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Profound reduction of somatic and visceral pain in mice by intrathecal administration of the anti-migraine drug, sumatriptan

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Abstract

Sumatriptan and the other triptan drugs target the serotonin receptor subtypes 1B, 1D, and 1F (5-HT_{1B/D/F}), and are prescribed widely in the treatment of migraine. An anti-migraine action of triptans has been postulated at multiple targets, within the brain and at both the central and peripheral terminals of trigeminal “pain-sensory” fibers. However, as triptan receptors are also located on “pain-sensory” afferents throughout the body, it is surprising that triptans only reduce migraine pain in humans, and experimental cranial pain in animals. Here we tested the hypothesis that sumatriptan can indeed reduce non-cranial, somatic and visceral pain in behavioral models in mice. Because sumatriptan must cross the blood brain barrier to reach somatic afferent terminals in the spinal cord, we compared systemic to direct spinal (intrathecal) sumatriptan. Acute nociceptive thresholds were not altered by sumatriptan pre-treatment, regardless of route. However, in behavioral models of persistent inflammatory pain, we found a profound anti-hyperalgesic action of intrathecal, but not systemic, sumatriptan. By contrast, sumatriptan was completely ineffective in an experimental model of neuropathic pain. The pronounced activity of intrathecal sumatriptan against inflammatory pain in mice raises the possibility that there is a wider spectrum of therapeutic indications for triptans beyond headache.

Keywords

Migraine; Headache; Inflammation; Serotonin; Sumatriptan; Intrathecal; Blood brain barrier

1. Introduction

The triptans, which target the serotonin receptor subtypes 1B, 1D, and 1F (5-HT_{1B/D/F}), are thought to exert their anti-migraine effects at multiple neural circuits that transmit “pain” messages. Thus triptans may activate pain-inhibitory control mechanisms within the brain [4], as well as inhibit the activation of trigeminal sensory neurons, by regulating cerebral blood flow in the periphery and reducing the release of “pain” neurotransmitters centrally [8,12,16,23,25,28,31,38]. The latter hypothesis is of particular interest because triptan receptor expression is widespread, found equally among trigeminal sensory afferents and afferents that innervate the rest of the body [37,44]. As this nonselective distribution is at odds with the

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prevailing view that triptans have selective actions for migraine/cranial pain [4,17,29], we explored the possibility of an analgesic action of sumatriptan on non-cranial pain, independent of the pain of headache.

We and others previously reported the predominant expression of the 5-HT_{1D} receptor subtype within a peptidergic sub-population of “pain-sensory” primary afferent nociceptors [37,41], which is consistent with a critical contribution of this receptor to the regulation of pain by triptans. The subcellular localization of 5-HT_{1D} receptors is of particular interest, because the receptor is undetectable at the plasma membrane of nociceptor terminals in the spinal cord dorsal horn. Rather, the receptors are concentrated within dense core vesicles (DCVs) of these synaptic terminals [37]. The pattern of expression parallels to that of the δ -opioid receptor [10,35,42], which is not only sequestered within DCVs in the spinal cord dorsal horn [14], but redistributes to the cell surface upon stimulation [3,9].

We studied the effects of systemic (subcutaneous; SC) or direct spinal (intrathecal; IT) injection of sumatriptan in behavioral models of both acute and chronic pain. Here we provide evidence that appropriate targeting of triptans can in fact generate profound relief of pain other than that associated with migraine.

2. Methods

We used wild type CD1 male mice (20–30 g), housed in a 12-h light–dark cycle. All experiments were approved by our Institutional Animal Care and Use Committee, and comply with the recommendations of the International Association for the Study of Pain. Experiments were performed during the day by the same experimenter in a temperature and humidity controlled environment.

