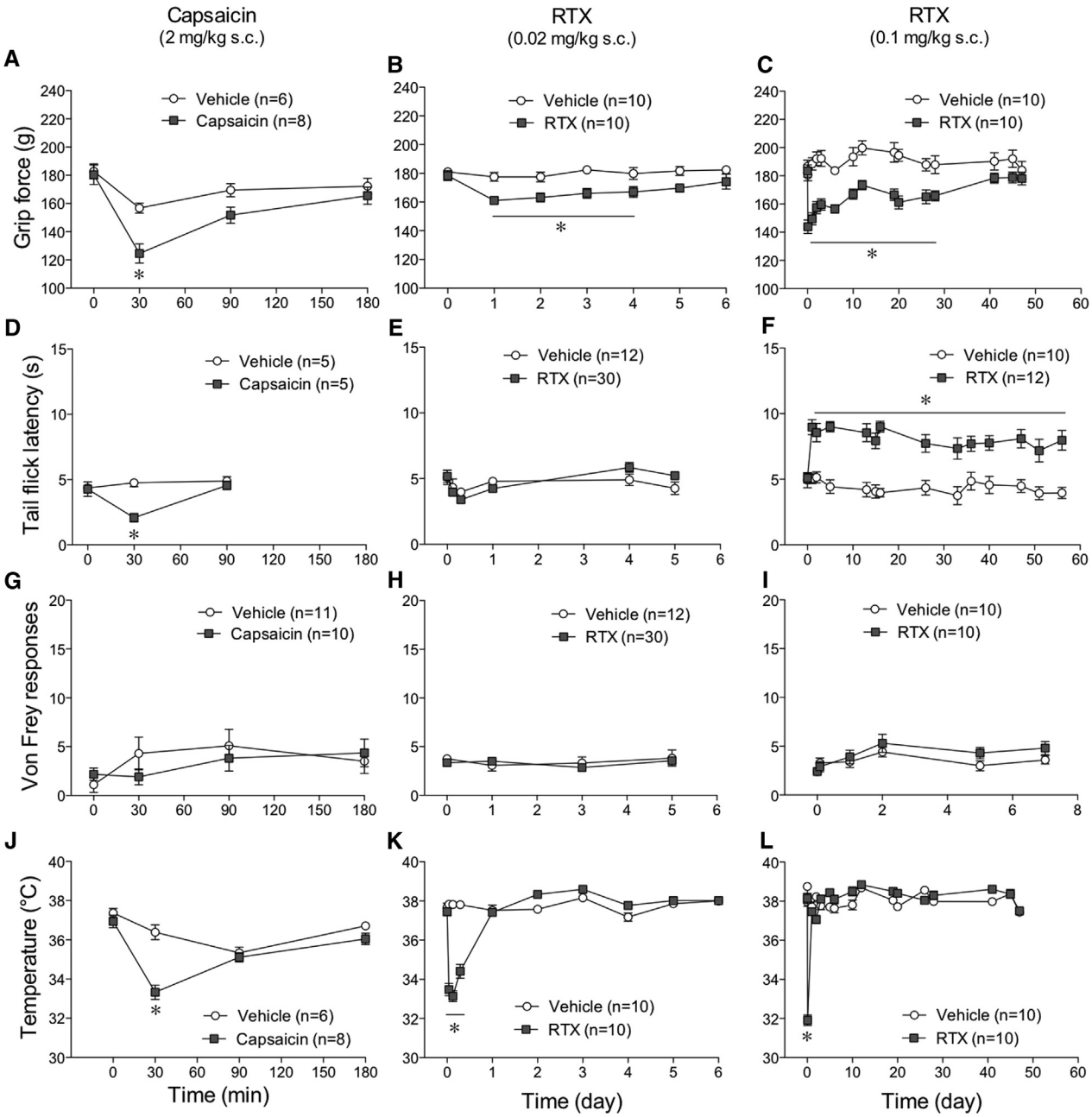


Figure 1. A single injection of capsaicin or RTX decreases grip force but causes variable responses in tail flick latencies, von Frey fiber responses, and body temperature. Mice were injected s.c. with 2 mg/kg of capsaicin **(A, D, G, J)**, .02 mg/kg of RTX **(B, E, H, K)**, or .1 mg/kg of RTX **(C, F, I, L)**. Grip force **(A, B, C)**, tail flick **(D, E, F)**, von Frey **(G, H, I)**, and body temperature **(J, K, L)** measurements were recorded. Statistical analyses were performed using a 2-way analysis of variance followed by Bonferroni post hoc analysis, where the * indicates $P < .05$ when compared to vehicle.

ligands. These data indicate that the decrease in grip force does not result from increased tactile sensitivity of the surface of their paws, which would have been reflected in an increased sensitivity to von Frey fibers.

Responses to a low dose of RTX (.02 mg/kg s.c.) differed from those produced by capsaicin in that the decrease in grip force (Fig 1B) persisted longer (4 days) in spite of its lack of effect on responses in the tail flick assay (Fig 1E) or on von Frey fiber sensitivity (Fig 1H). Biologic activity at this dose of RTX was confirmed by its ability to induce a transient decrease in body temperature that lasted for 7 hours (Fig 1K). A higher dose of RTX (.1 mg/kg s.c.) induced hyperalgesia that persisted for 28 days (Fig 1C), had no effect on von Frey fiber sensitivity (Fig 1I), and produced a persistent (>56 days) increase in tail flick responses (Fig 1F), consistent with desensitization of TRPV1 sites along thermal nociceptive pathways. The decrease in body temperature after injection of this higher dose of RTX (31.91 ± .26°C, n = 10) was only slightly greater than that following the lower dose (33.14 ± .28°C, n = 10) and lasted for the same amount of time (7 hours) as that following the lower dose (Fig 1L). The effect of RTX on grip force (28 days) was in marked contrast to

its much shorter duration of action on body temperature (<24 hours) and its much longer duration on thermal nociception (>56 days) when measured in the same groups of mice.

