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ANTIHYPERALGESIC EFFECT OF TETRODOTOXIN IN RAT MODELS OF PERSISTENT MUSCLE PAIN

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Abstract—Persistent muscle pain is a common and disabling symptom for which available treatments have limited efficacy. Since tetrodotoxin (TTX) displays a marked antinociceptive effect in models of persistent cutaneous pain, we tested its local antinociceptive effect in rat models of muscle pain induced by inflammation, ergonomic injury and chemotherapy-induced neuropathy. While local injection of TTX (0.03–1 μg) into the gastrocnemius muscle did not affect the mechanical nociceptive threshold in naïve rats, exposure to the inflammogen carrageenan produced a marked muscle mechanical hyperalgesia, which was dose-dependently inhibited by TTX. This antihyperalgesic effect was still significant at 24 h. TTX also displayed a robust antinociceptive effect on eccentric exercise-induced mechanical hyperalgesia in the gastrocnemius muscle, a model of ergonomic pain. Finally, TTX produced a small but significant inhibition of neuropathic muscle pain induced by systemic administration of the cancer chemotherapeutic agent oxaliplatin. These results indicate that TTX-sensitive sodium currents in nociceptors play a central role in diverse states of skeletal muscle nociceptive sensitization, supporting the suggestion that therapeutic interventions based on TTX may prove useful in the treatment of muscle pain.

Key words: mechanical hyperalgesia, inflammation, neuropathic muscle pain, delayed-onset muscle soreness, voltage-dependent sodium channels, clinical trials.

INTRODUCTION

For many decades tetrodotoxin (TTX), which is present in many poisonous animals including fishes from the *Tetraodontidae* family such as the pufferfish, has been used as a pharmacological tool to selectively block a subset of inward sodium currents (TTX-S I_{Na}) in electrophysiological recordings (Narahashi, 2008). Indeed, *in vitro* studies have shown that TTX is able to inhibit the conduction of action potentials in isolated nerve preparations (Muroi et al., 2011) and to block I_{Na} in neurons from sensory ganglia (Blair and Bean, 2002; Muroi et al., 2011). The current subsets identified by TTX have been demonstrated to depend on specific voltage-gated sodium channels (VGSC): TTX-sensitive (TTX-S) sodium channels, such as α-subunit of mammalian voltage-gated sodium channels (Nav) Nav1.1, Nav.1.3, Nav1.6 and Nav1.7 which are blocked by TTX at nanomolar concentrations, and TTX-resistant (TTX-R) sodium channels, such as Nav1.8 and Nav1.9 which are blocked by TTX only at micromolar concentrations (Dib-Hajj et al., 2009). This potent sodium channel block can explain the classical local symptoms of exposure to this toxin (e.g., *fugu* poisoning), including oral numbness, tingling and anesthesia (Bane et al., 2014; You et al., 2015). These properties are consistent with the strong antinociceptive effect exhibited by TTX in a number of *in vivo* pre-clinical (Lyu et al., 2000; Marcil et al., 2006; Nieto et al., 2008) and clinical (Hagen et al., 2008, 2011; Shi et al., 2009; Song et al., 2011) studies. Importantly, while the expression of VGSC varies between sensory neurons contributing to different pain symptoms (Minett et al., 2014), the antinociceptive effects of TTX have, however, been mainly studied in models of cutaneous pain.

While chronic muscle pain is an extremely common and disabling group of syndromes, which lack effective therapy, it has received much less attention than cutaneous pain. This is probably due to the fact that clinical entities related to chronic muscle pain, such as neuropathic muscle pain, are still not well characterized. Because of this scarcity of preclinical muscle pain models, most of the preclinical screening of new analgesic drugs is performed in models assessing cutaneous nociception. TTX-S VGSC have been reported to be present in dorsal root ganglion (DRG) nociceptors innervating the skeletal muscle (Ramachandra et al., 2012; Ramachandra and Elmslie, 2014), and nociceptive spinal monosynaptic reflexes are attenuated after exposure of sensory fibers innervating the skeletal muscle to TTX as observed in *in vivo* preparations (Schomburg et al., 2012). Furthermore, large-diameter sensory neurons, likely innervating the skeletal muscle, exhibit *de novo* expression of TTX-S VGSC after

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Abbreviations: D-PBS, Dulbecco's phosphate-buffered saline; DRG, dorsal root ganglion; ERK, extracellular regulated kinase; IL-6, interleukin 6; I_{Na}, inward sodium currents; mN, milli Newton; Nav, α-subunit of mammalian voltage-gated sodium channels; PKC, protein kinase C; TTX, tetrodotoxin; TTX-R, tetrodotoxin resistant; TTX-S, tetrodotoxin sensitive; VGSC, voltage-gated sodium channels.

