Antinociceptive effects of tetrodotoxin (TTX) in rodents

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Background. Tetrodotoxin (TTX) is a powerful sodium channel blocker extracted from the puffer fish. The analgesic effects of TTX were investigated in different animal pain models.

Methods. Wistar rats were submitted to the formalin test and to partial ligation of the sciatic nerve (Seltzer’s model). Swiss Webster mice were used in the writhing test. Rodents were divided into six groups receiving a s.c. injection of either 0.9% NaCl, TTX 0.3, 1, 3, or 6 μg kg⁻¹, or morphine (5 mg kg⁻¹). Substances were injected 30 min before 2.5% formalin injection into the hind paw, acetic acid administration intraperitoneally or neuropathic pain testing consisting of mechanical allodynia (von Frey filament) and thermal hyperalgesia (Plantar test).

Results. TTX decreased pain behaviour in the formalin test at the highest dose and in the writhing test at 3 and 6 μg kg⁻¹. It also diminished mechanical allodynia and thermal hyperalgesia with an ED₅₀ of 1.08 (0.89) and 0.62 (0.33) μg kg⁻¹, respectively. Observation of the rats after TTX injection did not show any motor deficit, respiratory distress or sedation. Morphine was also effective in relieving pain in all three tests but with signs of considerable sedation.

Conclusion. Systemic injections of TTX diminished pain behaviour in a dose-dependent manner in models of inflammatory, visceral and neuropathic pain without causing adverse events, whereas morphine analgesia was associated with heavy sedation. TTX is a very promising substance for the treatment of various types of pain but needs further evaluation.

Pain is the most common symptom encountered in clinical practice. The use of currently available drugs to treat pain is often associated with important side-effects and poor response in certain conditions. The development of new drugs to treat pain in patients, using new mechanisms of action is therefore essential in order to improve quality of life and decrease suffering.

Tetrodotoxin (TTX) is among the deadliest poisons known to man, and can be isolated from many tissues (liver, intestines, gonads, skin, kidney, blood) of the puffer fish or fugu (family of Tetraodontidae). It is also a reversible selective inhibitor of sodium channel conductance.¹² TTX is the most potent of the substances that can interfere with the production of action potentials and it blocks propagation of impulses in excitable membranes. It has a steep dose–response relationship and its lethal dose LD₅₀ after s.c. injection in mice has been estimated to be 10 μg kg⁻¹.¹

Pharmacokinetic studies have shown that TTX injected s.c. reaches a peak plasma concentration after 20 min with an elimination half-life varying from 30 min in the heart to 3–4 h in the kidneys and liver.¹ Only one study has been performed in animals to test TTX antinociceptive properties in neuropathic pain conditions.³ The authors showed that topical TTX applied to dorsal root ganglion neurones was able to decrease mechanical allodynia. Because of its adverse effects and toxicity such as

1 These authors contributed equally to this work.

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numbness, nausea, vomiting and paralysis, TTX was not tested in clinical practice, although a recent report suggested that it is analgesic in patients with refractory cancer pain. TTX is commonly used in many laboratories to characterize sodium channels and to study their role in normal physiology and disease and to characterize the type of sodium channels implicated in the molecular mechanisms of pain. For a recent classification of sodium channels and their role in hyperalgesia, see Ref. 5. As a potent sodium channel blocker, TTX also acts as a local anaesthetic and therefore inhibits the nociceptive pathway. Taking into account the similarities concerning the mechanism of action of TTX and other medications used in the treatment of neuropathic pain, and the fact that sodium channels are involved in the pathophysiology of inflammatory and visceral pain, we hypothesized that TTX exerts antinociceptive effects and decreases pain behaviour in animal pain models. Moreover, we speculated that there is a dose-dependent effect and that no major adverse effect could result from well-chosen doses based on the literature.

In order to assess the role of TTX in different pain conditions, we therefore decided to test its antinociceptive effects in inflammatory, visceral and neuropathic models of pain.

Materials and methods

Animals

This research protocol was approved by the Animal Ethics Committee of the Université de Montréal and all procedures conformed to the guidelines of the Canadian Council for Animal Care. Male Wistar rats and Swiss Webster mice (Charles River, St-Constant, Québec, Canada), 170–220 and 20–30 g, respectively, at the time of testing were housed three per cage with a bedding of wood sawdust (8–16 mm) (Pro Chip, Canada) and maintained on a 12 h dark–light cycle with free access to food (18.0% protein, 4.5% fat, 5.5% fibre, 7.0% ash, 2.5% added minerals) (Charles River, Canada) and water. During periods of testing, rodents were brought to the testing room 1 day before testing and kept there in natural daylight.