Tolerance Developed to TRPV1 Agonist Activity

When delivered daily at a dose of 2 mg/kg s.c., tolerance to the effects of capsaicin, including the decrease in body temperature and musculoskeletal hyperalgesia, was not observed (Figs 2A, 2D, 2F). This may be due to the short duration of capsaicin's effect and the long interval (24 hours) between challenges.

We then tested the ability of a lower dose of RTX (.02 mg/kg s.c.) to desensitize TRPV1 receptors along musculoskeletal pathways and thus relieve muscle pain. The dose was chosen for its lack of thermal antinociception and shorter musculoskeletal hyperalgesia compared to the high dose of RTX (.1 mg/kg s.c.). RTX at the lower dose had no effect on tail flick latencies, even when delivered at 6- to 10-day intervals. However, the low dose of RTX produced musculoskeletal hyperalgesia, and tolerance developed to this hyperalgesic effect by the third injection when administered at 6- to 10-day intervals (Fig 2B). This variable interval was required to allow mice to recover from hyperalgesia produced by the previous injection before delivering subsequent injections. In the same mice, the low dose of RTX decreased body temperature, but no tolerance developed to this effect when injected 4 times at 6- to 10-day intervals (Fig 2G).

After the musculoskeletal hyperalgesia dissipated following injection of the higher dose of RTX, a second injection failed to produce hyperalgesia. This indicates that tolerance developed to the hyperalgesic effect of the higher dose of RTX after a single injection (Fig 2C). In contrast, tolerance to the decrease in body temperature produced by the higher dose was not apparent until the fourth injection (Fig 2H). At that time, the high dose

