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ROLE OF MENINGEAL MAST CELLS IN INTRATHECAL MORPHINE EVOKED GRANULOMA FORMATION

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Abstract

Background—Intrathecal morphine forms granulomas that arise from the adjacent arachnoid membrane. We propose that these inflammatory cells exit the meningeal vasculature secondary to meningeal mast cell degranulation.

Methods—Three sets of experiments were accomplished in dogs. 1) *Ex vivo Meningeal mast cell degranulation*. Histamine release was measured *ex vivo* from canine dura incubated with opiates. 2) *In vivo cutaneous mast cell degranulation.* Flare areas on the dog abdomen were measured after subcutaneous opiates. 3) *In vivo granuloma pharmacology.* Dogs with lumbar intrathecal catheters received infusion of intrathecal saline or intrathecal morphine. Intrathecal morphine dogs received: i) No other treatment (Control); ii) Twice daily subcutaneous naltrexone; iii) Intrathecal co-infusion of cromolyn; or, iv) Twice daily subcutaneous cromolyn for the 24–28 day study course.

Results—1) Morphine but not fentanyl evoked dural histamine release, which was blocked by cromolyn but not naloxone. 2) Wheal/flare was produced by subcutaneous morphine, methadone, hydromorphone, but not fentanyl, and was unaffected by naltrexone but prevented by cromolyn. 3) Granulomas occurred in all dogs receiving intrathecal morphine (15/15); subcutaneous naltrexone had no effect on granulomas (6/6), but was reduced by concurrent intrathecal cromolyn (0/5) or twice daily subcutaneous cromolyn (1 of 5).

Conclusions—The pharmacology of cutaneous/dural MC degranulation and intrathecal granulomas are comparable, not mediated by opioid receptors, and reduced by agents preventing MC degranulation. If an agent produces cutaneous MC degranulation at concentrations produced by intrathecal delivery, the agent may initiate granulomas.

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INTRODUCTION

By histopathology and MRI, intrathecal infusion of high concentrations of morphine in canine and ovine models had no effect upon spinal parenchyma, but led to an aseptic collection of inflammatory cells (granuloma). [1–4] Preclinical work shows granulomas have several attributes. i) Incidence is concentration dependent [2–5] and not observed after repeated bolus delivery. [6] ii) Using serial MRI's in the dog, a mass may begin to form proximal to the catheter tip in an interval as short as 10 days; and, termination of morphine resulted in a progressive reduction in mass size over the ensuing 14 days.[5] iii) The mass arises from the dura-arachnoid layer of the meninges adjacent to the infusion catheter tip [3]. These observations are consistent with many human case series that have been reviewed. [7–910]

An important question relates to the mechanism of this cellular accumulation. Bacterial presence has been reported; but, appears to be a rare, secondary, event (see [3,11]). Solutions are typically prepared as to have cerebrospinal fluid compatible osmolarity and pH.[3,12,13] Finally, infusion of vehicle (saline)[2–4] or a variety of drugs (see, for example [14–16]) fails to produce a granuloma in the dog. Substitution of saline for morphine infusate, after a mass has been established, reduces granuloma size. [5] Thus, the best characterized component appears to be morphine itself. We showed that several opioid molecules, including hydromorphone and methadone, resulted in intrathecal masses; whereas reduced, if any, granulomas were noted with high concentrations of fentanyl or the mu-opioid peptide [D-Ala[2], N-MePhe[4], Gly-ol[5]]-enkephalin (DAMGO), casting doubt as to whether this effect is opioid receptor mediated [12] (but see[17]). Whether the morphine granuloma is prevented or reversed by opiate antagonism is unknown.

Local accumulation of inflammatory cells arising from the meninges and its accessibility to the intrathecal infusate led us to consider that the mechanism might be a local drug effect on a meningeal target. The comparability of this phenomenon to plasma extravasation in other dural systems led us to consider the role of meningeal mast cells. Our hypothesis relating mast cell function to the morphine-induced granuloma is based on four considerations. 1) Mast cells are distributed in dura arachnoid. [18–20] 2) Dural mast cell activation leads to local vasodilatation and release of chemoattractants that enhance cell migration. [21,22] 3) Opiates degranulate cutaneous mast cells in skin, but not reliably mast cells in lung, intestine, heart or blood basophils. [23,24] 4) As certain opiates (morphine) but not all (fentanyl) degranulate skin mast cells leading to dilation and plasma extravasation[25–29] and because the ability of an opiate antagonist to prevent such degranulation is controversial,[30,31] the role of opiate receptors in this phenomena appears unlikely. This profile suggests similarities to that of intrathecal morphine-evoked granulomas and is consistent with the hypothesis that granulomas involve a mast cell contribution. This hypothesis led to a series of studies to characterize opiate effects in *ex vivo* and *in vivo* models on mast cell degranulation and the contribution of this action to the granuloma initiated by continuous intrathecal morphine in the canine model.

MATERIALS AND METHODS

All studies described herein were accomplished under method protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. There are two principal components to this series of studies*: ex vivo* and *in vivo.*

Ex vivo studies

These studies sought to determine the pharmacology of meningeal mast cell degranulation. For these studies, mixed-breed hounds (2 male/3 female; approximately 25–35 kg) undergoing terminal experiments examining cardiac function were employed.

Meningeal harvest—The animals were deeply anesthetized to the minimum of a surgical plane anesthesia sufficiently deep to perform a laminectomy (sodium pentobarbital, 35 mg/kg IV, or propofol, 5–10 μg/kg/min, IV). The lumbar and thoracic vertebral bodies were exposed, cardiac arrest was initiated and the dura immediately harvested from the lumbar and thoracic levels. The dural fragments were immediately placed in an oxygenated iced Krebs ringer's solution. The dural segments were cut into small fragments weighing approximately 10 mg. These were placed in individual Eppindorf tubes filled with Krebs ringer (1mL) that were oxygenated by bubbling with 95% O_2/5% CO_2 and warmed in a 37° C heating block. After an initial 20 min incubation, the solution was removed by aspiration and replaced. At this time, an aliquot of the test agent(s) was pipetted into each chamber. After a 30 min incubation, the solution was aspirated into individual containers and frozen until assay. The dural fragments were then gently blotted dry and weighed. The fragment was then placed in formalin and refrigerated. The dural fragment was mounted on a cryostat and tangential 10 μ sections were taken and mounted on glass slides.

Assay of Mast cell mediators—Each incubation sample (100μL) was assayed for histamine content. Histamine was analyzed using ELISA (Research Diagnostics, Inc, Flanders, NJ). The limit of detection of this assay was approximately 0.3 ng/mL. The intra- and interassay precision was <11.0 and <12.0 %CV, respectively (3–30 ng/mL).

Dural mast cell visualization—Mast cells in the dural sections were stained with Alcian Blue or with Naphthol-AS-D-chloroacetate esterase staining, a Fast Red staining technique, following methods provided by manufacturer (Sigma-Aldrich Corp, St. Louis, MO). Samples were surveyed under a microscope at 20X. In each fragment, 5 stained sections were then examined at 60X in a 100μ field taken in the center of each fragment.

In Vivo Studies – intrathecal infusion

Study design—To characterize the effects of intrathecal drug treatments on granuloma formation, groups of animals were prepared with chronic lumbar intrathecal catheters. They were then assigned to be entered into protocols. The groups and number of animals are listed in Table 1.

Animals—Beagle dogs (Ridglan Farms Inc., Mt. Horeb, WI, or equivalent), 12 to 16 months of age (approximately 9–16 kilograms), were individually housed in runs with wood shavings and given *ad libitum* access to food and water. Animals were adapted for a minimum of 10 days prior to surgery. A nylon vest for pump placement was placed on each dog 48 hours prior to intrathecal catheter placement for acclimation.

Bolus subcutaneous drug delivery for systemic delivery—Drugs were injected subcutaneously in a shaved region of the nape of the neck using a 25 ga needle.

Continuous intrathecal drug delivery

i. Surgical preparation. Dogs were prepared with chronic intrathecal catheters by the surgical placement of the intrathecal catheter approximately 72 hours prior to dosing. Antibiotic sulfamethoxazole-trimethoprim (240mg tablet, 15–25mg/kg, oral, twice daily) was given 48 hours before and after surgery. Dogs received

atropine (0.04mg/kg, IM) prior to sedation with xylazine (1.5mg/kg, IM). After intubation, anesthesia was maintained under spontaneous ventilation with 1.0–2.0% isoflurane and 60% N_2O/40% O_2. Intraoperatively, animals were continuously monitored for oxygen saturation, inspired and end tidal values of isoflurane, CO_2, N_2O and oxygen, and heart and respiratory rates. Surgical areas were shaved and prepared with chlorhexadine scrub and solution. Using sterile technique, the dura of the cisterna magna was exposed. Through a small incision (1–2mm), the intrathecal catheter was inserted and passed caudally a distance of approximately 40–42 cm to a level corresponding to the L_{2-3} segment. The catheter was fabricated of polyethylene or polyurethane tubing (0.61 mm OD), packaged, and sterilized by E-beam irradiation. Dexamethasone sodium phosphate (0.25mg/kg, IM) was administered just after catheter placement. The external catheter was tunneled to exit at the left scapular region. Upon closure of the incision, isoflurane was removed and the animal recovered. Butorphanol tartrate (Tobugesic®; 0.04mg/kg, IM; Fort Dodge Animal Health, New York, NY) was administered upon recovery and as necessary to relieve post-operative discomfort. Following recovery, a nylon vest was placed on the animal and an infusion pump (Minimed 507; Medtronic, Northridge, CA) was secured in the vest pocket where it was connected to the externalized end of the intrathecal catheter.

