Role of Meningeal Mast Cells in Intrathecal Morphine–evoked Granuloma Formation

Tony L. Yaksh, Ph.D.,* Jeffery W. Allen, Ph.D.,† Samantha L. Veesart, L.A.T.,‡ Kjersti A. Horais, B.S.,§ Shelle A. Malkmus, B.S., R.V.T.,‡ Miriam Scadeng, M.D.,‖ Joanne J. Steinauer, B.S.,‡ Steve S. Rossi, Ph.D.#

ABSTRACT

Background: Intrathecal morphine forms granulomas that arise from the adjacent arachnoid membrane. The authors 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); and (3) *in vivo* granuloma pharmacology. Dogs with lumbar intrathecal catheters received infusion of intrathecal saline or intrathecal morphine. Intrathecal morphine dogs received (1) no other treatment (control); (2) twice-daily subcutaneous naltrexone; (3) intrathecal co-infusion of cromolyn; or (4) twice-daily subcutaneous cromolyn for the 24- to 28-day study course.
Results: Morphine but not fentanyl evoked dural histamine release, which was blocked by cromolyn but not naloxone. Wheal/flare was produced by subcutaneous morphine, methadone, hydromorphone, but not fentanyl, and was unaffected by naltrexone but prevented by cromolyn. Granulomas occurred in all dogs receiving intrathecal morphine (15 of 15); subcutaneous naltrexone had no effect on granulomas (six of six) but was reduced by concurrent intrathecal cromolyn (zero of five) or twice-daily subcutaneous cromolyn (one of five).
Conclusions: The pharmacology of cutaneous/dural mast cell degranulation and intrathecal granulomas are comparable, not mediated by opioid receptors, and reduced by agents preventing mast cell degranulation. If an agent produces cutaneous mast cell degranulation at concentrations produced by intrathecal delivery, the agent may initiate granulomas.

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 that 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

BY histopathology and magnetic resonance imaging, intrathecal infusion of high concentrations of morphine in canine and ovine models had no effect on spinal

◆ This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

* Professor of Anesthesiology and Pharmacology, ‡ Staff Research Associate, # Project Scientist, Anesthesiology Research, Department of Anesthesiology, University of California, San Diego, La Jolla, California. † Study Director, Anesthesiology Research, Department of Anesthesiology, University of California, San Diego, La Jolla, California. Current address: MPI Research, Mattawan, Michigan. § Senior Quality Assurance Specialist, Anesthesiology Research, Department of Anesthesiology, University of California, San Diego, La Jolla, California. Current address: Santarus Inc., San Diego, California. ‖ Associate Professor, Department of Radiology, University of California, San Diego, La Jolla, California.

Received from the Department of Anesthesiology, University of California, San Diego, La Jolla, California. Submitted for publication July 25, 2012. Accepted for publication October 26, 2012. This project was supported by National Institutes of Health grant NIDA-15353 (to Dr. Yaksh), Bethesda, Maryland. The Alfred Mann Foundation (Santa Clarita, California) provided a number of the MiniMed pumps (Medtronic, Northridge, California) used in these investigations. This work has been reported as an abstract and presented, in part, at the Annual Meeting of the Society of Toxicology, Baltimore, Maryland, March 21 2004.

Address correspondence to Dr. Yaksh: Anesthesiology Research, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0818. tyaksh@ucsd.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

 Anesthesiology 2013; 118:664-78

parenchyma but led to an aseptic collection of inflammatory cells (granuloma).[1–4] Preclinical work shows that granulomas have several attributes, as follows: (1) the incidence is concentration dependent[2–5] and not observed after repeated bolus delivery[6]; (2) using serial magnetic resonance imaging 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 size of the mass over the ensuing 14 days[5]; and (3) 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.[7–10]

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 Yaksh *et al.*[3] and Lehmberg *et al.*[11]). Solutions are typically prepared to have cerebrospinal fluid–compatible osmolarity and pH.[3,12,13] Finally, infusion of vehicle (saline)[2–4] or a variety of drugs (see, *e.g.*, Sabbe *et al.*,[14] Yaksh *et al.*,[15] and Chiari *et al.*[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, casting doubt as to whether this effect is opioid receptor mediated[12] (but see Johansen et al.).[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]; and (4) because 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 phenomenon 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 in this article 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 (two male and three female animals, weighing approximately 25–35 kg) undergoing terminal experiments examining cardiac function were used.