We diluted sumatriptan succinate, 12 mg/ml (Glaxo-SmithKline) in preservative-free saline for injection in a suitable volume. For systemic administration, SC sumatriptan was given at 300 and 600 μ g/kg. The lower systemic dose is sufficient to inhibit neurogenic plasma extravasation produced by electrical stimulation of the trigeminal ganglion [8], or by hindpaw injection of Complete Freund’s Adjuvant (CFA) or capsaicin [12]. The systemic doses used in this study thus well-exceed the amount of drug required for the inhibition of peripheral afferent activation [8,21,23,25,31,38]. In fact, Kayser and colleagues found that this was an effective dose for normalizing mechanical hyperalgesia after chronic constriction of the infraorbital nerve [29]. For localized injection to the CNS, we administered sumatriptan intrathecally, at 0.006, 0.02, 0.06, or 0.6 μ g in a total volume of 5.0 μ l. The effective intrathecal dose of 0.06 μ g is approximately 1/100th of the 300 μ g/kg systemic dose, so that any observed effect is almost certainly via a central target. IT injections were performed with a 30 gauge, 1/2-inch needle at the L4-5 lumbar interspace on lightly restrained, unanesthetized mice [19]. Animals that exhibit motor impairments after the injection were excluded from study. For all drug tests, nociceptive thresholds were measured immediately prior to as well as at 30, 60, 90, 120 and 240 min after drug administration.

In all nociceptive tests, mice were habituated to the test room and apparatus for 60 min on the day prior to the test and again immediately prior to the test. Mechanical nociceptive thresholds were determined using a modification of the “up and down” method [13] with calibrated Semmes–Weinstein monofilaments (North Coast Medical, Morgan Hill, CA). The starting filament was 3.61 (0.4 g), and the upper limit cutoff was 4.31 (2 g). To avoid further sensitization of animals with repeated testing, we set a lower limit cutoff in which four consecutive positive reactions with filaments of decreasing intensity would be scored as zero. Five animals were used in each treatment group.

Acute thermal thresholds were measured with the hot-plate test, set at 52.5 °C. We defined response latency as the time to the first nocifensive behavior, such as licking or jumping, with a cut off value of 50 s. This test was performed 60, 120, and 240 min after administration of drug. Thermal hypersensitivity to carrageenan was measured by the withdrawal latency to focused radiant light using a PAW Thermal Stimulator (UC San Diego Department of Anesthesia), with a cut off value of 20 s. Paw withdrawal latencies were determined immediately prior to and 24 h after carrageenan injection, and at the indicated times after drug administration. The mean of three consecutive trials was recorded for each animal.

To screen for sedative and other adverse sensorimotor effects, mice were tested on a Rotarod (Ugo Basile, Comerio, Italy). We measured the time in which mice were able to balance on a rod rotating on its axis at a constant velocity of 15 rpm. The total duration of each trial was 300 s. On the day prior to the test, animals accommodated to the task with three separate training trials. One hour prior to the test, the indicated dose and route of sumatriptan, saline or morphine was administered. A single trial was used for each dose and route; reported times represent % change from the baseline value for each animal $\pm$ SEM.

2.1. Models of persistent inflammation

Although we previously demonstrated changes in 5-HT_{1D} receptor expression after tissue injury using a different inflammatory agent, CFA, [2], as carrageenan induces a more rapid hypersensitivity (within hours) it was a more practical choice for these experiments. For the carrageenan model, we used a 27-gauge needle to make an intradermal injection of 20 μ l 3% carrageenan lambda (Sigma), dissolved in saline, in a lightly restrained, awake animal. Nociceptive thresholds were measured before carrageenan (pre), and at 24 h after injection (carra), and then at specified times after the injection of sumatriptan. Although previous reports show that sumatriptan pre-treatment can have peripheral anti-inflammatory effects in the rat hindpaw [12,18], in the present experiments we administered sumatriptan in animals with well-established hindpaw inflammation. We used calipers to measure hindpaw diameter prior to injection of carrageenan, 24 h post-injection, and again at 30, 60, and 120 min after sumatriptan, and found no changes in paw diameter after sumatriptan injection, regardless of dose or route of administration (data not shown).

For the formalin model, we injected 10 μ l of 2% formalin (Sigma) diluted in saline, into the plantar surface of the left hind paw of a lightly restrained, awake animal with a 27-gauge needle. Formalin induces biphasic pain behavior responses, divided into the phase 1 (0–10 min) and after interphase period with no pain behaviors, a phase 2 (10–60 min) [43]. Seven animals were used in each treatment group. We recorded the time spent licking and grooming the affected hindpaw, during both phases in 5-min bins. Animals received an injection of sumatriptan, morphine, or saline at the dose and route indicated 1 h prior to the start of the formalin test.