ii. Initiation of continuous intrathecal morphine infusion. All intrathecal infusions were carried out at 40μL/hr = 960μl/d = 1 mL/day. As previously reported, the initiation of infusion of 12 mg/d morphine in the canine model resulted in behavioral depression, agitation and motor signs.[3] It was found that a stepwise ramping of the infusion concentration from 3mg/d to 12/mg/d could be accomplished over a period of around 4–7 days and then continued to 24–28 days served to minimize adverse events. This protocol was followed in these studies.

iii. Behavioral Observations. Specific behavioral indices of arousal (depressed to agitated: −3 to +3), muscle tone (flaccid to rigid: −3 to +3) and coordination (normal to impaired: 0 to 3) were assessed daily. These indices have been previously validated.[32,33] For purposes of data presentation, overall sensorimotor impairment was presented as the cumulation of the absolute value of the observed muscle tone and coordination score at the time of euthanasia (e.g., 0 = no detectable sensorimotor abnormality; 6 being complete disability and requiring immediate euthanasia).

iv. In a limited number of animals during the initial intrathecal dose incrementation phase noted above, hind limb thermal escape latencies were assessed with the initiation of the 3 mg/mL/d and 12 mg/mL/d infusion (N = 4). In animals that were to receive concurrent dosing with naltrexone, escape latencies were assessed with the start of the 12.5 mg/mL morphine infusion and the initial dosing with naltrexone (0.9 mg/kg, subcutaneous) (N = 4). Hindpaw thermal escape latencies were assessed using a Hargreaves-type stimulator wherein the hindpaws are placed over a glass surface under which is a focused projection bulb. The latency to paw removal was the response. In the absence of a response within 20 sec, the stimulus was terminated and that latency assigned as the response.[34]

v. Necropsy. Dogs were deeply anesthetized with an intravenous dose of propofol (5–10 mg/kg/min, IV) or sodium pentobarbital (35mg/kg or to effect). Animals were exsanguinated by perfusion with saline (~4L) followed by 10% formalin (approximately 4L) delivered by roller pump at approximately 100 mmHg. The spinal column was exposed by laminectomy of the spinal canal. The condition of the spinal cord and overlying dura was noted and the location of the catheter tip *in situ*. The spinal cord was cut (taking care to keep the dura intact) and removed in

four blocks approximating the cervical, thoracic, lumbar (catheter tip region) and lower lumbar (below catheter tip).

vi. Histochemistry. Spinal blocks from perfusion fixed animals were paraffin embedded, sectioned at approximately 4–8 μm thickness and stained with hematoxylin-eosin. To assess mast cell condition, a fragment of dura was additionally stained with either Fast Red or Alcian Blue. To estimate cross-sectional area of the granuloma, hematoxylin-eosin sections are taken at the level of the largest girth of the mass (typically at or near the lumbar catheter tip) and the cross-sectional area of the granuloma and the spinal cord was manually outlined using Image- Pro Plus 5.1 software (Acton Manufacturing Center c/o Media Cybernetics, Inc., Acton, MA) to measure area in the outlined pixels. Pixel count was converted into mm^2.

vii. Post-Mortem magnetic resonance imaging. In several dogs volume estimates and images were obtained using postmortem magnetic resonance imaging of cords from dogs that received 12 mg/day morphine. After perfusion fixation, cords were immersed in DMSO. Images were acquired with a 7T magnetic resonance imaging scanner using 3D gradient echo sequence (FSPGR) TR/TE=10/2.9,FA 20, FOV 15mm, matrix 256×256, slice thickness 100 μm. Cross-sectional images were created and granulomatous tissue areas manually outlined. Using Amira software (Visualization Sciences Group, Burlington, MA), these serial sections were used to volumetrically reconstruct the granuloma and adjacent spinal cord and to calculate granuloma volume.

In Vivo Studies – Intradermal drugs to study flare formation

Male beagles (approximately 7–12 kg, N = 4) were anesthetized with IV propofol (5 μg/kg/min). Animals were intubated, body temperature maintained with an underbody heating pad and continuously monitored as described above. The chest and abdomen were shaven and surgically prepped. Intradermal injections of drug solutions were delivered in 50μL at T = 0 at 12–14 sites (6–7/side). The injection sites were marked with ink. The diameter of the redness around each injection site was measured across its longest and narrowest axis and recorded without reference to drug treatment at T = 0, 10, 30 and 60 min. Flare area immediately after injection and at intervals were calculated in mm^2 as an oval (3.14 x a x b where a = half length of long axis and b = half length of short axis). Animals were used at 5-day intervals. This protocol was similar to that previously described to assess flare in dogs.[35,36] Each animal was used 5–7 times.

Drugs

Preservative-free, Food and Drug Administration–approved formulations of morphine sulfate (Infumorph; Abbott, Abbott Park, IL), hydromorphone hydrochloride (Dilaudid-HP for Injection; Abbott), and saline vehicle (0.9% NaCl; Abbott) were used. Other opioids employed were prepared from the powder. D/L methadone HCl and fentanyl HCl were obtained from the National Institute of Drug Abuse. The opiate antagonists, naloxone HCl, naltrexone HCl, the mast cell degranulating compound 48/80 and the mast cell stabilizers Cromolyn Sodium salt and nedocromil sodium were obtained from Sigma Chemical (St. Louis, MO). All drugs were prepared in saline (0.9%) unless otherwise stated. Doses and concentrations employed are indicated in the text.

Statistics

Comparisons across treatment groups for granuloma size and for sensorimotor scores employed the nonparametric Kruskal Wallis with *post hoc* comparisons to the saline treatment group using Dunn's multiple comparison test. For the cutaneous flare,

comparisons were made across treatment using one way ANOVA with *post hoc* comparison to the saline (vehicle control) group using Dunnett's multiple comparison. For specific *post hoc* comparisons between the flare inducing agent alone and with a co-treatment, multiple two tailed t-tests were performed with a Bonferroni correction for alpha buildup performed for each set of tests. For analysis of thermal escape thresholds over time as a function of dose and treatment, a two-way repeated measures was performed with *post hoc* comparisons using the Bonferroni test. To relate degree of sensorimotor deficits with granuloma size, we undertook a linear regression of sensorimotor score at sacrifice versus cross-sectional area of the granuloma. Group comparisons having critical values corresponding to $p<0.05$ were considered to be statistically significant. All analyses employed the GraphPad Prism software package (v.4.0c for MAC OS X; GraphPad Prism Software Inc., La Jolla, CA).

RESULTS

In vivo spinal granuloma formation with intrathecal morphine

Granuloma formation—Previous work has shown that intrathecal infusion of saline has no untoward histopathologic reactions out though approximately 4 weeks (see FIGURE 1). In contrast, morphine at 12 mg/d (e.g. 12.5mg/mL delivered at 960μL/hr) in the dog led to the development of prominent granulomas inside 2 weeks in essentially all animals so infused.[3,12] FIGURE 1 displays representative granulomas from three of the last 15 dogs receiving 12 mg/d morphine for approximately 28 days. Using *ex vivo* MRIs, volumetric reconstruction of the granuloma of 4 dogs that received 12 mg/d for greater than 24 days revealed calculated mass volumes of 0.42, 0.39, and 0.31, 0.36 cm^3. As indicated by the typical volumetric reconstruction, the greatest girth of the mass was typically at the level of the catheter tip (FIGURE 2). In the animal imaged in FIGURE 2, dual catheters had been placed to permit infusion and sampling. As indicated, the granuloma developed around the infusion catheter and was largest at the tip of the infusion catheter (e.g. the drug delivery site). In the 15 dogs receiving intrathecal morphine (12 mg/d), the area of the granuloma at the level of largest girth was calculated. The median and inter quartile range of the maximum area of the pericatheter mass was 9.5 (8.1–14.0) mm^2. The corresponding median percentage of the cross-sectional area with quartiles was 27.1% (23.3–33.1), emphasizing the compressive nature of these masses. The distribution of the cross-sectional area and percent of the cross-sectional area of these masses is presented in FIGURE 3 and will be discussed further below. As reviewed previously,[3] examination of the granulomas revealed the presence of inflammatory cells with extensive immunoreactivity for CD68-ir macrophages and T cells (Leu4) (data not shown).

Meningeal mast cell degranulation with morphine—Staining of cryostat sections of dural fragments with Alcian Blue or Fast Red revealed in the cervical, thoracic and lumbar dura of a normal dog the significant presence of dense granule-containing profiles of mast cells (as in FIGURE 4A). Typically in any given section, while some mast cells were found in the dura mater, the majority appeared to be present in the arachnoid layer. As shown in FIGURE 4B, they frequently were found to distribute along the axis of the numerous small blood vessels present in the arachnoid layer. Harvest of dura from dogs that had received either no infusion (control) or approximately 28 days of saline vehicle revealed a large number of intact mast cell profiles. However, after 28 days of morphine (12 mg/day) and displaying a granuloma proximal to the catheter tip, proximal dura typically revealed relatively few intact profiles in the lumbar region. In contrast, examination of spinal dura harvested from upper thoracic (e.g. T5-T10) and cervical (C2–3) levels, where no granuloma was present, revealed significant numbers of intact granule-containing profiles (FIGURE 4C). Similar staining profiles were observed with Naphthol-AS-D-chloroacetate esterase staining, a Fast Red staining technique (not shown).