Meningeal Harvest. The animals were deeply anesthetized to the minimum of a surgical plane anesthesia sufficiently deep to perform a laminectomy (35 mg/kg sodium pentobarbital IV or 5–10 $\mu g \cdot kg^{-1} \cdot min^{-1}$ propofol IV). The lumbar and thoracic vertebral bodies were exposed, cardiac arrest was initiated, and the dura was 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 Eppendorf tubes filled with Krebs–Ringer's solution (1 ml) 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-μm sections were taken and mounted on glass slides.

Assay of Mast Cell Mediators. Each incubation sample (100 μl) was assayed for histamine content. Histamine was analyzed using enzyme-linked immunosorbent assay (Research Diagnostics, Inc., Flanders, NJ). The limit of detection of this assay was approximately 0.3 ng/ml. The intraaasy and interassay precision was less than 11.0 and less than 12.0% coefficient of variation, 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 the manufacturer (Sigma-Aldrich, St. Louis, MO). Samples were surveyed under a microscope at 20× magnification. In each fragment, five stained sections were then examined at 60× magnification in a 100-μm field taken in the center of each fragment.

Table 1. Summary of Intrathecal Infusion Treatment Groups

	Treatment Groups				
	Vehicle (1 ml/d)	Morphine (12.5 mg·ml^{-1}·d^{-1})	Morphine (12.5 mg·ml^{-1}·d^{-1}) + SQ naltrexone (0.9 mg·kg^{-1}·d^{-1})	Morphine (12.5 mg·ml^{-1}·d^{-1}) + IT cromolyn (12 mg·kg^{-1}·d^{-1})	Morphine (12.5 mg·ml^{-1}·d^{-1}) + SQ cromolyn (7.5 mg·kg^{-1}·d^{-1})
Group Size	5	15	6	5	5

SQ = subcutaneous; IT = intrathecal.

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 and weighing approximately 9–16 kg, were housed individually in runs with wood shavings and given *ad libitum* access to food and water. Animals were adapted for a minimum of 10 days before surgery. A nylon vest for pump placement was placed on each dog 48 hours before 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-gauge needle.

Continuous Intrathecal Drug Delivery. Continuous intrathecal drug delivery was performed as follows:

1. Surgical preparation. Dogs were prepared with chronic intrathecal catheters by surgical placement of the intrathecal catheter approximately 72 h before dosing. The antibiotic sulfamethoxazole–trimethoprim (240-mg tablet, 15–25 mg/kg orally, twice daily) was given 48 h before and after surgery. Dogs received atropine (0.04 mg/kg IM) before sedation with xylazine (1.5 mg/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 monitored continuously for oxygen saturation; inspired and end-tidal values of isoflurane, Carbon monoxide, nitrous oxide, and oxygen; and heart and respiratory rates. Surgical areas were shaved and prepared with chlorhexidine scrub and solution. Using sterile technique, the dura of the cisterna magna was exposed. Through a small incision (1–2 mm), the intrathecal catheter was inserted and passed caudally a distance of approximately 40–42 cm to a level corresponding to the L2–L3 segment. The catheter was fabricated of polyethylene or polyurethane tubing (0.61-mm OD), packaged, and sterilized by E-beam irradiation. Dexamethasone sodium phosphate (0.25 mg/kg IM) was administered just after catheter placement. The external catheter was tunneled to exit at the left scapular region. On closure of the incision, isoflurane was removed and the animal allowed to recover. Then, 0.04 mg/kg butorphanol tartrate (Tobugesic®; Fort Dodge Animal Health, New York, NY) was administered IM on recovery and as necessary to relieve postoperative discomfort. Following recovery, a nylon vest was placed on the animal and an infusion pump (MiniMed 507; Medtronic MiniMed, Northridge, CA) was secured in the vest pocket, where it was connected to the externalized end of the intrathecal catheter.
2. Initiation of continuous intrathecal morphine infusion. All intrathecal infusions were carried out at 40 μl/h = 960 μl/day = 1 ml/day. As reported previously, the initiation of infusion of 12 mg/day 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 3 mg/day to 12 mg/day could be accomplished over a period of approximately 4–7 days (and then continued to 24–28 days) and served to minimize adverse events. This protocol was followed in these studies.
3. 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: 0–3) were assessed daily. These indices have been validated previously.[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 = complete disability and requirement for immediate euthanasia).
4. 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^{-1}·day^{-1} and 12-mg·ml^{-1}·day^{-1} 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 subcutaneously) (n = 4). Hind-paw thermal escape latencies were assessed using a Hargreaves-type stimulator wherein the hind paws 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 s, the stimulus was terminated and that latency assigned as the response.[34]