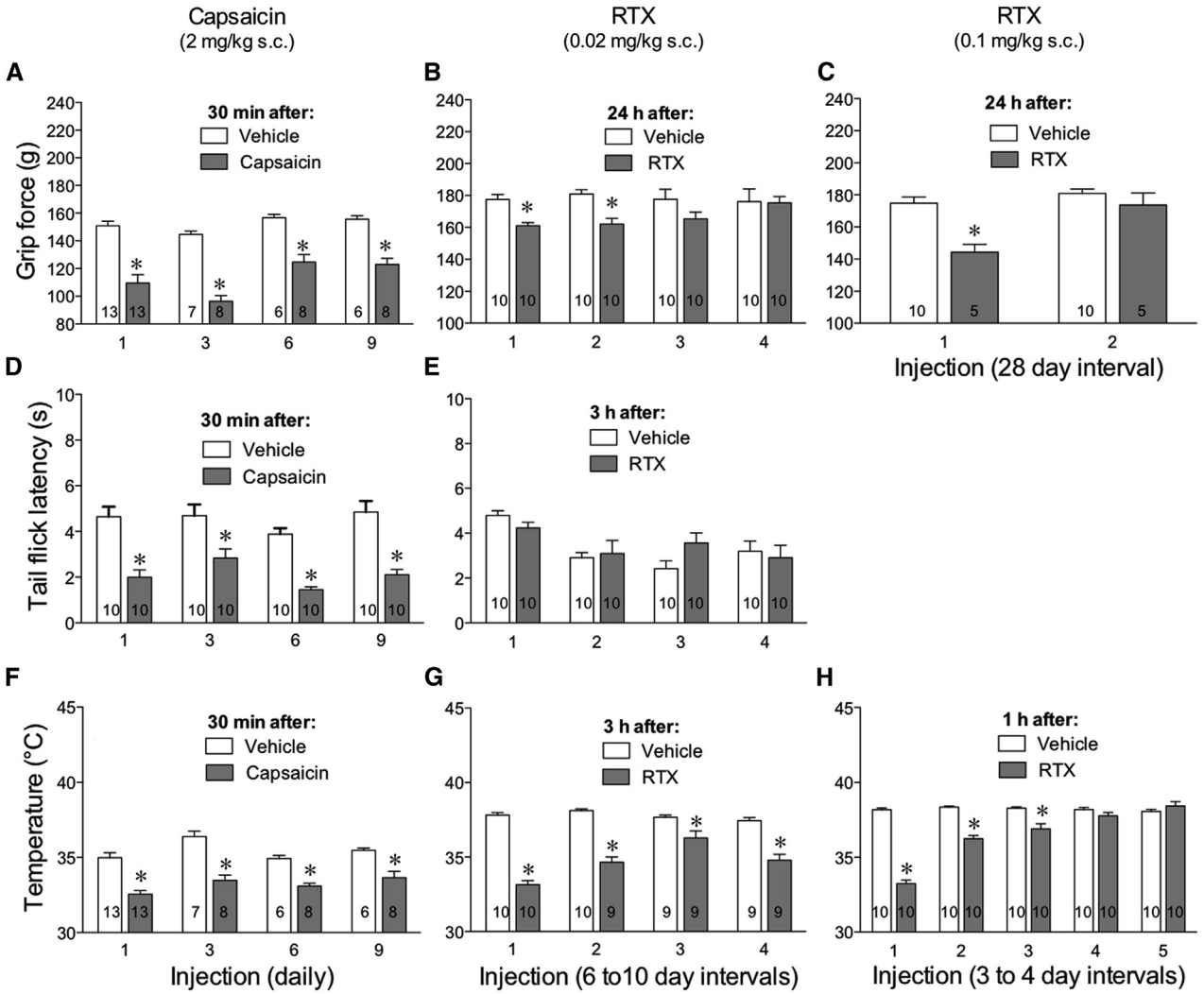


Figure 2. Unlike capsaicin, multiple injections of RTX cause tolerance to its effect on grip force but variable effects on tail flick and body temperature. Mice were injected daily with 2 mg/kg of capsaicin **(A, D, F)**, a low dose (.02 mg/kg) of RTX every 6 to 10 days **(B, E, G)**, or high dose (.1 mg/kg) of RTX **(C, H)**. Grip force **(A, B, C)**, tail flick **(D, E)**, and body temperature **(F, G, H)** measurements were recorded after each injection, as indicated. Statistical analyses were performed using the unpaired Student t-test, where the * indicates $P < .05$ when compared to vehicle on the same day.

of RTX injected s.c. increased body temperature (38.6 ± .22°C, n = 10) 24 hours after its fourth injection when compared to vehicle-injected control mice (37.8 ± .21°C, n = 10, Student t-test, $P = .012$). Together these data indicate that TRPV1 receptors supporting musculoskeletal hyperalgesia (grip force) do not desensitize as fast as those supporting thermal nociceptive fibers (tail flick); however, they desensitize much faster than those supporting thermoregulatory sensory fibers (body temperature).

After 4 injections of .02 mg/kg of RTX at 6- to 10-day intervals, mice were cross-tolerant to the hyperalgesic effect of capsaicin (Fig 3A) as well as to the high-dose RTX (Fig 3B). When tested in the tail flick assay for thermal nociception, 4 injections of the low dose of RTX prevented the thermal hyperalgesic effect of capsaicin (Fig 3C) and attenuated but did not prevent the thermal antinociceptive effect of high-dose RTX (Fig 3D). These data demonstrate that a low dose of RTX can desensitize TRPV1 receptors along musculoskeletal and thermal nociceptive pathways while sparing thermoregulatory pathways from complete TRPV1 receptor desensitization that leads to a tonic increase in body temperature (Fig 2G).

Morphine-Attenuated Hyperalgesia

To confirm that the decrease in grip force responses resulting from injection of capsaicin was due to hyperalgesia and not weakness, mice were injected with various doses of morphine 15 minutes prior to injection of capsaicin or vehicle and grip force was measured 30 minutes later (Fig 4A). Morphine prevented the capsaicin-induced decrease in grip force responses in a dose-dependent manner (Fig 4A).

To test the effect of morphine on RTX-induced hyperalgesia, we initially injected mice with RTX (.1 mg/kg s.c.); 24 hours later, after hyperalgesia was present, mice were injected i.p. with increasing doses of morphine or vehicle. Grip force measurements were recorded 45 minutes after morphine or vehicle. Morphine reversed the RTX-induced decrease in grip force (Fig 4B).

Musculoskeletal Hyperalgesia Is Inhibited by a TRPV1 Receptor Antagonist

To verify that RTX produces its hyperalgesic effect via the capsaicin-sensitive receptor (TRPV1), mice were pretreated with SB-366791, a TRPV1 antagonist[21] (.5 mg/kg i.p.), and 20 minutes later injected with capsaicin (2 mg/kg s.c.) or RTX (.1 mg/kg s.c.) or their vehicle. SB-366791 attenuated capsaicin- and RTX-induced musculoskeletal hyperalgesia in the grip force assay (Figs 5A and 5B). When the same dose of SB-366791 was injected 8 days after RTX (.1 mg/kg s.c.), it failed to reverse the hyperalgesic effect of RTX (Fig 5C).

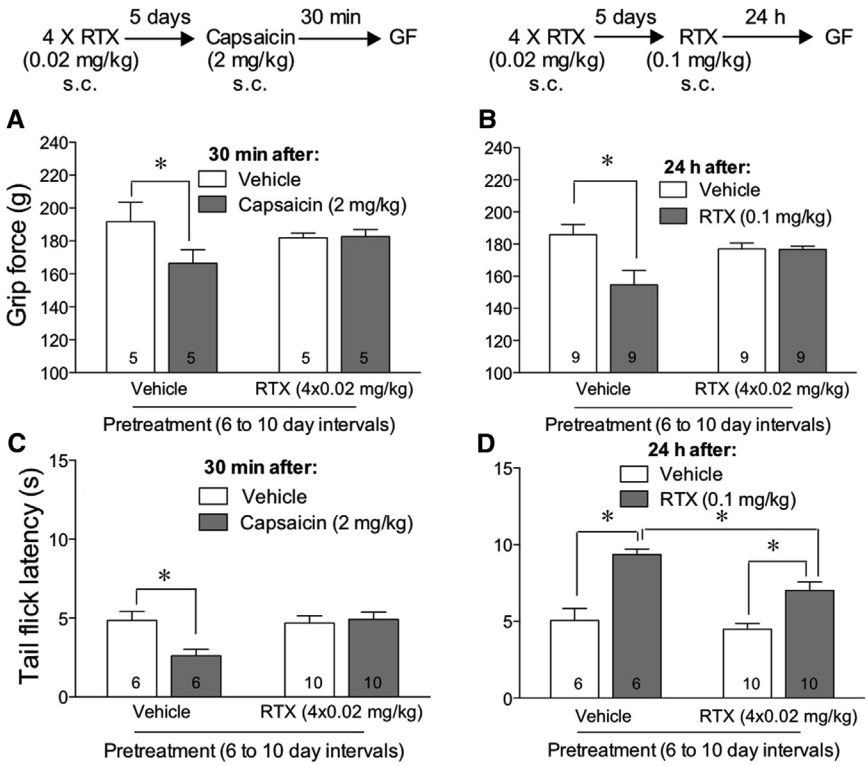


Figure 3. Repeated injections of a low dose of RTX decrease sensitivity of TRPV1 receptors to capsaicin and to RTX. Animals were injected s.c. 4 times with .02 mg/kg of RTX at 6- to 10-day intervals and 5 days later challenged with either 2 mg/kg of capsaicin **(A, C)** or .1 mg/kg of RTX **(B, D)**. Grip force **(A, B)** and tail flick **(C, D)** measurements were taken 30 minutes after capsaicin or 24 hours after RTX. Statistical analyses were performed using a 2-way analysis of variance followed by Bonferroni post hoc analysis, where the * reflects $P < .05$ when compared to the values indicated.