Drug administration

TTX was a gift from International Wex Technologies Inc. (Vancouver, Canada) and was supplied as a solution containing 15 μg ml⁻¹. Morphine sulphate was given by the CHUM pharmacy (solution of 10 mg ml⁻¹). All dilutions were made in 0.9% NaCl in such a way that each animal received the same volume of solution depending on their weight. Normal saline, TTX and morphine were all administered s.c. on the animals’ backs. Each block of experiments was conducted in a randomized and blinded manner by the same experimenter.

Formalin test

The formalin test is a well-established rat model of persistent somatic pain that was refined by Tjølsen and colleagues and Watson and colleagues. Two phases of the response are observed during the formalin test: an early phase starting immediately after injection and lasting for 5–15 min and a later phase 20–50 min after injection. While the stimulus for the early phase is a direct chemical stimulation of nociceptors, that for the later phase involves inflammation.

Animals were acclimatized to the testing environment (clear Plexiglas box) for 15 min or until explorative behaviour ceased. Care was taken while handling the animals in order to minimize stress. Fifty microlitres of 2.5% formalin was administered s.c., using a 27 gauge needle, into the dorsum of the right hind paw. After this injection, the animal was returned to the chamber, and its subsequent nociceptive behaviour observed. A mirror angled at 45° below the observation chamber provided the experimenter with an unobstructed view of the injected paw. Observation of the animal’s behaviour was made in consecutive 5 min periods for 60 min after formalin injection. The total time the animal spent in three distinct behavioural categories in each 5 min period were recorded: (i) injected paw has little or no weight on it; (ii) injected paw is elevated, not in contact with any surface; (iii) injected paw is licked, bitten or shaken. Nociceptive behaviour was quantified using the composite pain score–weighted scores technique (CPS–WST) as proposed by Watson and colleagues where behaviour (i) is discarded, behaviour (ii) weighted times one, and behaviour (iii) weighted times two. In addition, an overall CPS–WST was calculated for the first (0–15 min) and second (20–50 min) phases of the behavioural response. The animals were culled at the end of each experiment.

Experimental formalin groups

All animals (n=10 per group) received a s.c. injection 30 min before the formalin test of either 0.9% saline (control group), TTX at 0.3, 1, 3 or 6 μg kg⁻¹, or morphine at 5 mg kg⁻¹.

Writhing test

The writhing test is a widely used animal model of visceral pain consisting of intraperitoneal (i.p.) injection of an irritant that induces a syndrome called ‘writhing’, which consists of contractions of the abdomen, twisting and turning of the trunk, arching of the back and extension of the hind limbs.

Mice were acclimatized to the testing environment (clear Plexiglas box) for 15 min or until explorative behaviour ceased. Care was taken while handling the animals in order to minimize stress. Animals were allocated to different experimental groups (see below). For nociceptive testing, mice were injected i.p., using a 27 gauge needle, in the left lower quadrant of the abdomen with 0.1 ml per 10 g of body weight of a 0.6% acetic acid solution. After injection, the animals were returned to the chamber and their subsequent nociceptive behaviour observed: contractions of abdominal
musculature (writhes) were counted between 5 and 15 min after the injection. Mice were used once and then killed immediately.

**Experimental writhing groups**

All animals (n=10 per group) received a s.c. injection 30 min before i.p. acetic acid injection of either 0.9% saline (control group), TTX at 0.3, 1, 3 or 6 μg kg⁻¹, or morphine at 5 mg kg⁻¹.

**Neuropathic pain model**

Unilateral hind-limb neuropathy was achieved using the technique of partial sciatic nerve ligation (PNL) described by Seltzer and colleagues. Briefly, under isoflurane anaesthesia (1–2%) and aseptic conditions, the right sciatic nerve was exposed at high-thigh level and 1/3–1/2 of the dorsal thickness of the nerve was trapped in the ligature using an 8-0 monofilament nylon suture. The wound was then closed with 4-0 absorbable suture for the muscles and two staples for the skin. During sensory testing, animals were placed in elevated Plexiglas boxes (21x17x14 cm) with a 0.7 cm diameter mesh floor to test mechanical allodynia and a dry glass floor was used for thermal hyperalgesia. Rats were allowed to acclimatize for 15 min or until exploratory behaviour ceased. The time to complete one battery of tests was approximately 40 min.