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spinal nerve injury (Fukuoka et al., 2015). However, whether TTX is able to produce antinociceptive effects in models of persistent muscle pain remains to be determined. Thus, given the clinical and societal importance of persistent muscle pain and the promising profile of TTX as a putative analgesic, we explored its antinociceptive effects in models of nociceptive inflammatory, ergonomic and neuropathic muscle pain.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (initial weight 250–300 g; Charles River, Hollister, CA, USA) were used in these experiments. They were housed in the Laboratory Animal Resource Center facility at the University of California San Francisco, under environmentally controlled conditions (lights on 07:00–19:00 h; room temperature 21–23 °C) with food and water available *ad libitum*. Upon completion of experiments, rats were euthanized by CO_2 induced asphyxia followed by bilateral thoracotomy. Animal care and use conformed to NIH guidelines (NIH Guide for the Care and Use of Laboratory Animals) and to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. The University of California San Francisco Institutional Animal Care and Use Committee approved all experimental protocols. Concerted effort was made to minimize number and suffering of experimental animals.

Muscle inflammatory hyperalgesia

Rats were briefly anaesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals, St. Joseph, MO, USA) in 97.5% O_2. After the hair on hind limbs was clipped and disinfected with 70% isopropyl alcohol, carrageenan (λ-carrageenan, 1% in NaCl 0.9%) was injected into the belly of the gastrocnemius muscle, using a ½" 27G needle attached to an 100 μl microsyringe (Gastight®, Hamilton, Reno, NV, USA). The 100 μg/10 μl dose of carrageenan was determined in previous studies as sufficient to produce robust mechanical hyperalgesia in the rat's gastrocnemius muscle (Dina et al., 2008a, b).

Eccentric exercise-induced muscle hyperalgesia

The method used to eccentrically exercise the rat hind limb has been described previously (Kano et al., 2004; Taguchi et al., 2005; Alvarez et al., 2010). Briefly, isoflurane-anesthetized rats were placed in supine position on a heating pad (set to maintain core body temperature at 37 °C), and ophthalmic ointment applied to prevent corneal dehydration. The right hind paw was affixed to the foot bracket of a rodent exercise apparatus (Model RU-72, NEC Medical Systems, Tokyo, Japan) with 3 M Micropore® surgical paper tape, such that the angle of the knee and ankle joints was ~90° (paw 30° from vertical). The gastrocnemius muscle was stimulated via subcutaneous needle-type electrodes attached to a Model DPS-07 stimulator (Dia Medical System Inc., Tokyo, Japan) that delivered trains of rectangular electric pulses (100 Hz, 700 ms, 3 V) every 3 s, to give a total of 300 contractions. During these stimulus-induced contractions of the gastrocnemius muscle, the electromotor system rotated the foot to produce an extension of this muscle.

Oxaliplatin-induced neuropathic muscle pain

As previously reported (Joseph and Levine, 2009; Alvarez et al., 2011), oxaliplatin was freshly dissolved in normal saline at a concentration of 2 mg/ml and immediately administered by intravenous route (1 ml/kg) to isoflurane-anesthetized rats.

Drugs

Unless otherwise stated, all chemicals used in these experiments were obtained from Sigma–Aldrich (St. Louis, MO, USA). The stock solution of TTX (Abcam, Cambridge, MA, USA) was made by dissolving it in distilled water (1 μg/μl) and stored at −20 °C; further dilutions were made in Dulbecco's phosphate-buffered saline (D-PBS). Rats were briefly anesthetized with 2.5% isoflurane to facilitate the intramuscular injections (20 μl) of TTX or its vehicle (D-PBS) into the gastrocnemius muscle. The injection site was previously shaved and scrubbed with alcohol. Immediately after injection the skin puncture site was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold of the underlying injection site in the muscle could be repeatedly tested.

Testing of mechanical nociception

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a digital force transducer (Chatillon DFI2; Amtek Inc., Largo, FL, USA) with a custom-made 7-mm diameter probe. This width of the probe allows for selective evaluation of muscle pain (*vis-à-vis* overlying skin pain) (Alvarez et al., 2010). Rats were lightly restrained in a cylindrical acrylic holder with lateral slats that allow for easy access to the hind limb and application of the force transducer probe to the injection site in the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in milli Newton (mN), required to produce a flexion withdrawal reflex in the hind leg. Baseline withdrawal threshold was defined as the mean of 3 readings taken at 5-min intervals and magnitude of hyperalgesia calculated as percentage decrease from the baseline mechanical nociceptive withdrawal threshold.

Statistics

Group data are expressed as mean ± SEM of n independent observations. Statistical comparisons were made using GraphPad Prism 5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons were made by means of a one-or two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. A P value < 0.05 was considered statistically significant.