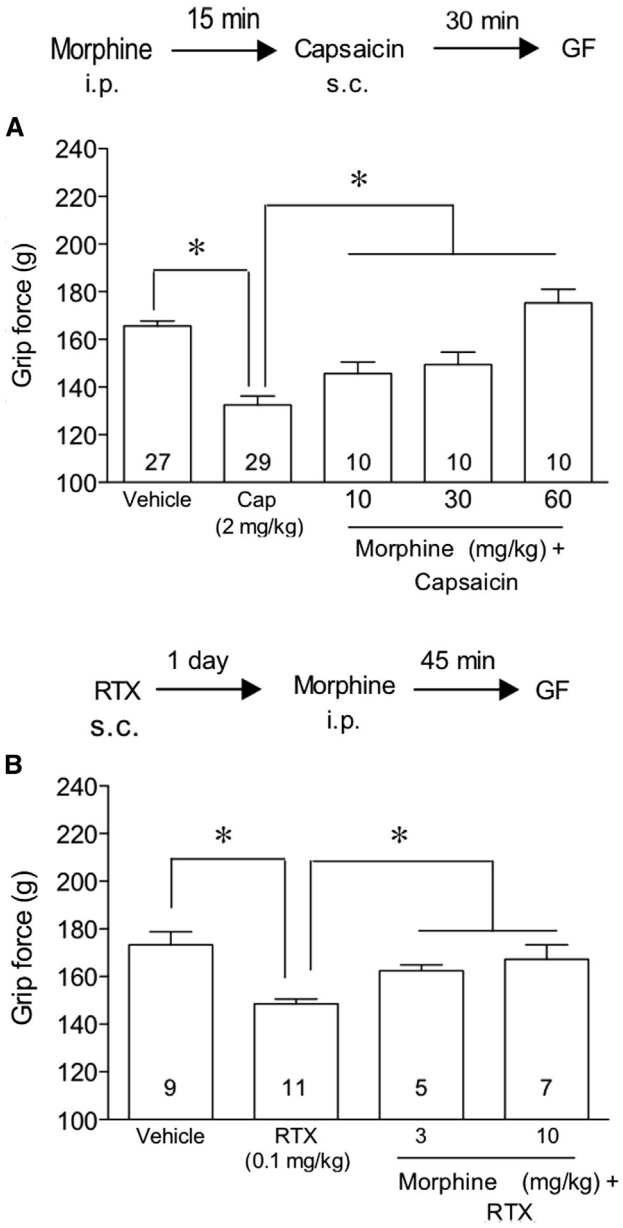


Figure 4. Morphine prevents and reverses the decrease in grip force produced by capsaicin **(A)** or RTX **(B)**. Grip force measurements were taken 45 minutes after the i.p. injection of morphine and 30 minutes after the s.c. injection of capsaicin (2 mg/kg) or 24 hours after the injection of RTX (.1 mg/kg). Statistical analyses were performed using a 1-way analysis of variance followed by Newman-Keuls post hoc analysis, where the * represents $P < .05$ when compared to the values indicated.

Intrathecal RTX

To assess the importance of centrally located TRPV1 receptors in musculoskeletal hyperalgesia, mice were injected i.t. with RTX (.125 μg/animal i.t.). Grip force values, von Frey fiber sensitivity, and tail flick latencies were measured at the times indicated in Fig 6. Intrathecal RTX decreased grip force values for 2 days (Fig 6A), suggesting musculoskeletal hyperalgesia. Von Frey fiber responses in RTX-pretreated mice did not differ from those in vehicle-injected control mice (Fig 6B), suggesting a lack of tactile hyperalgesia. Tail flick latencies in RTX-pretreated mice were increased (Fig 6C), reflecting thermal antinociception that persisted for the 14 days measured. In addition, RTX injected i.t. increased body temperature (38.33 ± .13°C, n = 19) 24 hours after its injection when compared to vehicle-injected control mice (37.50 ± .13°C, n = 21, Student t-test, $P = 8.5 \times 10^{-5}$).

When musculoskeletal hyperalgesia subsided after the i.t. injection of RTX, mice were challenged with RTX. Mice were tolerant not only to the hyperalgesic effect produced by a second injection of RTX injected i.t. (Fig 7A), but also to the hyperalgesic effect of a systemic dose of RTX (.1 mg/kg) injected s.c. (Fig 7B).