In vivo assessment of the cutaneous flare produced by intradermal opiates

Agonist degranulation—To determine the pharmacology of canine cutaneous mast cell degranulation, the flare producing effects of intradermal injections of Compound 48/80 and several opiates were assessed in the anesthetized dog. Subcutaneous injection of saline produced at most a small bleb at the injection site that typically resolved within 10 min with no incidence of reddening (flare). FIGURE 5A shows the visual image of the concentration-dependent fare produced by side by side subcutaneous injections of 50μL of increasing concentrations of Compound 48/80, morphine or saline (vehicle). FIGURE 5B summarizes the flare areas produced by subcutaneous Compound 48/80 (0.1–10 mg/mL), morphine (0.01–10 mg/mL) and methadone (0.1–10 mg/mL). These agents produced a maximum increase in flare area by 30 min that typically disappeared over the next several hours. In contrast, no change was observed with fentanyl. Ordering of the magnitude revealed that at the highest concentrations employed for morphine and methadone, effects were not different from those produced by the recognized mast cell degranulation agent Compound 48/80 ($p>0.05$). In contrast, fentanyl at the highest dose (2mg/mL) produced little flare, which, though different from saline, was difficult to discern. Higher doses of fentanyl were not examined, as at these concentrations, the multiple 50μL injections resulted in delivery of a dose equivalent to approximately 10–20 μg/kg and produced a decreased respiratory rate. (No clinically significant desaturation was, however, noted in those studies.)

Mast cell stabilization—To determine if these effects were mediated by mast cell degranulation, dogs were pretreated 60 min in advance with the mast cell stabilizers cromolyn (7.5 mg/kg, IM) or nedocromil (4.5 mg/kg, IM). As indicated in FIGURES 6 and 7, both agents completely blocked the flare response otherwise produced by all agents. In a dose ranging study, a lower dose of cromolyn (2.5mg/kg, IM) incompletely reduced the flare after Compound 48/80 and morphine (data not shown). Diphenhydramine (10 mg/kg, IM), the H1 blocking agent, was examined on the flare produced by high concentrations of Compound 48/80 and morphine and again this dose completely prevented the observed flare response produced by these agents (data not shown).

Opiate receptor antagonism—An important question was whether these effects from the opiates were affected by naloxone. Here, a very high dose of naloxone (10 mg/kg, IV) given 30 min prior to the agonists failed to block the flare change otherwise produced by each of the agents (FIGURE 8). The dose was evidently effective at blocking opiate receptor activation as the respiratory depression otherwise observed with the fentanyl was not observed.

Ex vivo dural mast cell degranulation

To extend the observations *in vivo* on the pharmacology of opiate-induced degranulation of mast cells as measured by the flare and to compare dural degranulation with cutaneous degranulation, we examined *ex vivo* histamine release from canine dural fragments.

Dural histamine release—As shown in FIGURE 9, Dural fragments incubated for 30 min with morphine resulted in a significant concentration-dependent degranulation of mast cells (FIGURE 9A–D) and an increase in histamine in the dural supernatant (FIGURE 9A) over the range of morphine concentrations of 10 to 100μM. As indicated, morphine in this preparation is at least as potent as the release evoked by 100μM of Compound 48/80, a recognized mast cell degranulating agent. Importantly, the morphine-evoked histamine release was blocked by co-incubation with cromolyn but not by naloxone. Similar but more limited studies examining meningeal histamine release were carried out with other opioids at the 100 μM dose. As shown in TABLE 2, hydromorphone and methadone, but not fentanyl, exposed in a concentration of 100 μM yielded significant increases in histamine release

different from saline and comparable to those observed with 100μM morphine. Histochemical examination of the mast cell population in the dural fragments after a 30 min incubation with vehicle revealed numerous complete mast cell profiles with dense intracellular granular staining (FIGURE 9A). Addition of morphine resulted in a prominent change in appearance of mast staining with an increasing fraction of the dural tissues showing varying degrees of disrupted cellular profiles with stain-free core indicative of degranulation (FIGURES 9C,D).

In vivo effects of opiate antagonism on granuloma formation

Naltrexone antagonism of intrathecally infused morphine evoked analgesia— The following studies were performed to define the *in vivo* antagonism by naltrexone of the analgesic effects of a granuloma producing dose of intrathecal morphine in the dog. The intrathecal infusion of morphine 3 and 12 mg/d resulted in an increase in hind paw thermal escape latency as compared to vehicle (saline). In a separate group, a single injection of naltrexone (0.9 mg/kg) prevented the increase over this 8 hr interval (FIGURE 10).

To determine the effects of opiate antagonism on intrathecal morphine-initiated spinal granuloma formation, animals were injected twice daily with the opiate-antagonizing dose of naltrexone (0.9 mg/kg, sq.). As indicated in FIGURE 3, in animals receiving 12 mg/d morphine for approximately 4 weeks with this twice daily dose of naltrexone, the appearance (see FIGURE 1) and incidence of granuloma formation was not different from that observed with morphine alone. Thus, the median cross-sectional area with quartiles in dogs receiving intrathecal morphine alone or with naltrexone was 9.5 (8.1–14.0) mm^2 and 21.9 (12.7–23.8) mm^2, respectively, and both were statistically different from vehicle ($p < 0.05$).

In vivo effects of cromolyn on granuloma formation

Intrathecal cromolyn—To determine the effects of a spinally-delivered mast cell stabilizer on intrathecal morphine-initiated spinal granuloma formation, animals received an intrathecal delivery of morphine 12 mg/d with an admixture of cromolyn yielding cromolyn infusion concentrations of 12 mg/d (N = 5). These doses were based on the *ex vivo* work (FIGURE 9), wherein equal doses of cromolyn were observed to prevent morphine-evoked mast cell degranulation. In this series, the incidence and the median area of the pericatheter masses in animals receiving intrathecal 12 mg/mL morphine over an interval of 24–28 days were reduced by concurrent intrathecal cromolyn (1 of 5 with granuloma, median percentage of the cross-sectional area with quartiles = 0, 0.0–1.1 mm^2), and were different from the intrathecal morphine alone group ($p<0.05$), but not different from the saline group ($p > 0.05$) (see FIGURE 3). Two dogs received an intrathecal infusion of cromolyn (12 mg/d) alone. In this limited series, no untoward effects on behavior were observed and hematoxylin-eosin staining showed no untoward signs (data not shown).

Subcutaneous cromolyn—To determine the effects of a systemically-delivered mast cell stabilizer on intrathecal morphine-initiated spinal granuloma formation, animals received an intrathecal delivery of morphine 12mg/d and twice daily dosing of cromolyn 7.5 mg/kg (N = 5). These doses were based on the *in vivo* degranulation work (FIGURE 6), wherein systemic cromolyn was observed to prevent intradermal morphine-evoked mast cell degranulation. In this series, the median percentage of the cross-sectional area and interquartile was 4.5 (0–4.8) mm^2 (in animals receiving subcutaneous cromolyn) over an interval of 24–28 days and were different from the intrathecal morphine alone group ($p<0.05$), but not different from the saline group ($p > 0.05$).

Behavior

As previously described,[3] the initiation of continuous infusion of 12 mg/d leads to an initial behavioral depression that is addressed by an incrementation of the infusion concentration over 3–7 days. Over the ensuing weeks, many animals receiving morphine, but not vehicle would display neurological signs that included hind limb spasticity, altered gait and an increased sensitivity to touch applied to the hind quarters. Bowel and bladder function were not impaired sufficiently to require early euthanasia. FIGURE 11 presents the sensorimotor score of all animals at the time of euthanasia. As indicated in FIGURE 11B, the animals receiving morphine only displayed a median score of 6 (maximum being 6) as compared to a median score of 0. Comparison across all treatment groups revealed a significant main effect, but only the morphine vs. saline comparison was significant. We plotted the motor score against the % cross-sectional area of the granuloma and observed a regression that, while statistically nonzero (slope with 95% CI: 0.047; 0.0189 – 0.075) with $r^2 = 0.253$, was modest. This suggests that the degree of motor deficit observed with the evolution of the granuloma correlated poorly with the actual compressive extent to of the granuloma. Thus, several animals with significant compression showed virtually no deficits at the time of euthanasia. This was a conclusion that we previously reached.[3]

DISCUSSION

Chronic intrathecal morphine initiates a volume-occupying accumulation of inflammatory cells deriving from the meningeal vasculature. We hypothesize that dural mast cell degranulation is a linkage between intrathecal opiates and the granuloma.

Opiate receptor activation and granuloma formation

In the dog, intrathecal infusion of approximately equi-analgesic concentrations of morphine, hydromorphone, or methadone, but not fentanyl, result in an intrathecal granuloma.[5] This drug effect discrepancy, along with failure of naltrexone in the present work to alter the granuloma, argues against a simple opioid receptor-mediated effect. While higher doses of naltrexone might be required to reverse a possible interaction, we note the following: i) naltrexone and its metabolite, 6-beta-naltrexol, have half-lives of several hours with the metabolite being longer[37] (but see[38]). 6 beta-naltrexol has opiate antagonist properties that may account for the long duration of blockade by the parent.[39] ii) The dose employed resulted in a significant antagonism of the analgesic effects produced by the granuloma-producing dose for an excess of 8 hrs. Accordingly, twice daily dosing would result in excess of 16 hrs of receptor coverage. In previous work we showed that repeated daily bolus morphine dosing resulted in no granulomas,[6] suggesting that periods between morphine injection were sufficient to presumably clear the agent and prevent granuloma formation, a cycle approximated by twice daily antagonist dosing. iii) We expect that size would be a sensitive index of granuloma formation and this was in fact numerically greater in the naltrexone group. Accordingly, we conclude that granuloma-inducing effects of intrathecal morphine is not dependent upon a “conventional” naltrexone-sensitive opiate receptor.