2.2. Spared nerve injury (SNI) model of neuropathic pain

We used a model of partial sciatic nerve injury in which we selectively ligated and cut the peroneal and sural nerves, sparing the tibial nerve [39]. Mice that did not develop mechanical allodynia on the fourth postoperative day (two out of 12 animals) were excluded from the study. On postoperative days 7 and 8, mechanical thresholds were obtained immediately before and 1 h after either IT saline or 0.06 μ g IT sumatriptan. Animals were injected in a blinded cross-over manner, in which half of the animals received sumatriptan on one day and saline on the other.

2.3. Acetic acid model of inflammatory visceral pain

To study the effect of sumatriptan in a model of visceral pain that is also associated with inflammation, we counted the number of abdominal stretches that occurred within 20 min of

intraperitoneal injections of dilute acetic acid (5.0 cc/kg of 0.6% acetic acid [11]). Mice were administered either IT saline, or SC or IT sumatriptan 60 min prior to the acetic acid injection. The observer scoring the behaviors was blinded to drug pre-treatment. Seven animals were tested in each group. The reported values represent the mean $\pm$ SEM of the group.

2.4. Data presentation and statistical analysis

There were five age and weight matched animals in each of the treatment groups in this study unless otherwise indicated. Data are represented as the means $\pm$ S.E.M. Mechanical and thermal threshold values were converted to the percentage of the maximum possible analgesic effect (%MPE), according to the formula $\%MPE = [(post\text{-}drug\ value - baseline\ value)/(cut\text{-}off\ value - baseline\ value)] \times 100$. Statistical significance was assessed with ANOVA statistics, with correction for multiple comparisons in post-hoc analysis. A *p*-value of <0.05 is considered significant and is indicated with an asterisk (*).

3. Results

We first tested the effect of systemic injection of sumatriptan on acute thermal pain thresholds. One test measured the latency of the reflex withdrawal of the hindpaw to a noxious heat stimulus applied to the hind-paw, and the second (the hot-plate test) involved a more complex behavior that is presumed to result from integrated spinal and supraspinal “pain” transmission circuits. We also measured mechanical nociceptive withdrawal thresholds with calibrated monofilaments. Fig. 1 illustrates that SC sumatriptan, at doses that inhibit neurogenic edema (i.e., regulate the release of transmitter from the peripheral terminals of nociceptors, ref [8, 12]), had no effect on acute pain behaviors. Because sumatriptan is thought to cross the blood brain barrier (BBB) inefficiently, we also studied the effects of direct IT injections. When administered by the IT route, we found that sumatriptan was still completely without effect in these tests of acute pain. By comparison, these tests of acute pain are very responsive to morphine. None of the intrathecal doses significantly interfered with motor function on a rotarod (Fig. 1D). Using the same methods as above, we incorporated a 15 min time point for acute mechanical thresholds, which is summarized in a Supplemental figure (Fig. S1).

3.1. Sumatriptan reduces tissue injury pain

In the present studies we used a model of persistent pain that triggers a massive exocytosis of DCVs [32]. The formalin test is ideal for this analysis as it consists of two transient and stereotyped phases of pain behavior: the first phase is comparable to acute pain and is thought to result from direct activation of nociceptors [33]; the second phase is a delayed inflammatory state, analogous to postoperative pain, which depends not only upon prolonged activity of nociceptors but also upon a first phase-induced central sensitization of pain transmission circuits within the spinal cord [1].

Fig. 2 illustrates that IT sumatriptan produced a profound reduction of pain behavior (analgesia) in the second phase of the formalin test. There was a modest effect of sumatriptan on first phase pain behavior, which is comparable to acute pain, at the highest intrathecal dose. However, IT sumatriptan prominently reduced pain behaviors in the second phase of the formalin test in a dose-dependent manner. In contrast to sumatriptan, morphine eliminated both first and second phase behaviors. The data for phase 1 and phase 2 behaviors in this experiment are presented in Table 1.

We assessed the utility of sumatriptan in a model of hypersensitivity associated with tissue injury and inflammation, in which innocuous stimuli evoke pain behaviors (allodynia). Intradermal carageenan is an ideal model for these experiments, as it produces local inflammation and a pronounced thermal and mechanical hypersensitivity, within 1 h of its

injection. Fig. 3 shows that intrathecal, but not systemic sumatriptan, completely reversed the thermal and mechanical hypersensitivity in this model of persistent pain. The antinociceptive effect was significant 30 min after injection of sumatriptan, lasted for approximately 1 h, and was dose-dependent. The behavior recorded after control injections of IT saline did not differ from that following SC sumatriptan. As expected, IT morphine produced a profound analgesia, with all animals reaching the cutoff latency (data not shown).