5. Necropsy. Dogs were anesthetized deeply with an IV dose of propofol (5–10 mg·kg^{-1}·min^{-1}) or sodium pentobarbital (35 mg/kg or to effect). Animals were exsanguinated by perfusion with saline (approximately 4 l) followed by 10% formalin (approximately 4 l) 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 and the location of the catheter tip were noted *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 the catheter tip).
6. Histochemistry. Spinal blocks from perfusion-fixed animals were embedded in paraffin, sectioned at approximately 4–8 μm thickness, and stained with hematoxylin and eosin. To assess mast cell condition, a fragment of dura was also stained with either Fast Red or Alcian Blue. To estimate cross-sectional area of the granuloma, hematoxylin and eosin–stained sections were 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 outlined manually 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 square millimeters.
7. Postmortem 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 dimethyl sulfoxide. Images were acquired with a 7-T magnetic resonance imaging scanner using three-dimensional fast spoiled gradient echo sequence (repetition time, 10 ms; echo time, 2.9 ms; flip angle, 20 degrees; field of view, 15 mm; matrix, 256 × 256; slice thickness, 100 μm). Cross-sectional images were created and granulomatous tissue areas outlined manually. 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 weighing approximately 7–12 kg (n = 4) were anesthetized with IV propofol (5 μg·kg^{-1}·min^{-1}). Animals were intubated, and body temperature was maintained with an underbody heating pad and monitored continuously as described above. The chest and abdomen were shaven and surgically prepared. Intradermal injections of drug solutions were delivered in 50 μl at time 0 at 12–14 sites (six or seven per 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 time 0 and at 10, 30, and 60 min. Flare area immediately after injection and at intervals were calculated in square millimeters as an oval ($3.14 \times a \times b$, 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 five to seven times.

Drugs

Preservative-free, U.S. 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 used were prepared from the powder. D/L-Methadone HCl and fentanyl HCl were obtained from the National Institute on Drug Abuse. The opiate antagonists naloxone HCl and 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 stated otherwise. Doses and concentrations used are indicated in the text.

Statistical Analysis

Comparisons across treatment groups for granuloma size and for sensorimotor scores used the nonparametric Kruskal-Wallis test with *post hoc* comparisons to the saline treatment group using the Dunn 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 the Dunnett multiple comparison test. 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 ANOVA was performed with *post hoc* comparisons using the Bonferroni test. To relate degree of sensorimotor deficits to 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. The GraphPad Prism software package (v.4.0c for MAC OS X; GraphPad Software Inc., La Jolla, CA) was used for all analyses.

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 through approximately 4 weeks (fig. 1). In contrast, morphine at 12 mg/day (*e.g.*, 12.5 mg/ml delivered

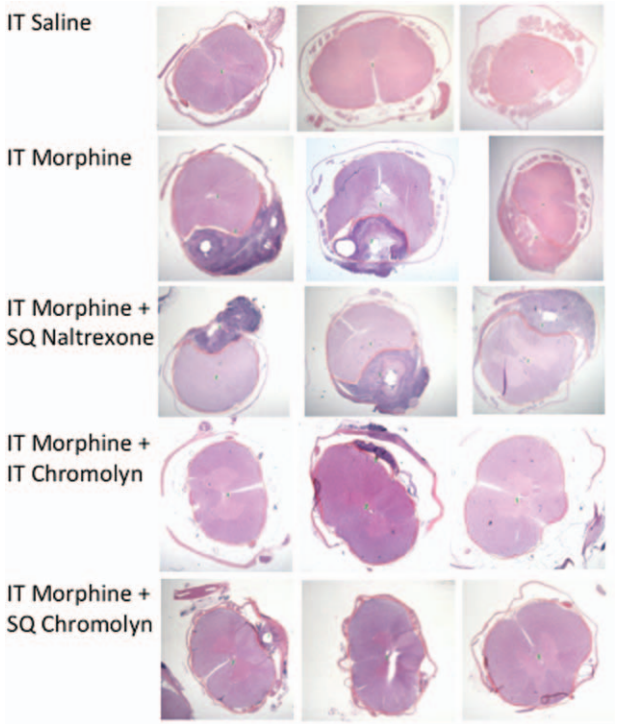


Fig. 1. Representative lumbar hematoxylin and eosin–stained sections from three of the 5–15 dogs that received 24–28 days of intrathecal (IT) saline (*above*), IT morphine (12 mg/ml) (*second row*), IT morphine (12 mg/ml) plus twice-daily naltrexone (*third row*), IT morphine (12 mg/ml) plus IT cromolyn (12 mg/ml) (*fourth row*), or IT morphine (12 mg/ml) plus twice-daily subcutaneous cromolyn (7.5mg/kg) (*below*). Dark reaction product indicates granuloma. SQ = subcutaneous.