Spinal Meningeal Mast cells

Mast cells are distributed in spinal dura-arachnoid,[19,20,40] and often aligned with local arachnoid vessels.[41] The meningeal mast cells are subject to degranulation by the local milieu.[42,43] Such activation releases low and high molecular weight compounds including vasodilators (histamine and serotonin), chemoattractants for T cells and mediators of increased permeability (tumor necrosis factor, tryptase and chymase)[44–46] of the meningeal and cerebral vasculature.[22,42,47,48] Tryptase is present in large amounts in all mast cells [49] and activates proteinase activated receptors (PARs)[46] that disrupt vascular endothelial barrier integrity.[50] This contribution to plasma extravasation and cell migration in dura led

us to consider the role of MMCs in the intrathecal accumulation of inflammatory cells forming the granuloma.

Opiates and mast cell degranulation

Opiates degranulate mast cells and release vasodilating substances, notably histamine.[25] In the skin where mast cells are located,[36] degranulation results in a local cutaneous vasodilation, a flare. Work focusing on the pharmacology of this cutaneous effect shows that while a variety of opioids produce flares, this effect is not associated with an opiate receptor. Thus, subcutaneous injection of some opiate molecules (morphine, hydromorphone, methadone) but not other agents (fentanyl) [28,51,52] result in histamine release/flare in a manner that is largely refractory to opiate antagonism, but readily prevented by cromanes (e.g., cromolyn and nedocromil).[25,29–31,51] Importantly, this structure activity series and failure of opiate antagonism to reverse the effects of dural or cutaneous mast cell degranulation suggests that classical opiate receptors play at most a minor role. Importantly, as shown here, meningeal mast cells are degranulated by morphine, but not by fentanyl and this effect is not antagonized by concentrations of naloxone which are larger than those required to block effects of opiate receptor activation in *in vitro* organ systems.[53] This similarity between dural and cutaneous mast cell activation is interesting, in that in contrast, mast cells from the heart, lung and the gastrointestinal tract are reported to not show a strong response to opiates.[24] The parallels between dura and cutaneous mast cells may reflect the fact that dura and skin arise embryologically from the somitic mesoderm and neural crest and their resident mast cells would potentially display similar pharmacological profiles.[54,55]

How do opiates act to degranulate mast cells? We note several possibilities. i) Mast cell degranulation may be initiated through activation of high-affinity receptors for the Fc region of IgE, through receptors on the cell surface.[56,57] ii) Degranulation may occur through a *receptor independent* interaction mediated by cationic charges that act as receptor mimetic agents to trigger mast cells, by interacting with sialic acid residues of the cell surface and then with Gi-like proteins, activating phospholipase C and intracellular calcium mobilization.[58–60] This pathway has been shown for morphine in mast cells.[61] Mast cell stabilizers such as cromolyn may act by specifically preventing such G-protein activation[61]; iii) Opiates interact with the MD2 component of toll-like receptor 4, leading to activation.[62–64] This effect is not stereospecific and is antagonized by the opiate receptor-inactive stereoisomer of naloxone.[64] Toll-like receptor 4 activation can initiate mast cell degranulation [65,66]. One *caveat* to this mechanism is that toll-like receptor activation is also apparently produced by fentanyl, which does not initiate a granuloma at therapeutic doses.[64]

Meningeal mast cell and spinal granuloma formation

Either co-intrathecal delivery of cromolyn or twice daily subcutaneous cromolyn resulted in a reduction in incidence and cross-sectional area of granulomas in dogs receiving continuous infusion of a granuloma-producing dose of morphine. Interestingly, intrathecal cromolyn in rats prevented dural mast cell degranulation otherwise noted after intraplantar carrageenan.[40] Cromolyn can prevent degranulation by a variety of stimuli.[67,68] Aside from preventing G-protein activation,[61] cromolyn also has other effects, including i) block of chloride transport in mast and nerve cells,[69] ii) reduced substance P binding and iii) reduced adenosine-induced plasma extravasation.[70] What role these other actions play in the observed phenomena is not known. Additional studies defining the dose dependency of the cromolyn effect and the actions of other, non cromane, mast cell stabilizers to confirm the role of the presumed target would be desirable.

Corollaries of mast cell contributions to granuloma formation

The possibility that mast cells may be an intervening mechanism raises several issues.

i. Ligands that do not degranulate mast cells would be predicted to not produce granulomas. Fentanyl does not produce granulomas in dogs at intrathecal doses up to 2mg/mL/d. Consistent with this hypothesis, intradermal alfentanil had minimal effects upon mast cell degranulation when delivered by continuous intrathecal infusion at the maximum tolerable dose of 20mg/mL/d and resulted in minimal dural reactions.[71] Anilinopiperidines are rarely employed for chronic intrathecal delivery because of their rapid clearance and associated significant plasma exposure in contrast to agents such as morphine with a low log P. As a *caveat* to the above commentary, we note in one case report that intrathecal fentanyl at a high daily dose of 2.7 mg/d (drug treatment history, infusate concentration and duration of exposure were not specified) was associated with an intrathecal mass.[72] In a second report, intrathecal sufentanil (17μg/d) in a patient with a history of prior exposure to several agents including fentanyl, baclofen and ziconotide also developed a granuloma.[73] These examples suggest the possible role of mechanisms in addition to potential contribution of mast cells, particularly at high concentrations.

ii. Agents that block mast cell degranulation in the skin should be efficacious in blocking the granuloma. The present spinal work is limited to cromolyn. Stabilizers such as nedocromil [74–77] have different potencies and should have similar granuloma-sparing effects at lower doses and different pharmacokinetics. Alternately, Syk tyrosine kinases are important in mast cell degranulation.[78] Inhibitors (e.g. BAY613606) reduce mast cell degranulation, histamine release and inflammation more potently than cromolyn.[79] Interestingly, intrathecal BAY613606 blocked dural mast cell degranulation.[40].

iii. We propose that a potential screening approach is to determine whether the agent produces flares after local subcutaneous delivery. We suggest that the pharmacology of the cutaneous mast cell resembles that of the meningeal mast cell. A *caveat* to the use of the cutaneous flare is the issue of concentration. We did not examine the possibility that the failure of fentanyl to produce a granuloma (or flare) might represent its rapid subcutaneous/intrathecal clearance, although the highest tolerable doses were employed. A second *caveat* is that if the local drug target requires a low concentration relative to that required to degranulate mast cells then the therapeutic use of the agent that produced cutaneous flare might not be confounded by granuloma formation. Ziconotide produces histamine release [80], yet intrathecal ziconotide in preclinical safety evaluations [81,82] or in humans[83] has not been reported to initiate granulomas. This observation may reflect the extremely low concentrations required of this agent for therapeutically effective degrees of calcium channel block. Conversely, as noted, while anilinopiperdines are not generally considered to degranulate mast cells, very high concentrations may have modest effects on vascular cells and this may reflect why they also may have some propensity to initiate a granuloma in humans.

In conclusion, while granuloma formation is often considered to be clinically infrequent, two observations are telling: i) there is an increasing incidence of observations/reports and in post-marketing surveillance reports describing these space-occupying masses and ii) amongst clinicians using intrathecal analgesics, 66% report a patient experiencing neurologic injury secondary to a granuloma (see for review[9]). Thus, this is not an effect to be dismissed. Our work seeks to address the mechanisms of this phenomenon. The present results must be considered as a contribution to a mechanistic understanding of the role of meningeal mast cells. The results do not exclude other mechanisms that may not engage mast cells. Further, the canine work demonstrates a mast cell effect with *pretreatment*. We do not know if blocking mast cell degranulation will reverse a granuloma. Finally, showing

an effect of cromolyn absolutely cannot be construed as a safety evaluation of the drug by either route or the doses employed. Further preclinical work [84,85] defining such parameters and safety is required to evaluate the risk-benefit of this or any adjunctive therapy.

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Summary statement

Intrathecal morphine infusion leads to a meningeally derived granuloma. The present work in the dog points to a close correlation between the origin of these intrathecal granulomas and the degranulation of meningeal mast cells.