Effect of RTX on Skeletal Muscle Afferents Expressing TRPV1 Receptor Immunoreactivity

To determine the effect of RTX on muscle afferents, mice were injected with RTX (.1 mg/kg s.c.) prior to or after injections of FG into the gastrocnemius muscle. In 1 group of animals, FG was injected 5 days before RTX (FG→TRX) to assess numbers of DRG (L3-L5) cells innervating muscle without the influence of RTX. In a second group of mice, FG was injected after RTX (RTX→FG) to test whether prior exposure to RTX affected the ability to detect DRG cells retrogradely labeled from muscle. When compared to vehicle-treated mice, the FG→RTX and the RTX→FG mice did not differ from each other in the total number of FG-labeled neurons, indicating that RTX did not produce its musculoskeletal hyperalgesia through degeneration of TRPV1-expressing neurons (Fig 8D). However, RTX downregulated TRPV1 receptor immunoreactivity in neurons innervating the gastrocnemius muscle by 35.5% as documented by the decrease in number of cells stained with both FG and TRPV1 receptor immunoreactivity in the group injected with RTX compared to those in vehicle-injected controls (Fig 8E).

Discussion

In healthy animals, TRPV1 receptors play a crucial role in thermal hyperalgesia[28,29,45,49] and in thermoregulation.[17,18] Following inflammation or nerve injury, they also mediate tactile mechanical hyperalgesia (von Frey fiber),[28,38,63,67,68] suggesting that these receptors are upregulated on injury.[2,37,72,73] Our results confirm the hypothermic effect of TRPV1 ligands, their antinociceptive effect in the tail flick assay, and their lack of effect on tactile mechanical sensitivity in healthy normal mice. In addition, we found a unique role for TRPV1 receptors in modulating musculoskeletal nociception as measured using the grip force assay in healthy normal mice. Specifically, administration of RTX induced a prolonged musculoskeletal hyperalgesia, even at a low dose that produced no thermal antinociception. These data document an important distinction between the modulation of mechanical nociception when measured using the von Frey fiber assay (tactile) compared to that when measured using the grip force assay (musculoskeletal). They also suggest that

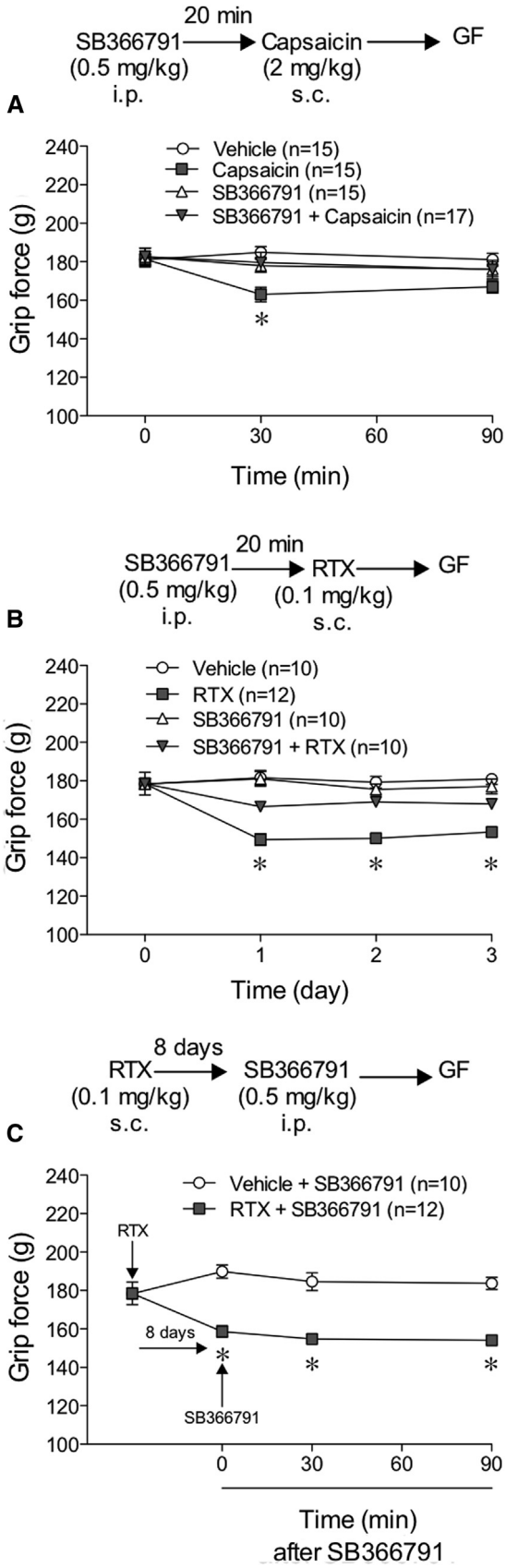


Figure 5. TRPV1 antagonist (SB-366791) prevents the musculoskeletal hyperalgesia produced by capsaicin and by RTX. Animals were injected with SB-366791 (.5 mg/kg i.p.) and 20 minutes later they were injected s.c. with either 2 mg/kg of capsaicin **(A)** or .1 mg/kg of RTX **(B)**. Grip force was measured 30 minutes after capsaicin and 24 hours after RTX. In panel **C**, mice were injected with SB-366791 at this same dose 8 days after the injection of RTX and grip force was measured at the times indicated. Statistical analyses were performed using a 2-way analysis of variance followed by Bonferroni post hoc analysis, where the * indicates $P < .05$ when compared to all the other values at that time.

muscle pain may be a side effect of some TRPV1 receptor ligands developed for their analgesic activity for cancer and inflammatory pain.