**Mechanical allodynia**

Mechanical allodynia was evaluated with von Frey hairs (Senselab aesthesiometer, Somedic, Sweden). The plantar surface of the paw was stimulated with a series of von Frey filaments of ascending forces (with a range that comprised between 0.06 and 23.96 g). For each filament, the stimulus was repeated five times with an interval of 1–2 s between each stimulation. The threshold was determined as the lower force that evoked a withdrawal response to one of the five stimuli.

**Thermal hyperalgesia**

Thermal hyperalgesia was assessed using an infrared noxious heat stimulus (Plantar test, Ugo Basile, Italy). The centre of a focused beam of radiant heat was applied to the plantar surface of the hind paw and the withdrawal latency time recorded. Results of each test are expressed as the mean of three withdrawal latencies (s). Three minutes was allowed between each test.

**Protocol for neuropathic pain experiments**

After surgery, the rats were allowed 11 days to recover. On the 12th day, rats were tested to verify that mechanical allodynia and thermal hyperalgesia were present and, on the 13th day, drugs were given s.c. 30 min before neuropathic testing. Mechanical allodynia and thermal hyperalgesia were evaluated in 36 rats allocated to six different groups: (i) 0.9% NaCl; (ii) TTX 0.3 μg kg⁻¹; (iii) TTX 1 μg kg⁻¹; (iv) TTX 3 μg kg⁻¹; (v) TTX 6 μg kg⁻¹; or (vi) morphine (5 mg kg⁻¹).

**Safety and adverse effects**

Due to a theoretical narrow therapeutic index and the known lethal effects of TTX at high doses, adverse effects were carefully monitored and recorded during pain tests. In particular, special attention was given to monitor signs of muscle paralysis (hind-limb or trunk paralysis), respiratory distress, sedation, or any abnormal or unusual animal behaviour.

**Statistical analysis**

All comparisons were performed using SigmaStat, Jandel Scientific Software, version 1.0. Data are expressed as mean (SEM). The overall CPS–WST₀,₁,₂ for the first and second phases of the behavioural responses in the formalin test and the number of writhes was compared using an ANOVA with Bonferroni test as post-hoc analysis. Assessment of neuropathy and the analgesic effects of TTX and morphine were evaluated using an ANOVA adapted for factorial experimental design. Each of the ANOVA mentioned above was assessed for mechanical allodynia and for thermal hyperalgesia on the ipsilateral side. The contralateral side was analysed separately. Dose–response curves and ED₅₀ values were determined using ALLFIT software. The critical level of significance was set at 5% (P<0.05).

**Results**

**Formalin test**

In the formalin test (Fig. 1A), only morphine was antinociceptive in the acute phase of the test whereas TTX was not at any of the concentrations tested (Fig. 1B). Furthermore, morphine and TTX at doses of 6 μg kg⁻¹ significantly attenuated pain behaviour in the inflammatory phase of the formalin test (Fig. 1C). However, at 3 μg kg⁻¹ of TTX, overall pain scores were less than the control group, yet failed to reach significance. The ED₅₀ for TTX was 3.0 μg kg⁻¹ for the formalin test.

**Writhing test**

TTX at doses of 3 and 6 μg kg⁻¹ and morphine produced significant antinociceptive effects in mice (Fig. 2). At a dose of 1 μg kg⁻¹, TTX reduced the number of abdominal contractions but this was not significant. The ED₅₀ for TTX was 0.94 μg kg⁻¹ for the writhing test.

**Neuropathic pain**

Animals tested 12 days after surgery showed a significant decrease in pain threshold compared with preoperative values for mechanical allostodynia and thermal hyperalgesia.
Mechanical allodynia

Von Frey values obtained in neuropathic conditions (ipsilateral paw) were not statistically different from those measured when rats received 0.9% NaCl or TTX 0.3 \( \mu g \text{kg}^{-1} \) (Fig. 3). However, TTX at 1, 3 and 6 \( \mu g \text{kg}^{-1} \), and morphine produced a significant antinociceptive effect. Moreover, pain scores when TTX was administered at 3 and 6 \( \mu g \text{kg}^{-1} \) returned to preoperative levels and for morphine reached a level even higher than before surgery. The antinociceptive effect of TTX was therefore dose-dependent with an \( ED_{50} \) of 1.08 (0.89) \( \mu g \text{kg}^{-1} \).