FINAL BOX SUMMARY

What we already know about this topic

- Intrathecal infusion of morphine is associated with granuloma formation in animals and humans, although the mechanisms for this effect and whether it can be prevented or predicted have not been examined

What this article tells us that is new

- In dogs, intrathecal infusion of morphine induced granuloma formation which was prevented by an inhibitor of mast cell degranulation, but not by naltrexone
- Subcutaneous injection of some, but not all opioids, induced mast cell degranulation, suggesting this may be a useful screen for potential granuloma formation with intrathecal infusion

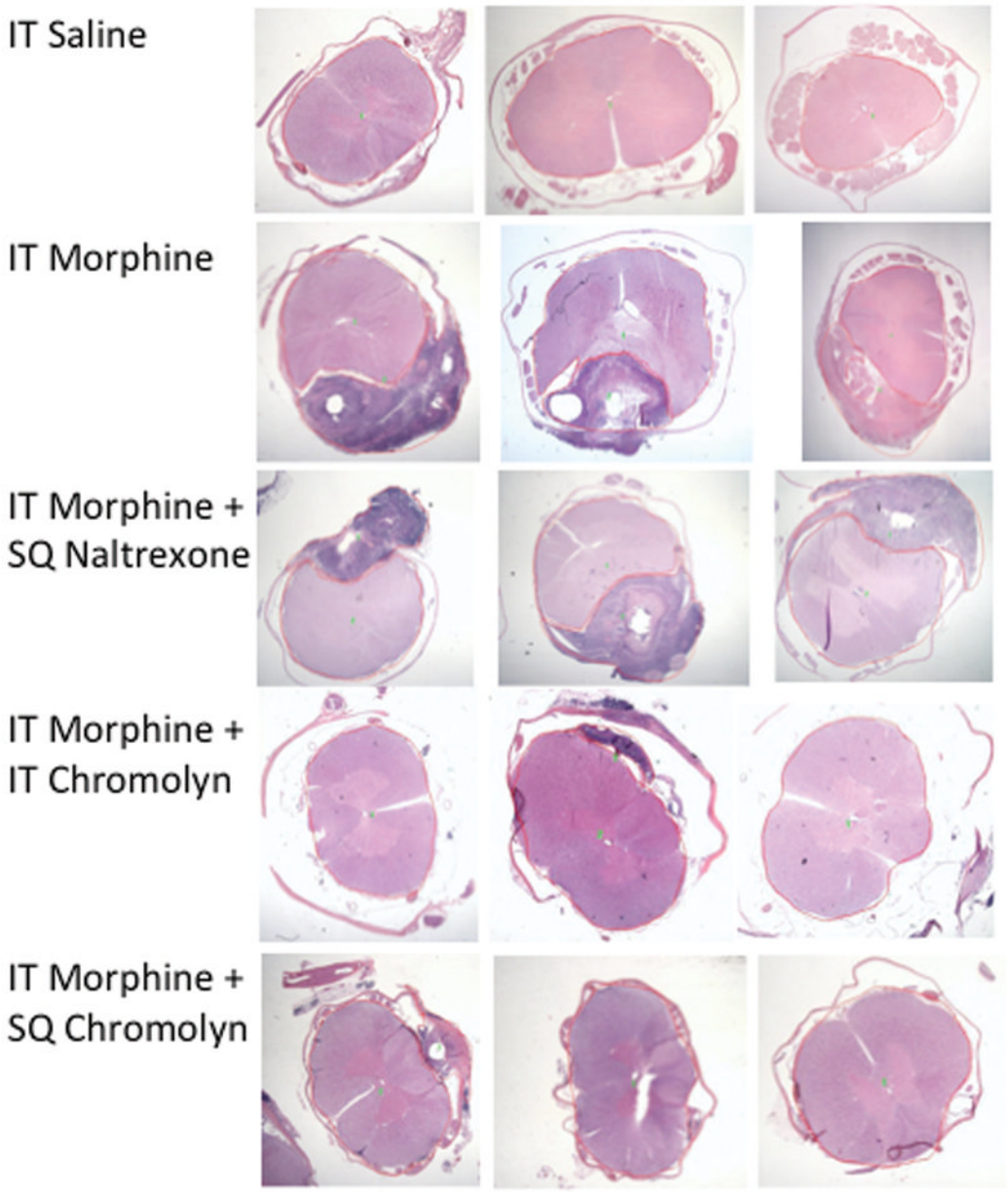


Figure 1.
Representative lumbar H&E sections from 3 of the 5–15 dogs that received: 24–28 days of intrathecal (IT) saline, IT Mor (12 mg/mL), IT Mor (12 mg/mL) + BID (twice daily) naltrexone; IT Mor (12 mg/mL) + IT cromolyn (12 mg/mL), or IT Mor (12 mg/mL) + BID subcutaneous cromolyn (7.5mg/kg). Dark reaction product indicates granuloma.

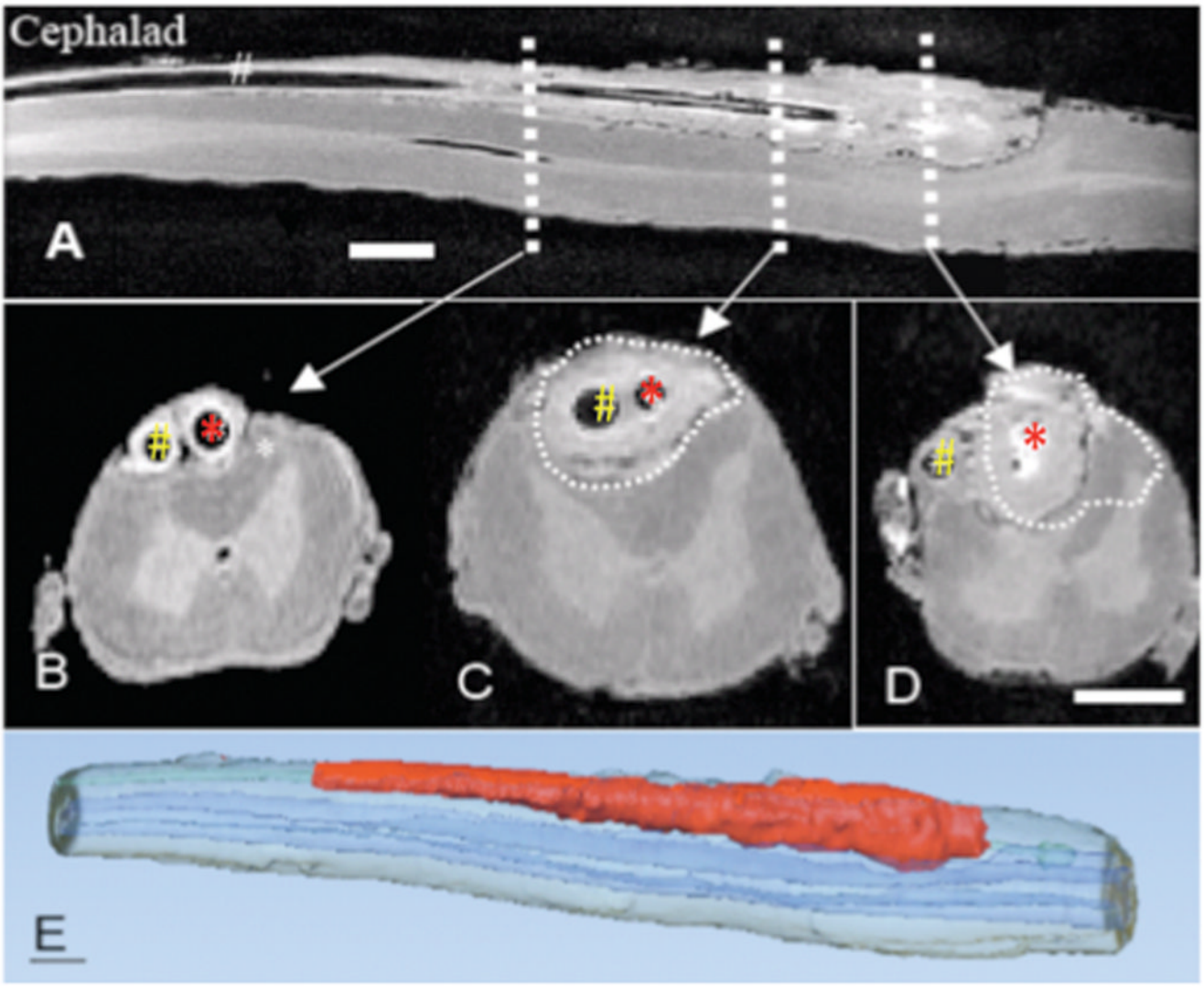


Figure 2.

Ex Vivo MRI (magnetic resonance imaging) taken *post mortem* through the lumbar spinal cord of the dog with sampling catheter (red arrow) and infusion catheter (white arrow) that received a 28-day infusion of intrathecal morphine sulfate (12 mg/d). A: Mid-sagittal plane through the lumbar cord. B,C,D: shows three transverse sections taken at the levels indicated. In Figure B, sampling catheter and infusion catheter indicated by red and white arrows, respectively. Size bars are approximately 0.3 cm. The C and D sections correspond to the levels in the longitudinal section that are proximal to the respective catheter tip. E: Three dimensional reconstruction of dog spinal cord from *ex vivo* MRI. Red and blue volumes indicate granulomatous mass and spinal cord, respectively.

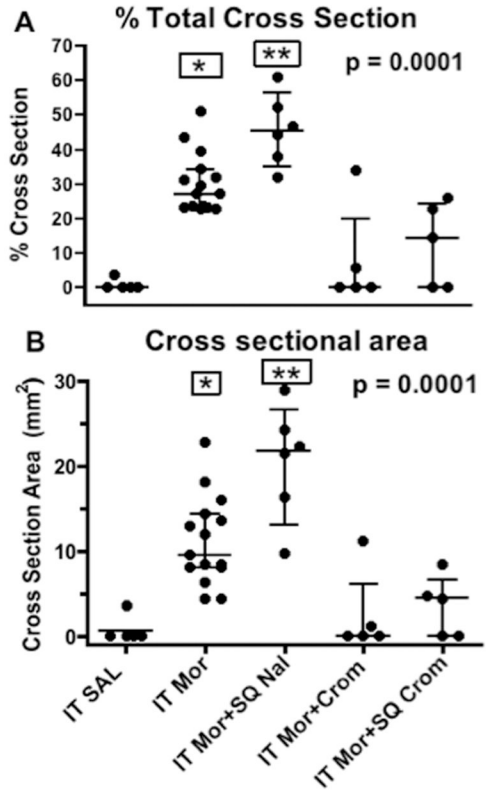
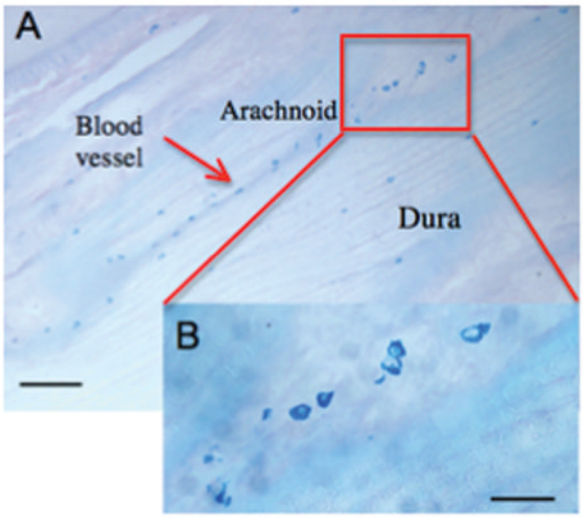


Figure 3.