TRPV1 receptors are usually activated by low doses of capsaicin, resulting in thermal hyperalgesia and hypothermia,[18,56] whereas higher doses desensitize these sites.[35] In contrast, RTX may briefly activate TRPV1 receptors but then desensitize them for much longer periods of time.[59] Sometimes, RTX desensitization is so rapid that little to no receptor activation is observed in models of thermal nociception.[58] Our data indicate that the rate of TRPV1 receptor desensitization depends on its anatomic location and function. For example, TRPV1 sites on thermoregulatory afferents were extremely resistant to desensitization, requiring 3 to 4 parenteral injections of even high doses of RTX. In contrast, TRPV1 sites along thermal nociceptive pathways were very sensitive to desensitization, requiring just 1 injection of RTX to produce a protracted (>56 days) thermal antinociception. TRPV1 receptors along musculoskeletal pathways are also desensitized after a single injection of RTX; however, the initial activation of the receptor appears to be responsible for the protracted hyperalgesia.

In our hands, musculoskeletal hyperalgesia produced by RTX was prevented by prior desensitization or pharmacologic antagonism of TRPV1 receptors. However, once established, hyperalgesia was no longer sensitive to TRPV1 receptor antagonists, suggesting that the hyperalgesic effect is maintained by a mechanism that no longer requires TRPV1 receptor activity. When initiated by TRPV1 receptor activity, activation of extracellular signal-regulated kinases (ERK) is believed to participate in the transition from acute hyperalgesia to chronic hyperalgesia.[55,74] ERK is generally activated in cases of peripheral inflammation and nerve injury.[10,55,74] For example, the ability of TRPV1 receptors to initiate but not maintain chronic hyperalgesia is characteristic of a rodent model of pancreatitis[55] in which cerulein-induced pancreatic hyperalgesia is attenuated by a TRPV1 antagonist only within 3 weeks of cerulein treatment. The failure of the antagonist to reverse hyperalgesia thereafter was attributed to increased ERK phosphorylation leading to enhancing expression of other pronociceptive modulators like brain-derived neurotrophic factor,[46,47] prodynorphin, and NK-1 receptors[27] rather than TRPV1 receptors.[55] A similar mechanism may be responsible for TRPV1 receptor–induced hyperalgesia in muscle.

Muscle pain differs from cutaneous pain in that muscle pain is deep, dull, and poorly localized whereas cutaneous pain is more localized.[44] This difference could be attributed to the variation in their innervation by large and

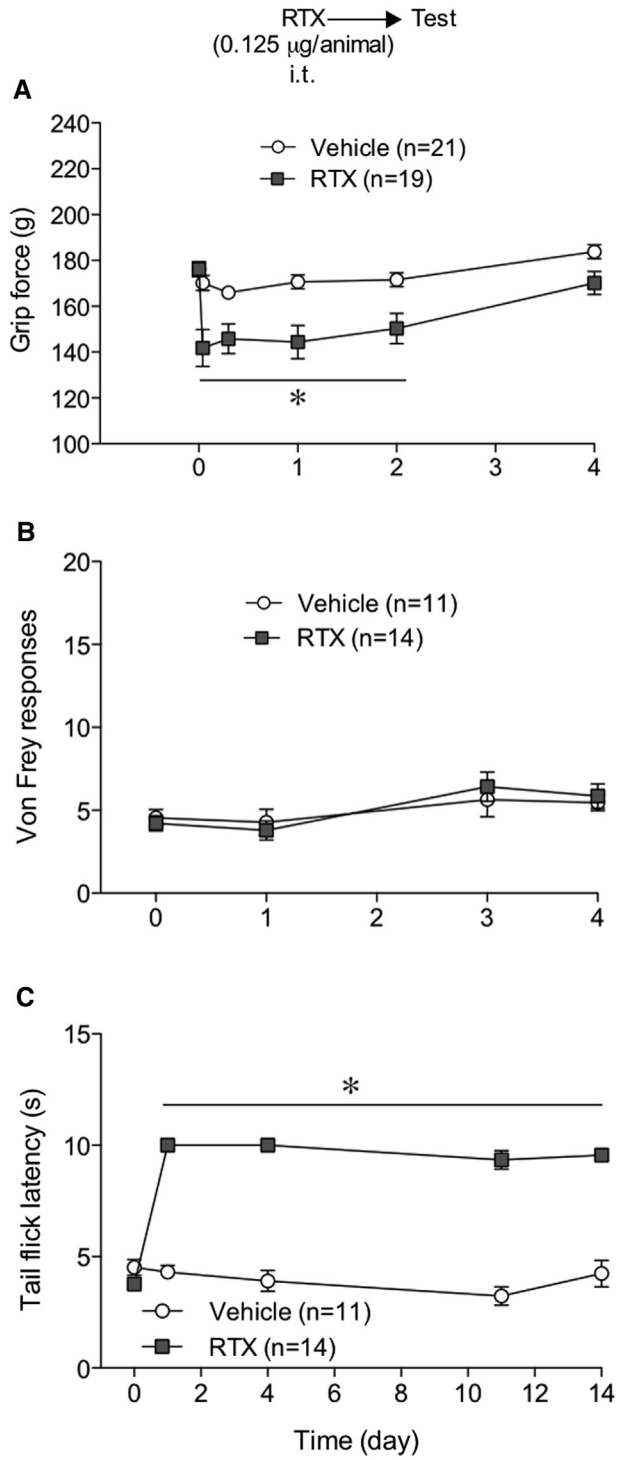


Figure 6. Intrathecal injection of RTX produced a transient (2-day) musculoskeletal hyperalgesia. Mice were injected i.t. with .125 μg/animal of RTX or vehicle, and grip force **(A)**, von Frey fiber **(B)**, and tail flick **(C)** responses were measured at the times indicated. Statistical analyses were performed using a 2-way analysis of variance followed by Bonferroni post hoc analysis, where the * indicates $P < .05$ when compared to vehicle controls measured at the same time.