For the contralateral paw, there was no statistical difference in mechanical allodynia testing between the day before surgery (pre-lesion baseline) and the days after surgery (days 12 and 13) except after the injection of morphine which produced a significantly higher level compared with that observed before surgery (Fig. 3, upper).

**Thermal hyperalgesia**

Plantar test values obtained in neuropathic conditions were not statistically different from those measured when rats received 0.9% NaCl, TTX 0.3 and 1 \( \mu g \text{kg}^{-1} \) (Fig. 4). TTX administered at doses of 3 and 6 \( \mu g \text{kg}^{-1} \), and morphine produced a significant antinociceptive effect. Moreover, pain scores when TTX was administered at 3 and 6 \( \mu g \text{kg}^{-1} \) returned to preoperative levels and for morphine reached a level even higher than before surgery. The antinociceptive effect of TTX was therefore dose-dependent with an \( ED_{50} \) of 0.62 (0.33) \( \mu g \text{kg}^{-1} \).
For the contralateral paw, there was no statistical difference in thermal hyperalgesia between the day before surgery and the days after surgery (days 12 and 13) except after morphine administration which produced a significantly higher threshold compared with that observed before surgery (Fig. 4, upper).

Safety and adverse effects

For all the pain experiments reported in this study, no animal showed signs of respiratory distress or displayed symptoms of motor impairment or paralysis. However, marked sedation was observed in the morphine group whereas there was no difference between saline and TTX groups even at high doses. No experiment had to be stopped because of animal distress.

Discussion

TTX, a powerful and lethal poison is a sodium channel blocker. TTX has been used extensively to characterize sodium channels and to study their role in normal physiology and disease. In this study, we showed for the first time that TTX possesses dose-dependent antinociceptive properties when administered systemically to rodents. These effects were not associated with animal distress or paralysis at the doses used. These antinociceptive effects were found in the inflammatory, visceral and neuropathic animal models of pain.

In this study, TTX was most effective in neuropathic pain conditions where it significantly reduced mechanical allodynia and thermal hyperalgesia. This is not surprising because of its effect on sodium channels. Functionally, sodium channels can be divided into TTX-sensitive and TTX-resistant channels based on their sensitivity to TTX. The precise mechanism of neuropathic pain is unknown but among several theories, the involvement of sodium channels such as Na\textsubscript{v}1.3\textsuperscript{17} and Na\textsubscript{v}1.8\textsuperscript{18,19} was recently noted. These sodium channels could be implicated in the spontaneous firing of the injured neuropathic nerves. Moreover, TTX could effectively block Na\textsubscript{v}1.3 which is TTX-sensitive but probably not Na\textsubscript{v}1.8 (unless at high concentrations), a TTX-resistant sodium channel preferentially expressed in small-sized nociceptive dorsal root ganglion neurones.\textsuperscript{6} Both channels seem to be implicated in neuropathic pain. In the Seltzer model of neuropathic pain, examining changes in expression and function of TTX-resistant and -sensitive sodium channels (by measuring current density in dorsal root ganglion neurones cultured from the treated animals) it was shown that Na\textsubscript{v}1.3 sodium channels were increased whereas Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 were not.\textsuperscript{17} Therefore, the
The mechanism of action of TTX in this study, where the same animal model of neuropathic pain was used, can be explained by these findings, that is, action on Nav1.3 sodium channels.

The hypothesis that accumulation of TTX-sensitive sodium channels in injured neurones plays a critical role in the generation of ectopic discharges and leads to mechanical allodynia has already been tested in a previous study. Using the spinal nerve ligation model of neuropathic pain in rats, the authors showed that topical TTX (but not systemic at similar dose) in the dorsal root ganglia at nM concentrations reduced mechanical allodynia and they suggested therefore, that TTX-sensitive subtypes of sodium channels play an important role in maintaining allodynia. Interestingly, TTX action was not associated with conduction blockade of action potentials in the dorsal root ganglia. Therefore, the actual mechanism of action of TTX needs to be further evaluated at the molecular level.