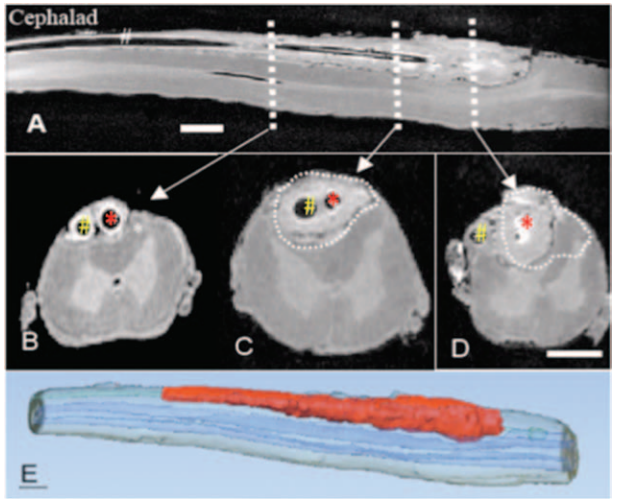


Fig. 2. *Ex vivo* postmortem magnetic resonance imaging scans through the lumbar spinal cord of the dog with sampling catheter (*red arrow*) and infusion catheter (*white arrow*) that received a 28-day infusion of intrathecal morphine sulfate (12 mg/day). (*A*) Midsagittal plane through the lumbar cord. (*B–D*) Three transverse sections taken at the levels indicated. In *B*, the sampling catheter and the infusion catheter are 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* magnetic resonance imaging scan. Red and blue volumes indicate granulomatous mass and spinal cord, respectively.

at 960 μl/h) in the dog led to the development of prominent granulomas within 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/day morphine for approximately 28 days. Using *ex vivo* magnetic resonance imaging, volumetric reconstruction of the granuloma of four dogs that received 12 mg/day for greater than 24 days revealed calculated mass volumes of 0.42, 0.39, 0.31, and 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 (fig. 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/day), the area of the granuloma at the level of largest girth was calculated. The median of the maximum area of the pericatheter mass was 9.5 mm^2 (interquartile range [IQR], 8.1–14.0 mm^2). The corresponding median percentage of the cross-sectional area was 27.1% (IQR, 23.3–33.1%), emphasizing the compressive nature of these masses. The distribution of the cross-sectional area and percentage of the cross-sectional area of these masses is presented in figure 3 and 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 (fig. 4A). Typically, in any given section, although 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–C3) levels, where no granuloma was present, revealed significant numbers of intact granule-containing profiles (fig. 4C). Similar staining profiles were observed with