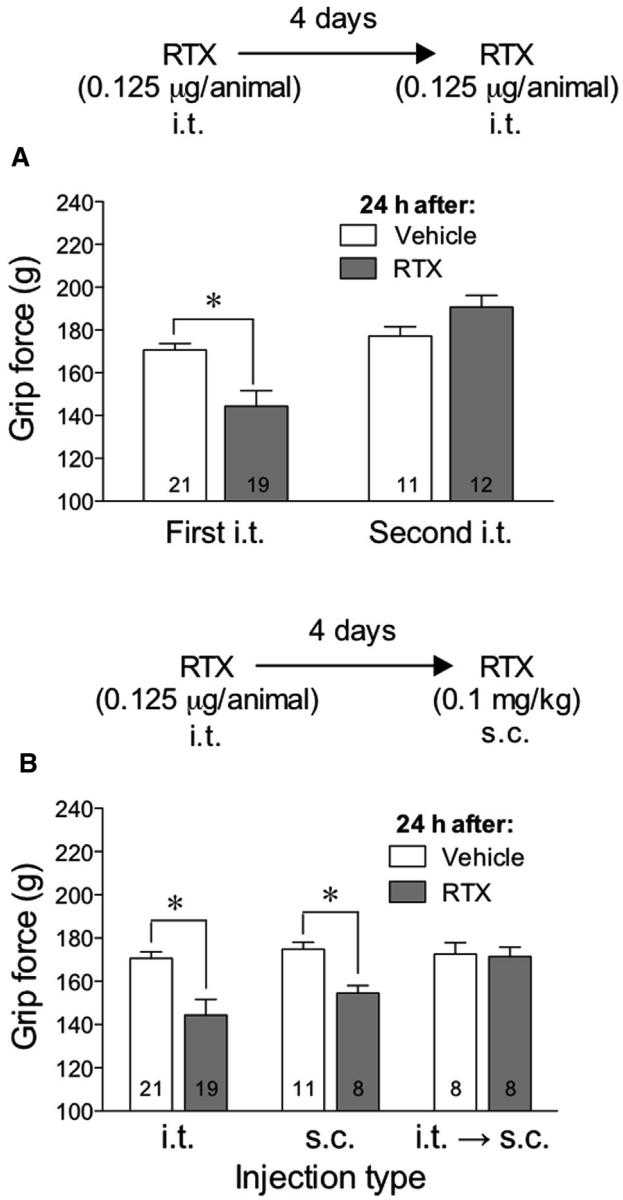


Figure 7. Intrathecal injection of RTX resulted in tolerance to the hyperalgesic effect of RTX injected i.t. or s.c. In panel **A**, mice were injected i.t. with .125 μg/animal of RTX or vehicle 4 days prior to a second challenge with the same i.t. dose of RTX or vehicle and grip force measured 24 hours after each injection. In panel **B**, mice were injected with .125 μg/animal of RTX i.t. and 4 days later challenged with .1 mg/kg of RTX or vehicle injected s.c. and grip force measured 24 hours later. Statistical analyses were performed using an unpaired Student t-test, where the * indicates $P < .05$ when compared to vehicle-injected controls.

small fibers. For instance, about 47% of afferents innervating muscles have myelinated axons compared to 24% of those innervating the skin.[11] Approximately 40% of afferents from muscle are TRPV1 receptor-immunopositive compared to an estimated 16% of afferents projecting to skin.[11] This may account for the fact that muscle afferents are twice as responsive to TRPV1 receptor activation than those from skin.[42] Furthermore, TRPV1 receptors innervating muscle are expressed by sensory neurons with larger soma compared to those innervating skin.[11] One might speculate that these differences in innervation may have contributed to the concurrent appearance of musculoskeletal hyperalgesia and thermal antinociception after administration of RTX. The uniqueness of afferents projecting to muscle and their associated TRPV1