3.2. Sumatriptan does not influence nerve injury-induced pain

What is the spectrum of pain conditions amenable to control by sumatriptan? Because the pathophysiological mechanisms that underlie nerve injury-induced hyperalgesia involve changes in primary afferents and spinal cord dorsal horn that are distinct from those of chronic inflammation [5], we turned to an experimental form of nerve injury that models a neuropathic pain condition in patients. In this model of nerve injury pain, we transected two of the three branches of the sciatic nerve, sparing the tibial branch, which permits behavioral testing of the plantar surface of the hindpaw. Mice demonstrate a pronounced mechanical hypersensitivity of the partially denervated hindpaw, within two days of the denervation [39]. In contrast to the profound analgesic action of sumatriptan for inflammatory pain, sumatriptan was completely without effect on the mechanical hypersensitivity produced by nerve injury, regardless of dose or route of delivery. As expected, IT morphine produced a profound analgesia, to cutoff latencies (Fig. 4).

3.3. Sumatriptan reduces visceral inflammatory pain

With a view to determining whether the effect of IT sumatriptan extends to other non-somatic models of inflammation, we also examined mice in a standard model of visceral pain, namely, intraperitoneal injection of dilute acetic acid. Fig. 5 shows that the number of abdominal stretches produced by intraperitoneal acetic acid was not significantly reduced by systemic sumatriptan. However, there was a significant antinociceptive effect after intrathecal sumatriptan, with the 0.06 μ g dose reducing the number of abdominal stretches by approximately 80%.

4. Discussion

We report the novel finding that sumatriptan, a purportedly selective anti-migraine drug, can significantly reduce the pain of inflammation in non-cranial regions of the body, when given intrathecally in mice. Importantly, systemic administration of sumatriptan was without effect, even at doses 200-fold greater than the effective intrathecal dose, demonstrating the potent analgesic effect of sumatriptan in models of tissue injury pain when administered intrathecally. To our knowledge this is the first exploration of this route of administration for sumatriptan as an analgesic, and may open up an important avenue of therapy for those with intractable inflammatory pain.

In the unstimulated baseline state, intrathecal sumatriptan was completely ineffective against acute thermal or mechanical pain thresholds (Fig. 1), establishing that the failure of systemic sumatriptan to reduce acute pain was not merely due to its limited ability to cross the BBB. In fact, this lack of baseline analgesic activity by sumatriptan is consistent with other studies of systemic triptan on acute pain behaviors [15,40] and with the prevailing view that triptans have no clinical utility in the treatment of acute pain.

In contrast to acute pain, intrathecal sumatriptan produced a selective and profound inhibition of the second phase of the formalin test (Fig. 2), as well as the hypersensitivity associated with tissue inflammation (Fig. 3). In fact, intrathecal sumatriptan not only completely reversed thermal hyperalgesia but also revealed an analgesic effect (i.e. latencies exceeded those at

baseline, Fig. 3A). Also, despite the dramatic and complete reversal of hypersensitivity of the carrageenan-treated hindpaw, sumatriptan did not affect pain thresholds in the unstimulated contralateral hindlimb. This localized action of sumatriptan to the area of tissue injury is consistent with the functional availability of receptors only in afferents stimulated by noxious inputs.

High doses of systemic sumatriptan did produce a modest but statistically insignificant reduction of pain behavior in the formalin test (Fig. 2 and Table 1). There are also reports of modest anti-hyperalgesic effects at comparable systemic doses soon after plantar injection of carrageenan [7], and comparable analgesic effects at extremely high systemic doses of sumatriptan [20,24]. At these high systemic doses there may be sufficient access of sumatriptan to the critical CNS sites (e.g. superficial dorsal horn [27]), which are much more readily reached by the intrathecal route. Second, and consistent with its ability to inhibit neurogenic inflammation in the periphery, systemic sumatriptan may also have an anti-inflammatory action at the peripheral terminals of nociceptors, thus reducing the afferent drive to the dorsal horn [8,12].