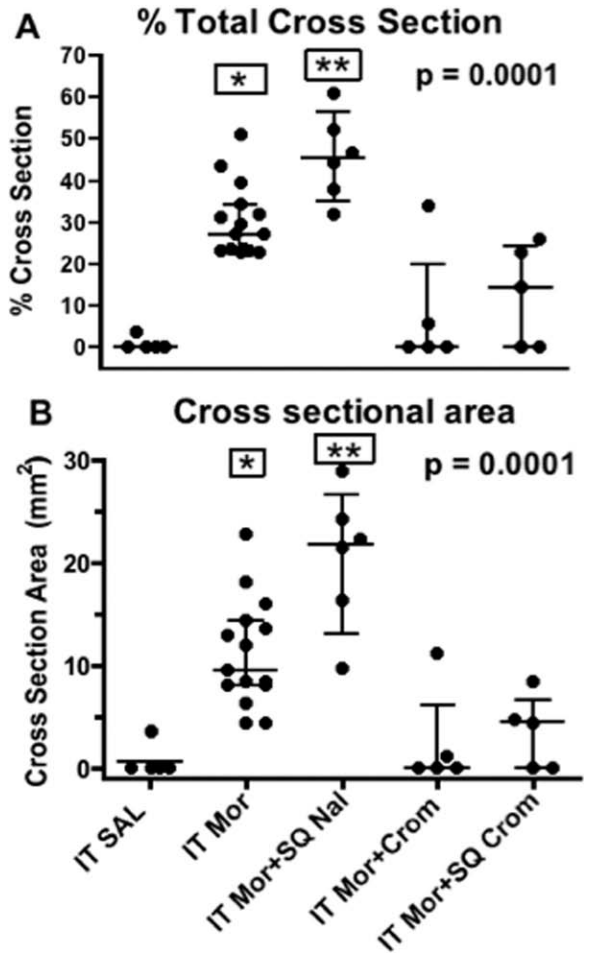


Fig. 3. Scattergram of (*A*) percentage cross-sectional area of mass to total spinal area and (*B*) cross-sectional area (in square millimeters) of the granulomatous mass at the catheter tip for dogs receiving intrathecal (IT) saline, IT morphine (12 mg/day), IT morphine (12 mg/d) plus subcutaneous naltrexone twice daily (0.9 mg/kg), IT morphine (12 mg/day) plus IT cromolyn (12 mg/day), or IT morphine (12 mg/day) plus subcutaneous cromolyn twice daily (7.5 mg/kg). Each point represents a single dog receiving approximately a 28-day IT infusion. *Error bars* indicate the median with 25th and 75th percentiles. 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 one-way ANOVA.) *Post hoc* comparison to the saline treatment group using Dunn multiple comparison: * $P < 0.01$; ** $P < 0.001$. Crom = cromolyn; Mor = morphine; Nal = naltrexone; SAL = saline; SQ = subcutaneous.

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 flare produced by side-by-side subcutaneous injections of 50 μl 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 used 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 (2 mg /ml) produced little flare, which, although different from saline, was difficult to discern. Higher doses of fentanyl were not examined, as at these concentrations, the multiple 50-μl injections resulted in delivery of a dose equivalent to approximately 10–20 μg/kg and produced a decreased respiratory rate. However, no clinically significant desaturation was noted in those studies.

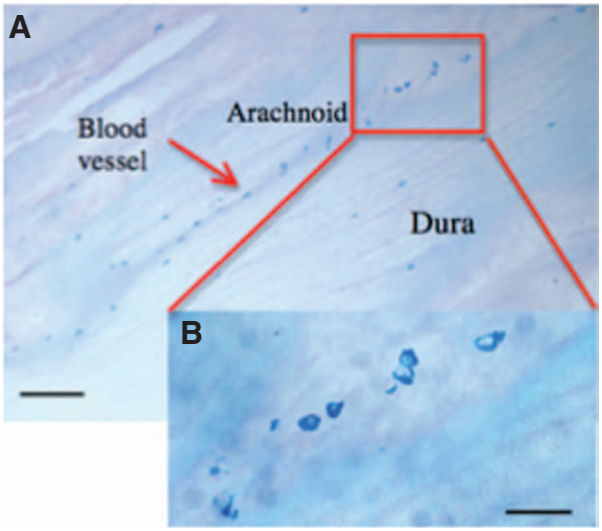
Mast Cell Stabilization. To determine whether these effects were mediated by mast cell degranulation, dogs were pretreated 60 min in advance with the mast cell stabilizers cromolyn (7.5 mg/kg IM) or nedocromil (4.5 mg/kg IM). As indicated in figures 6 and 7, both agents completely blocked the flare response otherwise produced by all agents. In a dose-ranging study, a lower dose of cromolyn (2.5 mg/kg IM) incompletely reduced the flare after Compound 48/80 and morphine (data not shown). Diphenhydramine (10 mg/kg IM), the H_1-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 before the agonists, failed to block the flare change otherwise produced by each of the agents (fig. 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



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 (40 μL/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 40 μL/hr		

Fig. 4. (*A*) Section showing Alcian Blue–stained lumbar dura mast cells in control animal at low power (*bar* = 100 μm). (*B*) Inset showing enlargement of marked area at high power (*bar* = 30 μm). 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 three animals receiving IT morphine (12 mg/day).