The formalin test is a commonly used animal model of tonic and inflammatory pain. In the formalin test, an interesting aspect is that two different types of stimulus are used in the same test to study possibly varying antinociceptive effects of a drug in the two phases of the test. It may also be claimed that this test is a better model of clinical pain than the hot-plate and tail-flick tests. In this test, TTX had no effect in the first phase (acute pain) but displayed a significant analgesic effect on inflammatory pain (second phase) at high doses. The mechanisms involved in inflammatory pain are not completely understood but it has been shown that sodium channel immunoreactivity within primary sensory neurones is dramatically increased. Furthermore, in a rat model of inflammatory pain, Na1.8 expression in dorsal root ganglion neurones increased along with an increase in TTX-resistant sodium current amplitude. Therefore, antinociceptive effects of TTX may not be completely explained by action on these sodium channels.

The writhing test is a well-known animal model of inflammatory and visceral pain. It is useful in studying possible antinociceptive effects of a compound. However, its main disadvantage is its lack of specificity, as many drugs without certain antinociceptive effects can effectively inhibit the writhing response. TTX was able to decrease visceral pain dose-dependently without any side-effects. The mechanism of action of TTX in visceral pain is difficult to explain. Indeed, it has been shown that Na1.8, a TTX-resistant sodium channel, was involved in the activation of afferent nerves after chemical irritation of the bladder, in a rat model of visceral pain. Plasticity in TTX-resistant and TTX-sensitive sodium channels might

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**Fig 4** Thermal hyperalgesia evaluation in rats using the plantar test on the ipsilateral (operated paw, main figure) and contralateral (upper figure) paws the day before surgery (J0), 12 days after surgery (J12) and on the 13th day (treatment) when animals received a s.c. injection of 0.9% NaCl, TTX at 0.3, 1, 3 or 6 μg kg⁻¹, or morphine (5 mg kg⁻¹). Data are expressed as mean (SEM) (n=6). *P<0.05 for treatment with 0.9% NaCl vs different treatments.
Contribute to sensory and reflex changes in various pathological conditions and therefore, from the results obtained in the formalin and writhing tests, it is suggested that TTX acted on some TTX-sensitive sodium channels in order to be analgesic. Indeed, Na$_{v}$.1.7 sodium channel is TTX-sensitive and present in virtually all dorsal root ganglion neurones.

To date, TTX has not been used systemically to treat pain probably because of the risk of potentially dramatic adverse effects (paralysis, respiratory distress and even death). However, it seems to be safe if concentrations are kept below 10 $\mu$g kg$^{-1}$ in rodents.

Although TTX is a potent neurotoxin, it has been used safely and effectively for severe refractory cancer pain. Indeed, an interim analysis of the results of a phase IIa trial in 22 subjects receiving escalating doses of 15, 30, 45 or 60 $\mu$g of TTX i.m. over four consecutive days showed that 17 (68%) of the 25 TTX treatments administered elicited a full or partial analgesic response. Furthermore, the safety of i.m. TTX in this group of patients suffering from inadequately controlled severe cancer pain was reported recently, although in abstract form. The results from this trial showed that adverse events consisted of transient perioral numbness or tingling and nausea. Ataxia, which resolved spontaneously, was reported in two patients. TTX, now administered s.c., is undergoing a phase IIb/III clinical trial with Health Canada.

In our animal study, TTX displayed ED$_{50}$S close to 1 $\mu$g kg$^{-1}$ in all pain tests except for the formalin test where the ED$_{50}$ was 3.0 $\mu$g kg$^{-1}$. This latter dose is closer to the LD$_{50}$ dose reported in the literature and in preliminary studies [11.1 (10.50–11.70) $\mu$g kg$^{-1}$ (95% CI) in rats after i.m. injection and 9.0 (8.2–9.8) $\mu$g kg$^{-1}$ (95% CI) in mice after i.p. injection].

From results obtained in the three pain tests presented in this study, it can be suggested that morphine, was administered at too high doses. Indeed, in the formalin test, morphine pain scores were excessively low and the animals were mostly asleep and moving little whereas in the TTX groups, animals moved freely throughout the experiment. The same was true for the writhing test. Finally in neuropathic pain conditions, the administration of morphine led to a significant increase in pain threshold markedly above preoperative values. Moreover, this was also the case for the contralateral paw, indicating excessive morphine dose.

In conclusion, TTX provides dose-dependent analgesia in inflammatory, visceral and neuropathic pain conditions in rodents, without displaying adverse effects such as sedation, respiratory distress or paralysis at the doses used. The applicability of TTX to human pain is currently being tested.

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