The greater efficacy of intrathecal over systemic sumatriptan in reversing inflammation-induced pain emphasizes that the blood brain barrier may be a critical factor in triptan action against somatic and visceral pain associated with inflammation. Our behavioral results, in fact, are consistent with reports that osmotic perturbation of the BBB [28,38] or administration of more lipophilic triptans [16,22,23] enhances the inhibitory effect of systemic sumatriptan on the activity of neurons in the trigeminal nucleus caudalis, though there is not complete agreement as to the extent that these drugs access the CNS [6,27].

In distinct contrast to its effects in the setting of tissue injury, sumatriptan had no effect on mechanical hyperalgesia after nerve injury, regardless of dose or route of administration (Fig. 4). One possibility for the lack of effect of sumatriptan is that the mechanical allodynia characteristic of this nerve injury model is mediated by myelinated afferents [36], which do not express the 5-HT_{1D} receptor [37]. A contribution of increased spontaneous activity from injured unmyelinated afferents, by contrast, would also not be regulated by a presynaptic action of sumatriptan because nerve injury dramatically downregulates these receptors in the central terminals of these afferents [2]. Our results are somewhat in agreement with Kayser and colleagues, who reported that systemic triptans fail to reduce the hyperalgesia produced by chronic constriction of the sciatic nerve. Surprisingly, those authors also found that systemic sumatriptan reduces pain after chronic constriction of the infraorbital nerve [29]. As triptan receptors are expressed in both trigeminal (i.e. infraorbital) and sciatic nerve terminals [2,37, 41,44], we have no explanation for this differential action by triptans. Our studies, of course, do not exclude a differential action by the triptans on trigeminal nociceptors [21,30], nor do they rule out the possibility that triptans reduce pain by activating other CNS antinociceptive control circuits [4].

The fact that sumatriptan only influenced pain behavior generated by nociceptors sensitized by prior tissue injury, taken together with the requirement of an intrathecal route of administration, argues strongly that the central terminal of the primary afferent nociceptor is a major target of sumatriptan for the relief of inflammatory pain. As studies using subtype-selective receptor antagonists disagree as to the relative importance of 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors in the action of triptans [7,21,26], it will be of great interest to address this question more directly, for example, by the use of antagonists or assessing the pain-relieving effects of triptans in mouse lines with genetic deletions of the individual triptan receptors.

In summary our studies demonstrate that intrathecal sumatriptan has a significant antinociceptive action in mouse behavioral models of non-migrainous, somatic and visceral

inflammatory pain. The far greater effect of the intrathecal over the systemic route of administration suggests that the antinociceptive effects after IT injection involve regulation of the central terminals of primary afferent nociceptors. While recognizing that mouse models of somatic and visceral pain do not completely model any clinical pain condition, our studies raise the possibility that intrathecal triptans will have utility in the treatment of a variety of non-migrainous pain conditions in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors have applied for a use-patent involving the intrathecal administration of sumatriptan for chronic pain. Supported by the NIH-NINDS Grants NS 14627, NS 48499, and NS 47113.

Abbreviations

BBB	blood brain barrier
5-HT	serotonin
DCVs	dense core vesicles
SC	subcutaneous
IT	intrathecal
5-HT _{1D} -IR	5-HT _{1D} receptor immunoreactivity

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.pain.2008.06.002.

Fig. 1.

Sumatriptan does not affect baseline pain thresholds. Tests of nociception by (A) hot-plate test, and (B) radiant heat to the hindpaw (Hargreaves test), or (C) mechanical pain using calibrated monofilaments, demonstrate no significant changes in threshold over the time course of the test after systemic (SC) or intrathecal (IT) administration of sumatriptan. SC doses were at 300 $\mu\text{g}/\text{kg}$ (SC 300) and 600 $\mu\text{g}/\text{kg}$ (SC 600), and IT doses were 0.06 (IT 0.06) and 0.60 μg (IT 0.60). A positive control of 10 nmol IT morphine sulfate (MSO_4) produced a robust analgesic response in these tests. Much higher concentrations of sumatriptan can increase nociceptive responses, presumably by the nonselective activation of 5-HT_{1A} receptors [20,34], so the lack of a nociceptive effect at the doses administered in these studies argues against the unintended activation of 5-HT_{1A} receptors. (D) These doses or routes of administration showed no evidence of confounding deficits due to sedation or sensorimotor incoordination on the Rotarod test.