significant concentration-dependent degranulation of mast cells (fig. 9, A–D) and an increase in histamine in the dural supernatant (fig. 9A) over the range of morphine concentrations of 10 to 100 μM. As indicated, morphine in this preparation is at least as potent as the release evoked by 100 μM 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 to a concentration of 100 μM yielded significant increases in histamine release different from saline and comparable to those observed with 100 μM 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 (fig. 9A). Addition of morphine resulted in a prominent change in appearance of mast cell staining, with an increasing fraction of the dural tissues showing varying degrees of disrupted cellular profiles with stain-free core indicative of degranulation (fig. 9, C and 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/day) resulted in an increase in hind-paw thermal escape latency as compared with vehicle (saline). In a separate group, a single injection of naltrexone (0.9 mg/kg) prevented the increase over this 8-h interval (fig. 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 subcutaneously). As indicated in figure 3, in animals receiving 12 mg/day morphine for approximately 4 weeks with this twice-daily dose of naltrexone, the appearance (fig. 1) and incidence of granuloma formation was not different from that observed with morphine alone. Thus, the median cross-sectional area in dogs receiving intrathecal morphine alone or with naltrexone was 9.5 mm^2 (IQR, 8.1–14.0 mm^2) and 21.9 mm^2 (IQR, 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 dose of 12 mg/day morphine with an admixture of cromolyn, yielding cromolyn infusion concentrations of 12 mg/day (n = 5). These doses were based on the *ex vivo* work (fig. 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 morphine (12 mg/ml) over an interval of 24–28 days were reduced by concurrent intrathecal cromolyn (one of five with granuloma; median percentage of the cross-sectional area with quartiles, 0 mm^2 [IQR, 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 ($P > 0.05$) (fig. 3). Two dogs received an intrathecal infusion of cromolyn (12 mg/day) alone. In this limited series, no untoward effects on behavior were observed, and hematoxylin and eosin staining showed no untoward signs (data not shown).