Scatter gram of A: % of cross-sectional area of mass to total spinal area and B: cross-sectional area (mm^2) of the granulomatous mass at catheter tip for dogs receiving intrathecal (IT) saline, IT Morphine (Mor: 12mg/d), IT Mor (12 mg/d) + subcutaneous naltrexone (Nal: twice daily (BID): 0.9mg/kg); IT Mor (12 mg/d) +IT cromolyn (Crom: 12 mg/d) or IT Mor (12 mg/d) + subcutaneous cromolyn (Crom: BID 7.5mg/kg). Each point represent a single dog receiving approximately a 28-day IT infusion. Error bars indicate the median with 25^{th} and 75^{th} %. For numbers of animals in each group see Table 1. Kruskal Wallis analysis across treatment groups showed a significant main effect: $p<0.0001$. (Similar results were observed with a 1-way ANOVA.) *Post hoc* comparison to the saline treatment group using Dunn's multiple comparison: * $p<0.01$; **:$p<0.001$.



C Incidence of intact mast cells in spinal dural fragments taken at cervical, thoracic and lumbar levels in IT vehicle or morphine infused dogs

TREATMENT	Cervical	Thoracic
IT saline (40μL/hr)	12	7
IT saline (40 μL/hr	8	8
IT saline (40 μL/hr)	13	10
Control (no infusion)	7	6
MEAN ± SD	10.5±2.4	8.5±1.3
IT Morphine*	10	8
IT Morphine*	11	6
IT Morphine*	7	8
MEAN ± SD	9.3±2.1	7.3±1.2

*12 mg/mL 40μL/hr; IT: Intrathecal

Figure 4.

A: Section showing Alcian Blue stained lumbar dura mast cells in control animal at low (bar = 100μ). B. Inset showing enlargement of marked area at high (bar = 30μ) power. Note alignment of cells in arachnoid with blood vessel. C: Table presenting counts of intact Alcian Blue staining profiles in cervical, thoracic and lumbar dura in three animals receiving intrathecal (IT) saline or 3 animals receiving IT Morphine (12mg/d).

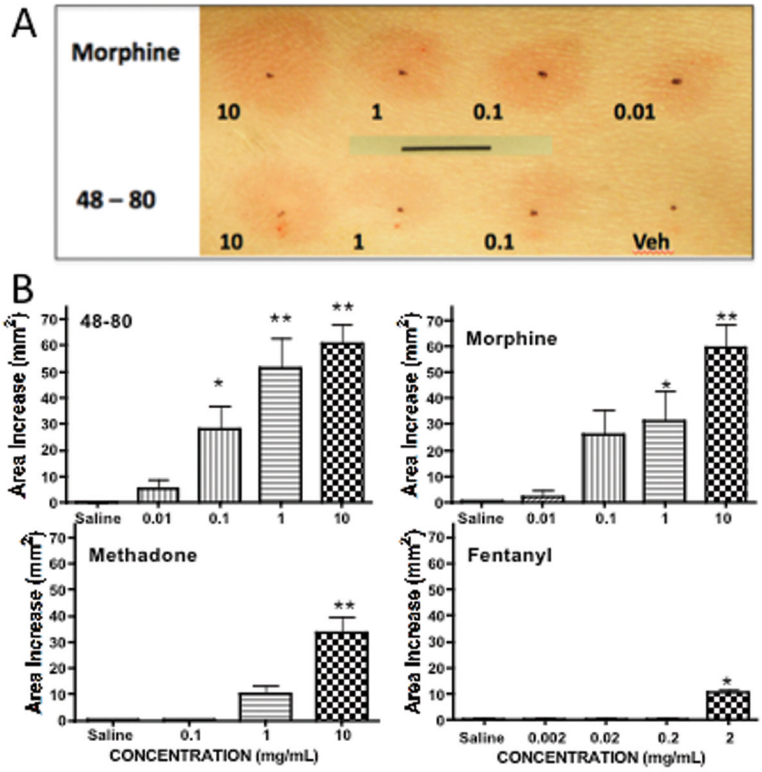


Figure 5.

A: Representative photograph of dog abdomen showing cutaneous flares taken at 30 min after the subcutaneous injection of different concentrations (mg/mL) of morphine or 48–80 in 50μL. Bar indicates 10 mm. B: Histograms showing the flare area at approximately 30 min after subcutaneous injection of different concentrations of compound 48–80, morphine, methadone or fentanyl. Each histogram presents the mean ± SEM of 4–6 subcutaneous injections. 1-Way ANOVA analysis of each drug across its treatment doses *showed* a significant main effect for each drug: $p<0.0001$. *Post hoc* using Dunnett's multiple comparison: * $p<0.05$ vs. saline, ** $p<0.01$ vs. saline.

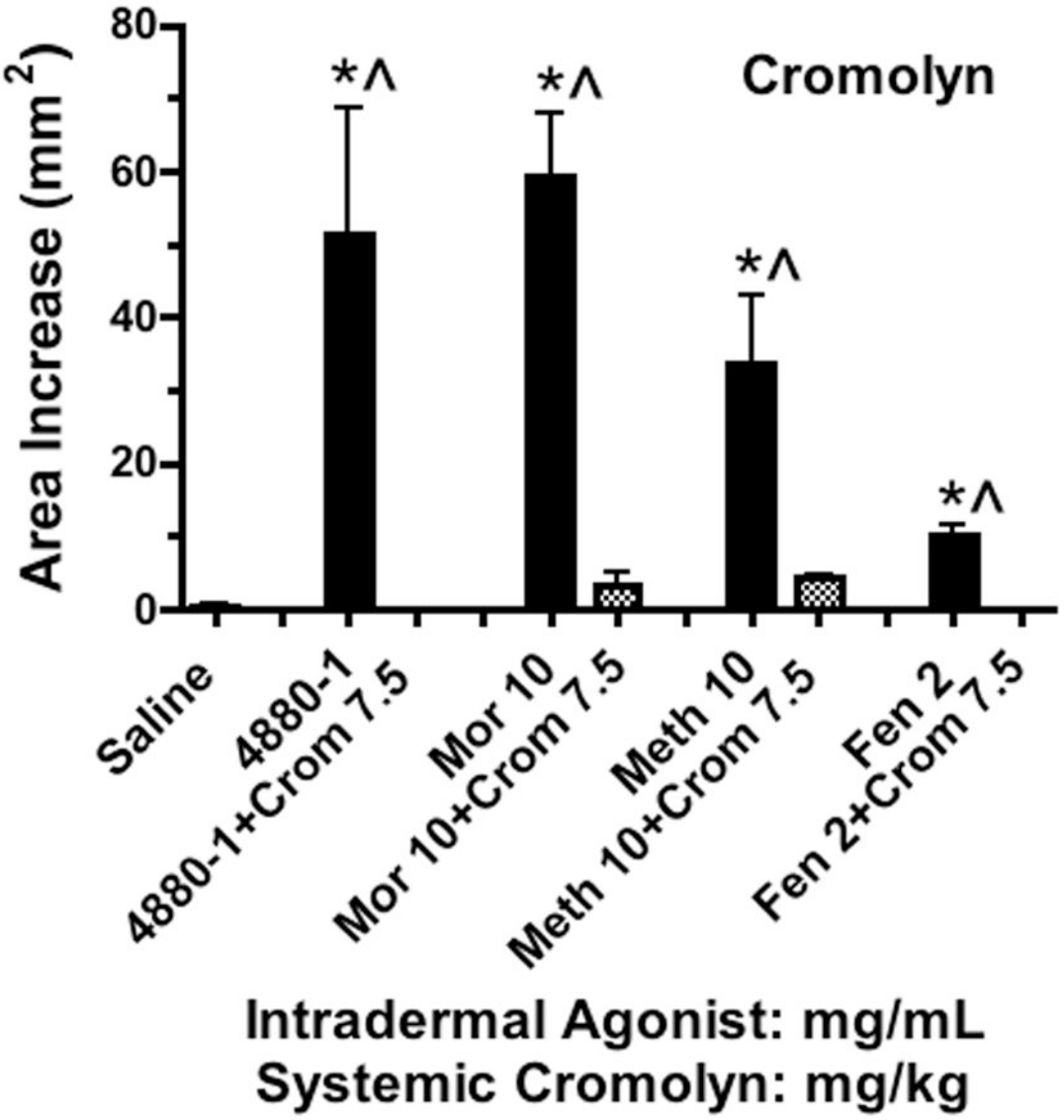


Figure 6.

Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of 48–80, morphine, methadone or fentanyl (mg/mL)in 50μL in animals pretreated with cromolyn (7.5 mg/kg). Each histogram presents the mean ± SEM of 3–5 subcutaneous injections. 1-Way ANOVA analysis across treatments showed a significant main effect ($p<0.0001$). **Post hoc* using Dunnett's multiple comparison: * $p<0.05$ vs. saline. ^ *Post hoc* t-test with Bonferroni correction: drug alone vs. drug + cromolyn: $p<0.05$.