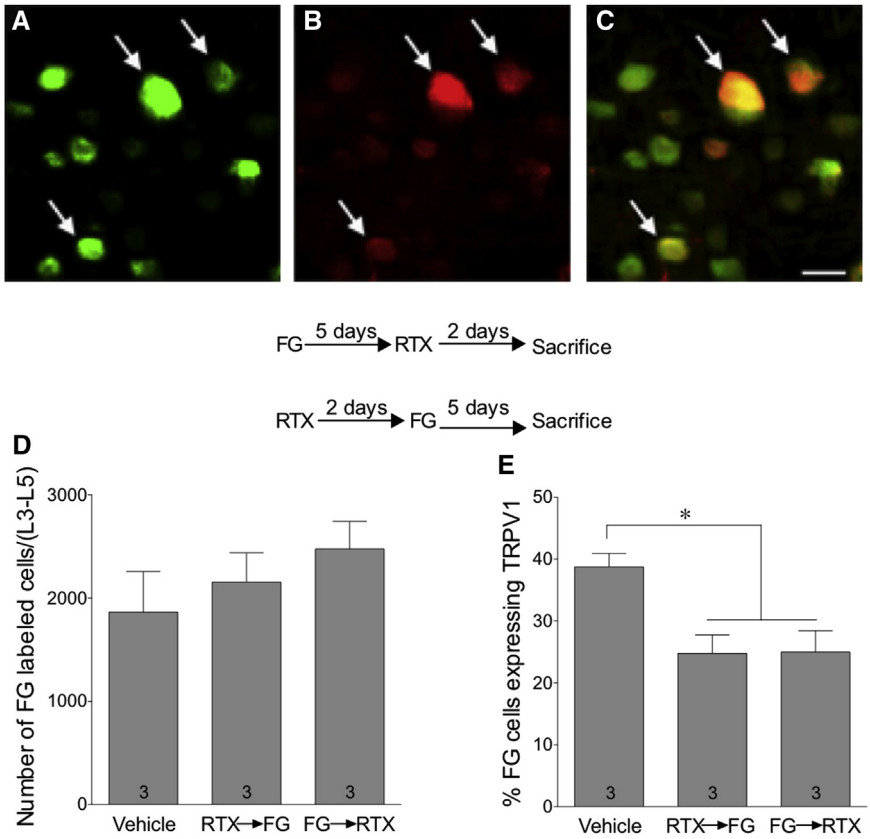


Figure 8. RTX decreased TRPV1 receptor immunoreactivity in afferents projecting to skeletal muscle without causing their degeneration. Mice were injected with FG 4% (10 μL in the gastrocnemius muscle) either 5 days before (FG → RTX) or 2 days after (RTX → FG) injection of RTX (.1 mg/kg s.c.). Representative pictures from an L4 DRG of a vehicle-injected mouse showing cells that are **(A)** retrogradely labeled with FG (green), **(B)** immunoreactive for TRPV1 receptors (red), **(C)** and double-labeled for both FG and TRPV1 receptors (yellow). Arrows indicate FG-labeled cells that are also immunopositive for TRPV1 receptors. Scale bar = 20 μm. The total number of FG-labeled projections in the DRG (L3-L5) was counted **(D)**, as well as the percentage of FG projections that were TRPV1 receptor immunopositive **(E)**. Statistical analyses were performed using a 1-way analysis of variance followed by Newman-Keuls post hoc analysis, where the * indicates $P < .05$ when compared to vehicle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

populations may explain the involvement of TRPV1 receptors in some types of musculoskeletal hyperalgesia, including cancer[19,29,32] and nerve injury,[38,63,67,68] but their lack of involvement in others, including carregeenan[26] and stress-induced hyperalgesia.[1]

The rate of desensitization of TRPV1 receptor populations may depend on the strategic survival advantage of maintaining their activity. For example, mammals tend to operate optimally at temperatures dangerously close to lethality.[54] For this reason, it is important to maintain normal thermoregulatory function, including sensitivity to increased ambient temperatures that may be deleterious to survival. Because the total absence of TRPV1 receptor activity leads to hyperthermia,[17,18] the most advantageous survival strategy is to suppress the development of tolerance to these ligands. Consistent with this, desensitization to the hypothermic effect of RTX required 3 parenteral injections of .1 mg/kg of RTX compared to the single injection that was sufficient to desensitize thermal nociceptors. One might speculate that the slow development of tolerance to RTX by TRPV1 sites in muscle may similarly reflect the importance of detecting muscle pain, helping avoid damage by overuse. The previously reported ability of capsazepine to abolish mechanical hyperalgesia produced by eccentric exercise[16] is in agreement with the role for TRPV1 receptor activity in muscle fatigue.

Histologically, RTX is known to reduce TRPV1 receptor immunoreactivity in lumbar DRGs.[3,9,24] However, it is unclear whether RTX destroys TRPV1 receptor–expressing afferents or simply downregulates these receptors in muscle. To address this, we traced muscle afferents innervating the gastrocnemius muscle using FG and then stained their cell bodies for TRPV1 receptors. We found that RTX did not affect the total number of afferent fibers projecting to muscles; however, RTX reduced the percentage of muscle afferent cells in DRGs expressing the TRPV1 receptor by 35.5% compared to vehicle-treated mice. This argues that RTX did not produce its musculoskeletal hyperalgesia via the degeneration of muscle afferents. Instead, the decrease in percentage of cells that were double stained suggests downregulation of TRPV1 receptors on these afferents. Still, it is unclear whether musculoskeletal hyperalgesia results from that desensitization or a persistent activation of the remaining TRPV1 receptors on these fibers. The lack of hyperalgesia after injection of

a TRPV1 antagonist and the temporal overlap between RTX-induced thermal antinociception and musculoskeletal hyperalgesia suggest that musculoskeletal hyperalgesia is due to a persistent activation of muscle nociceptors on which TRPV1 sites reside rather than to their desensitization.

Central TRPV1 receptors are important for pain transmission.[15,38] When RTX is injected i.t., it causes a long-lasting thermal antinociception in models of inflammatory[3,4,26] and cancer pain.[6,29] Central RTX also decreases TRPV1 receptor, calcitonin gene-related peptide, and substance P expression on nerve terminals in the spinal cord without affecting their expression in DRGs.[3,26] RTX also prevents activation of the ERK pathway in spinal cord–injured mice.[14] Because the receptor population appears to differ in the cord than in the periphery, we studied the effect of i.t. RTX on musculoskeletal hyperalgesia to see if spinal receptors respond differently to RTX than those in the periphery. Initially, RTX injected i.t. produced an acute (2 days) musculoskeletal hyperalgesia; however, when that subsided, mice were fully tolerant to the hyperalgesia produced by either central or systemic injections of RTX, indicating receptor desensitization. As a result of this shorter hyperalgesic episode, central targeting of TRPV1 receptors may provide a better method for pain therapy than parenteral injections, consistent with previous proposals.[3,6,15,26,28,38,57]

In conclusion, activation and desensitization of TRPV1 receptor populations on afferent fibers lead to different effects depending on their location and the type of tissue they innervate. In healthy normal control mice, tactile mechanical nociception is not mediated by TRPV1 activity whereas musculoskeletal nociception is enhanced by peripherally administered RTX, inducing a surprisingly protracted hyperalgesia. TRPV1 receptors in the spinal cord desensitize more rapidly than those in the periphery, minimizing the musculoskeletal hyperalgesia and making this a better route for clinical pain relief for these drugs.

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