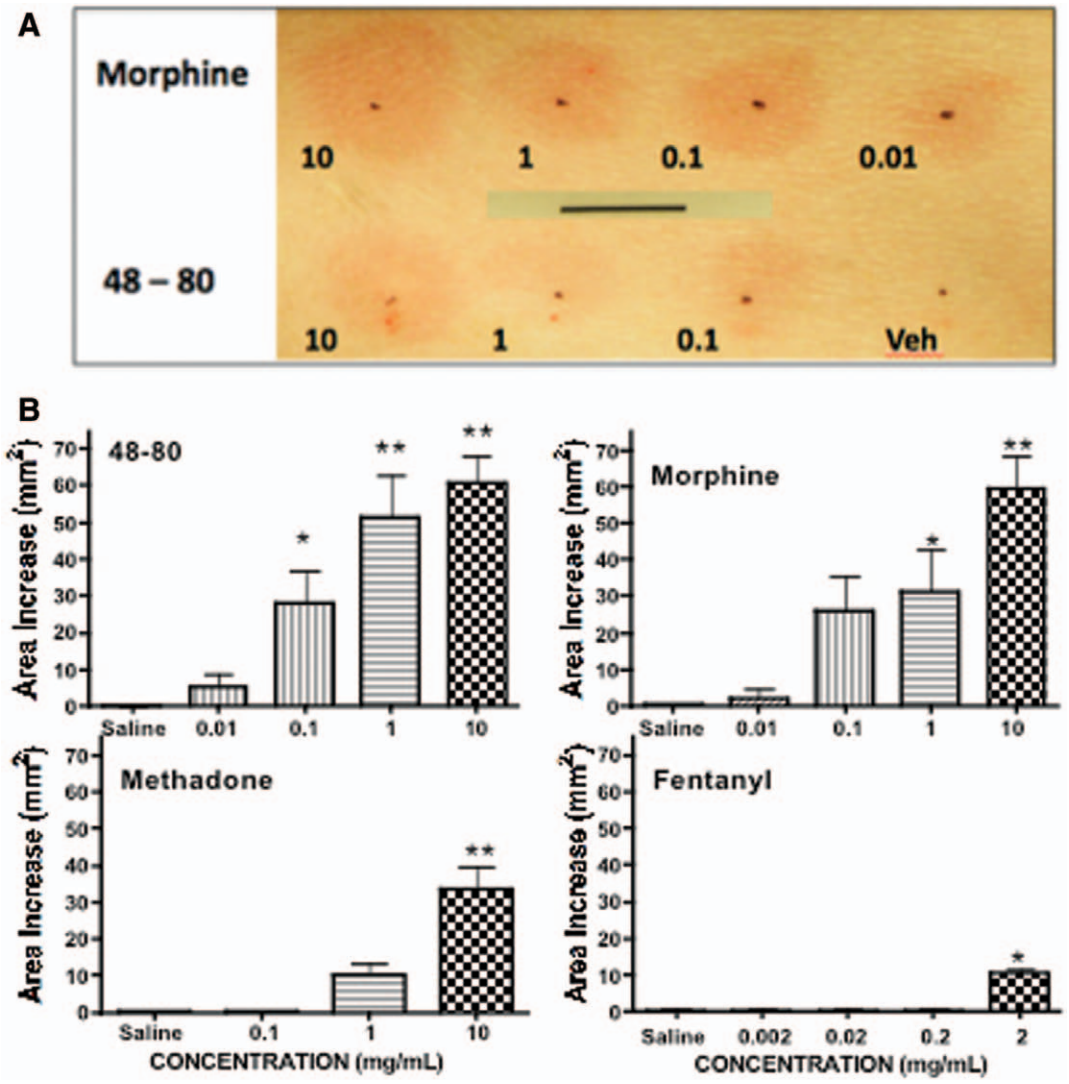


Fig. 5. (*A*) Representative photograph of dog abdomen showing cutaneous flares taken at 30 min after the subcutaneous injection of different concentrations (in milligrams per milliliter) of morphine or Compound 48/80 in 50 μl. *Bar* = 10 mm. (*B*) Histograms showing the flare area at approximately 30 min after subcutaneous injection of different concentrations of Compound 48/80, morphine, methadone, or fentanyl. Each histogram presents the mean ± SEM of four to six subcutaneous injections. One-way ANOVA of each drug across its treatment doses showed a significant main effect for each drug ($P < 0.0001$). *Post hoc* using Dunnett multiple comparison: *$P < 0.05$ *versus* saline; **$P < 0.01$ *versus* saline.

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 12 mg/day morphine and twice-daily dosing of 7.5 mg/kg cromolyn (n = 5). These doses were based on the *in vivo* degranulation work (fig. 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 was 4.5 mm^2 (IQR, 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 described previously,[3] the initiation of continuous infusion of 12 mg/day 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 neurologic signs that included hind-limb spasticity, altered gait, and an increased sensitivity to touch applied to the hindquarters. 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, 6) as compared with a median score of 0. Comparison across all treatment groups revealed a significant main

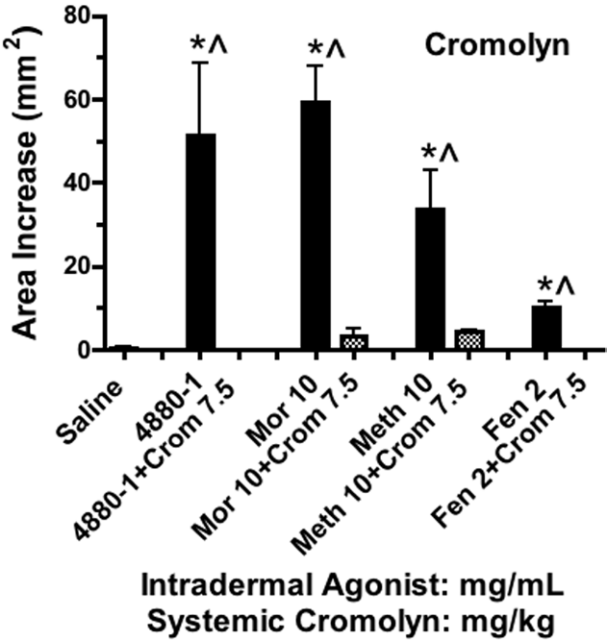


Fig. 6. Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of Compound 48/80, morphine, methadone, or fentanyl (in milligrams per milliliter) in 50 μl in animals pretreated with cromolyn (7.5 mg/kg). Each histogram presents the mean ± SEM of three to five subcutaneous injections. One-way ANOVA across treatments showed a significant main effect ($P < 0.0001$). *Post hoc* using Dunnett multiple comparison: * $P < 0.05$ *vs.* saline. ^ *Post hoc t* test with Bonferroni correction, drug alone *vs.* drug plus cromolyn: $P < 0.05$. Crom = cromolyn; Fen = fentanyl; Mor = morphine.