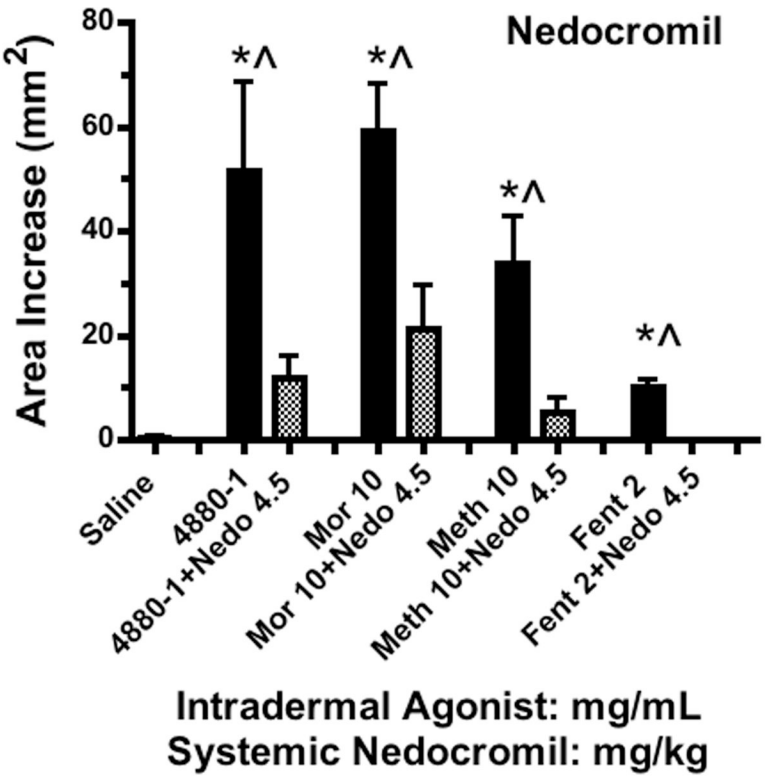


Figure 7.

Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of 48–80, morphine, methadone or fentanyl (mg/mL) in animals pretreated with nedocromil (4.5 mg/kg). Each histogram presents the mean ± SEM of 3–5 subcutaneous injections. 1-Way ANOVA analysis across treatments *showed* a significant main effect ($p<0.0001$). **Post hoc* using Dunnett's multiple comparison: * $p<0.05$ vs. saline. ^ *Post hoc* t-test with Bonferroni correction: drug alone vs. drug + nedocromil: $p<0.05$.

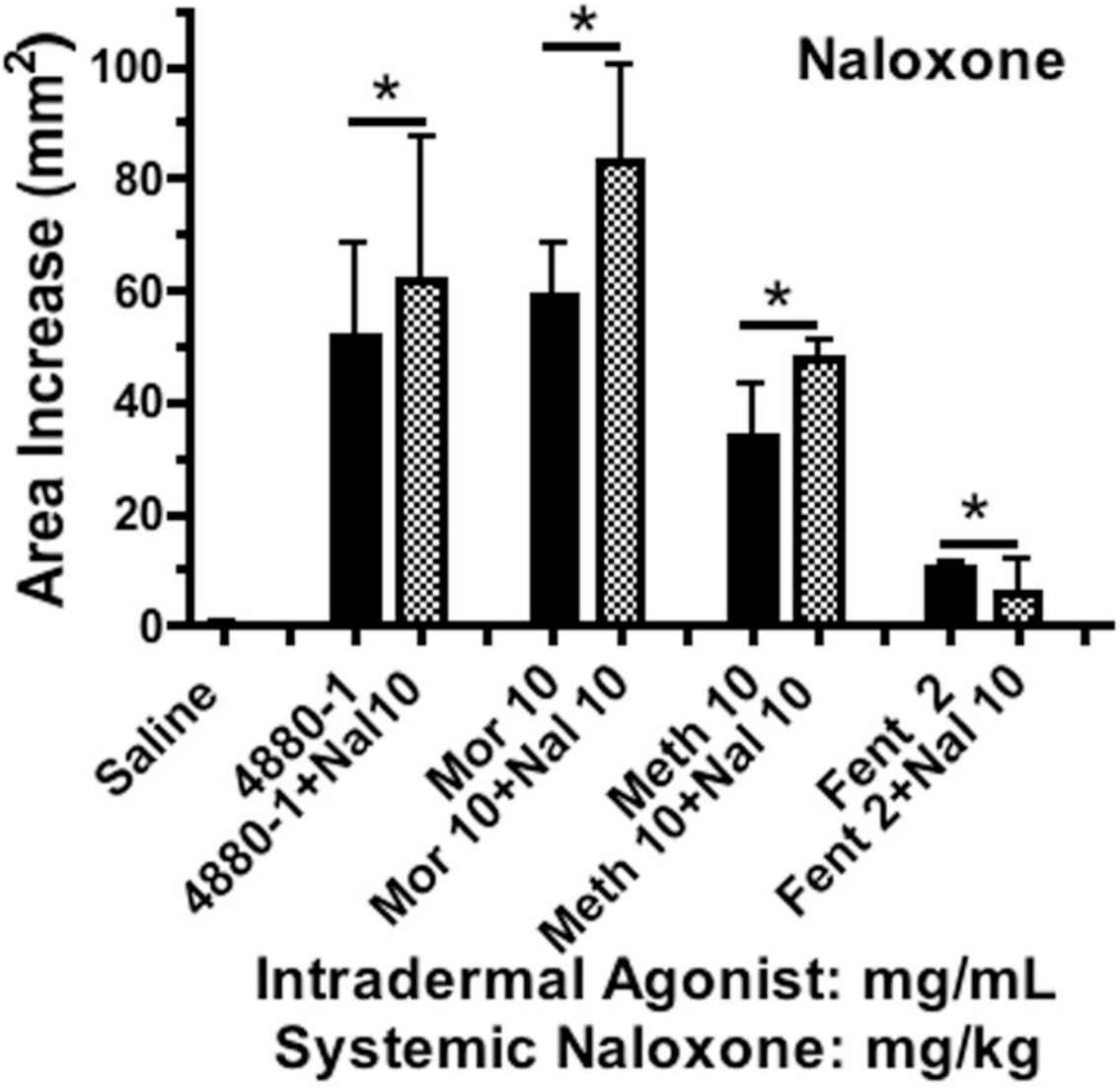


Figure 8.

Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of different concentrations of 48–80, morphine, methadone or fentanyl (mg/mL) in animals pretreated with naloxone (10mg/kg). Each histogram presents the mean ± SEM of 3–5 subcutaneous injections. 1-Way ANOVA analysis across treatments showed a significant main effect ($p<0.0001$). **Post hoc* using Dunnett's multiple comparison: * $p<0.05$ vs. saline. *Post hoc* t-test with Bonferroni correction: drug alone vs. drug + naloxone were not different ($p >0.05$).

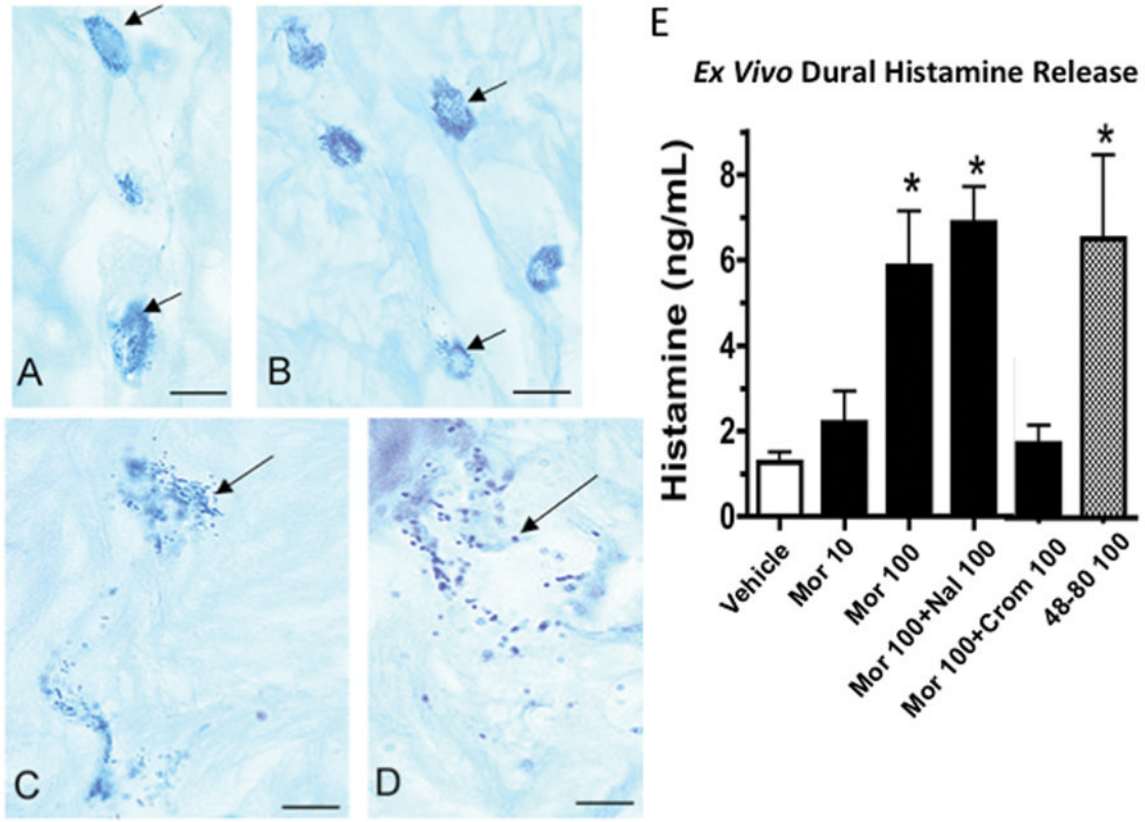


Figure 9.