Fig. 2.

Intrathecal sumatriptan selectively and profoundly reduces the second phase of formalin-induced pain. The formalin test began 1 h after the administration of saline, sumatriptan, or morphine. The time course of hindpaw licking in 5 min bins (A), and the cumulative time spent licking in phase 1 (0–10 min) and phase 2 (11–60 min) (B) show that both IT saline- and SC sumatriptan-injected animals displayed stereotypical biphasic behaviors, but that intrathecal (IT) administration of sumatriptan selectively and dose-dependently reduced the amount of second phase behaviors. SC doses of sumatriptan were 300 (SC 300) and 600 $\mu\text{g}/\text{kg}$ (SC 600). IT doses of sumatriptan were 0.006 (IT 0.006), 0.06 (IT 0.06) and 0.60 μg (IT 0.60). A positive control of 10 nmol IT morphine sulfate (MSO4) produced a robust analgesic response in this test. The phase 1 and phase 2 data summarized in this figure is shown in Table 1.

Fig. 3.

Sumatriptan modulates inflammation-induced hypersensitivity when given intrathecally. The time course of sumatriptan responses after sensitization by carrageenan is shown to thermal (top) and mechanical (bottom) stimulation. The pre-test baseline is shown at left (pre). Thermal (A) and mechanical (B) thresholds are greatly reduced at 24 h after injection of carrageenan to the left hindpaw (carra). Responses are shown over a time course (from 30 to 240 min) for a range of doses after the administration of SC or IT sumatriptan. Responses of the contralateral hindpaw (contra) remained unchanged throughout the procedure. Reduction of thermal (C) and mechanical (D) hyperalgesia by IT sumatriptan is dose-dependent, shown at 30–90 min after administration of drug (doses are as indicated in Figs. 1 and 2). Values are given as percent of the maximal possible effect (%MPE).

Fig. 4.

Sumatriptan is without effect in a model of neuropathic pain. The spared sciatic nerve injury model establishes a mechanical allodynia ipsilateral to the injury that is stable and fully developed at 7 days post-nerve transection (SNI 7d). Neither IT sumatriptan 0.6 μ g (IT suma) nor IT saline (IT saline) reduced SNI-induced hypersensitivity, 30–120 min after administration of drug, whereas IT morphine (IT morphine) produced a significant analgesia. Consistent with the lack of effect of IT sumatriptan on acute nociceptive processing, nociceptive thresholds of the unaffected contralateral hindpaw (contra) were not changed.

Fig. 5.

Intrathecal sumatriptan reduces visceral pain produced by dilute acetic acid. Saline or sumatriptan was administered 60 min prior to the intraperitoneal injection of dilute acetic acid. Sumatriptan dosing and routes are indicated as in Fig. 2. Animals were monitored for 20 min after the injection. Although systemic administration of sumatriptan, at both the high and low dose, reduced the number of abdominal stretches following acetic acid injection, these effects were not statistically significant. By contrast, intrathecal sumatriptan significantly reduced the number of abdominal stretches.

Intrathecal sumatriptan selectively and profoundly reduces the second phase of formalin-induced pain

Table 1

	Phase I			Phase II		
	Time (s)	SEM I	p-value	Time (s)	SEM II	p-value
Saline	111	14	0.5895	155	35	
SC 300	104	9	0.0972	156	49	0.97650
SC 600	88	9	0.1036	65	14	0.02500
IT 0.006	89	11	1.1036	79	314	0.05730
IT 0.06	69	3	0.0031	24	18	0.00150*
IT 0.6	60	11	0.0004*	22	13	0.00130*
MSO4	2	2	<0.0001*	0	0	0.00020*

The phase I and phase 2 response times for the experiment in Fig. 2 are presented with SEM data with a summary of the statistical analysis. Statistical significance was assessed with ANOVA statistics, with Bonferroni Dunn correction for multiple comparisons in post-hoc analysis. A p-value of <0.0024 versus saline is considered significant and is indicated with an asterisk (*).

* Bonferroni Dunn $p < 0.0024$; significant versus saline.