Histology shows single Alcian Blue stained mast cells (see arrows) found in dog lumbar meninges harvested after exposure to A: vehicle, B: 10μM morphine, C/D: 100μM morphine (Bar = 20μ) E. *Ex vivo* histamine release from dural fragments after treatment as indicated (Mor: morphine; Nal: naloxone; Crom: cromolyn). Doses indicated are in μM indicated in legend. Each bar presents the mean ± SEM of 4–8 samples. 1-Way ANOVA analysis across treatments *showed* a significant main effect: $p<0.0001$. *Post hoc* using Dunnett's multiple comparison: * $p<0.05$ vs. vehicle (saline).

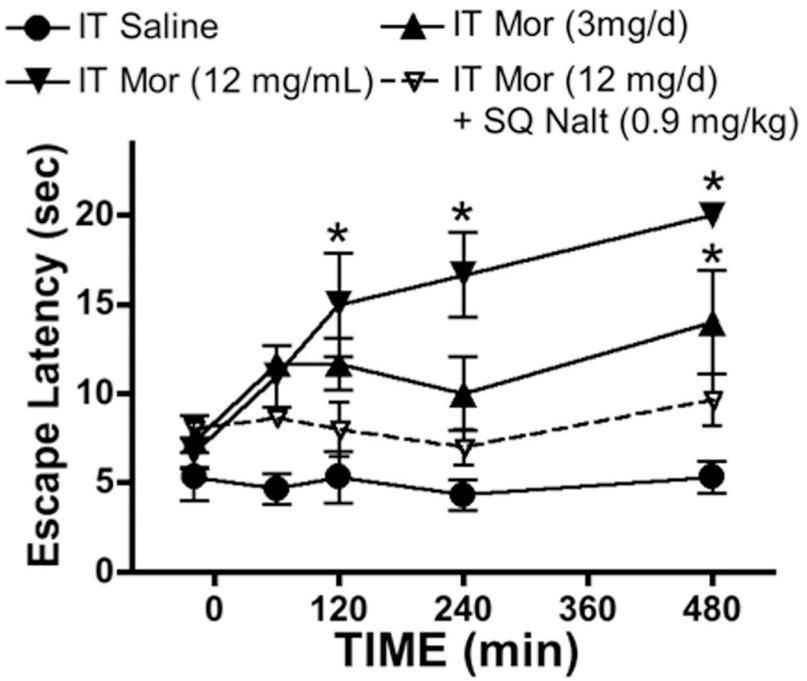


Figure 10.

Thermal escape latency (sec) in dogs receiving continuous infusion of the doses (mg/mL/d) indicated of morphine (Mor) alone or morphine with bolus subcutaneous delivery of naltrexone (Nalt: 0.9 mg/kg). Each line represents the mean ±SEM for 4 dogs. Data were analyzed with a 2-way repeated ANOVA across time. Main effects for time and treatments were significant ($p = 0.0002$ and $p = 0.0007$, respectively). Individual comparisons across treatments at each time point vs. saline were accomplished with a *post hoc* Bonferroni test. * $p<0.05$ vs. saline.

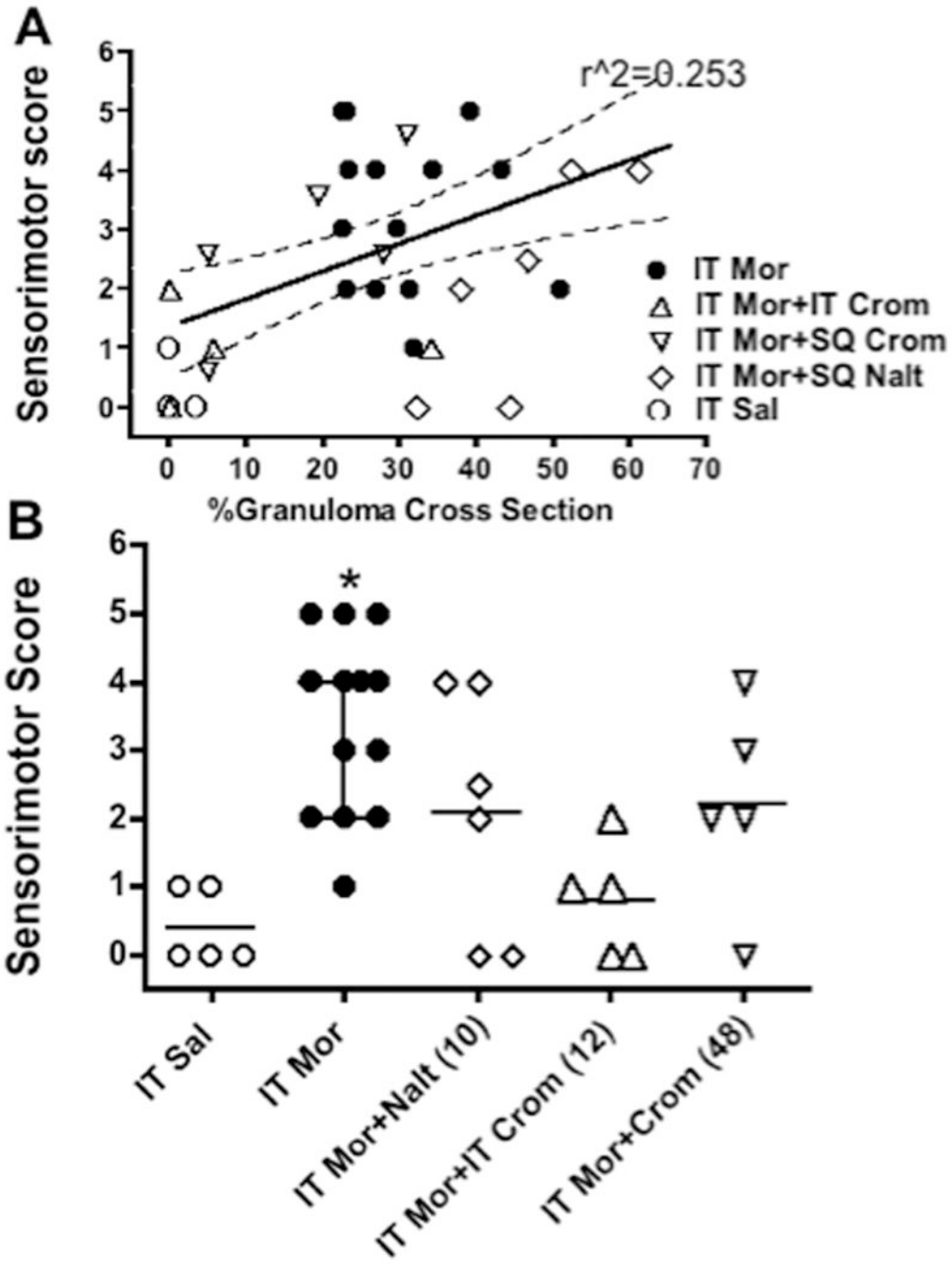


Figure 11.

A: Regression line (with 95% confidence intervals) of sensorimotor scores vs. % cross-sectional area of granuloma in individual dogs. Higher scores indicate increased side effects. See text. Symbols indicate treatment received by the individual dog. (Regression line slope was 0.047 ± 0.014 with an r^2 of 0.253). B: scattergram showing senori-motor score plotted vs. treatment. *Kruskal-Wallace comparison across groups was significant (p = 0.0025). Post hoc* comparison with a Dunn's multiple comparison: * p<0.05 vs. saline.

TABLE 1

Summary of Intrathecal infusion treatment groups

	TREATMENT GROUPS				
	Vehicle (1ml/d)	**Morphine (12.5 mg/mL/d)**	**Morphine (12.5 mg/mL/d) + SQ naltrexone (0.9mg/kg/d)**	**Morphine (12.5 mg/mL/d) + IT Cromolyn (12 mg/kg/d)**	**Morphine (12.5 mg/mL/d) + SQ Cromolyn (7.5 mg/kg/d)**
Group Size	5	15	6	5	5

Abbreviations: mg/mL/d: milligrams/milliliter/day; SQ: Subcutaneous; IT: intrathecal

Table 2

Histamine release *Ex Vivo* from dural fragments by agents

DRUG*	Histamine (ng/mL)	Release (ng/mL) (mean ± SD)	Significance (vs. Saline)#
Saline^	1.5, 1.8,1.0, 0.8, 0.9	1.2 ± 0.4	
Morphine^	3.9, 6.1, 5.1, 4.6, 10.6	6.1 ± 2.7	p<0.01
RS-Methadone	5.8, 3.2, 4.7	4.6 ± 1.3	P<0.05
Hydromorphone	6.1, 5.9, 3.8	5.3 ± 2.3	P<0.05
Fentanyl	1.1, 2.1, 2.2	1.8 ± 0.6	p>0.05

*100 μM; 30 min incubation,

^data same as in figure, p for comparison;

#One-way ANOVA across treatment groups (p = 0.0018) with comparison vs. saline using Dunnetts.