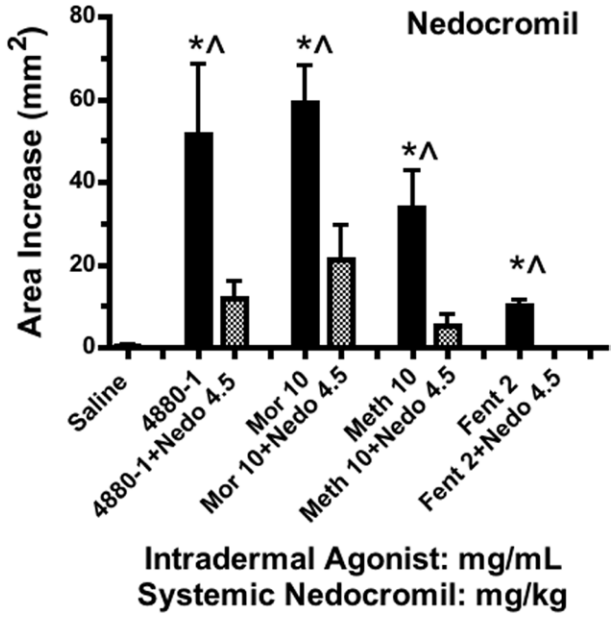


Fig. 7. Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of Compound 48/80, morphine, methadone, or fentanyl (in milligrams per milliliter) in animals pretreated with nedocromil (4.5 mg/kg). Each histogram presents the mean ± SEM of three to five subcutaneous injections. One-way ANOVA across treatments showed a significant main effect ($P < 0.0001$). *Post hoc* using Dunnett multiple comparison: * $P < 0.05$ vs. saline. ^ *Post hoc t* test with Bonferroni correction, drug alone *vs.* drug plus nedocromil: $P < 0.05$. Fent = fentanyl; Meth = methadone; Mor = morphine; Nedo = nedocromil.

effect, but only the morphine *versus* saline comparison was significant. We plotted the motor score against the percentage cross-sectional area of the granuloma and observed a regression that, although statistically nonzero (slope, 0.047; 95% CI, 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 of the granuloma. Thus, several animals with significant compression showed virtually no deficits at the time of euthanasia. This was a conclusion that we reached previously.[3]

Discussion

Chronic intrathecal morphine initiates a volume-occupying accumulation of inflammatory cells derived 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 equianalgesic concentrations of morphine, hydromorphone, or methadone, but not fentanyl, results 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. Although higher doses of naltrexone might be required to reverse a possible interaction, we note the following. First, naltrexone and its metabolite 6-beta-naltrexol have half-lives of several hours, with that of the metabolite being longer[37] (but see Garrett and el-Koussi[38]). 6-Beta-naltrexol has opiate antagonist properties that may account for the long duration of blockade by the parent.[39] Second, the dose used resulted in a significant antagonism of the analgesic effects produced by the granuloma-producing dose for an excess of 8 h. Accordingly, twice-daily dosing would result in an excess of 16 h 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. Third, 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 the granuloma-inducing effects of intrathecal morphine are not dependent on 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

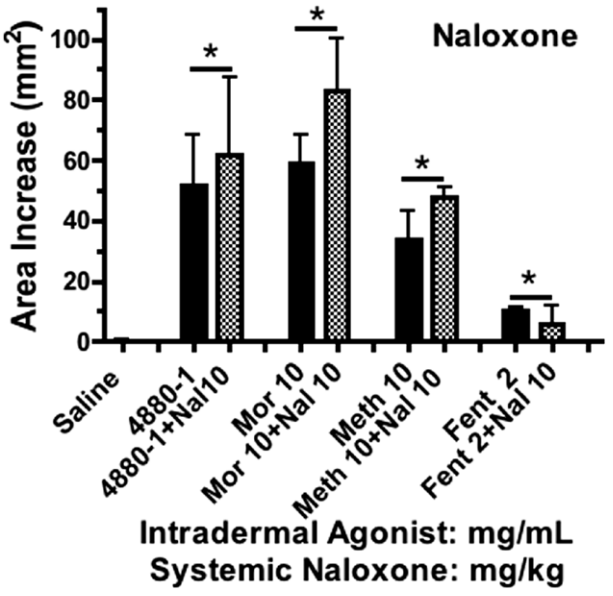


Fig. 8. Histograms showing the cutaneous flare area at approximately 30 min after subcutaneous injection of different concentrations of Compound 48/80, morphine, methadone, or fentanyl (in milligrams per milliliter) in animals pretreated with naloxone (10 mg/kg). Each histogram presents the mean ± SEM of three to five subcutaneous injections. One-way ANOVA across treatments showed a significant main effect ($P < 0.0001$). *Post hoc* using Dunnett multiple comparison: * $P < 0.05$ *versus* saline. *Post hoc t* test with Bonferroni correction: drug alone *versus* drug plus naloxone were not different ($P > 0.05$).

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[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 meningeal mast cells 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 (*i.e.*, a flare). Work focusing on the pharmacology of this cutaneous effect shows that although 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] results 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 that 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 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 pharmacologic profiles.[54,55]

How do opiates act to degranulate mast cells? We note several possibilities:

1. 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]
2. 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]
3. 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 the 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 (1) block of chloride transport in mast and nerve cells,[69] (2) reduced substance P binding, and (3) 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, nonchromane mast

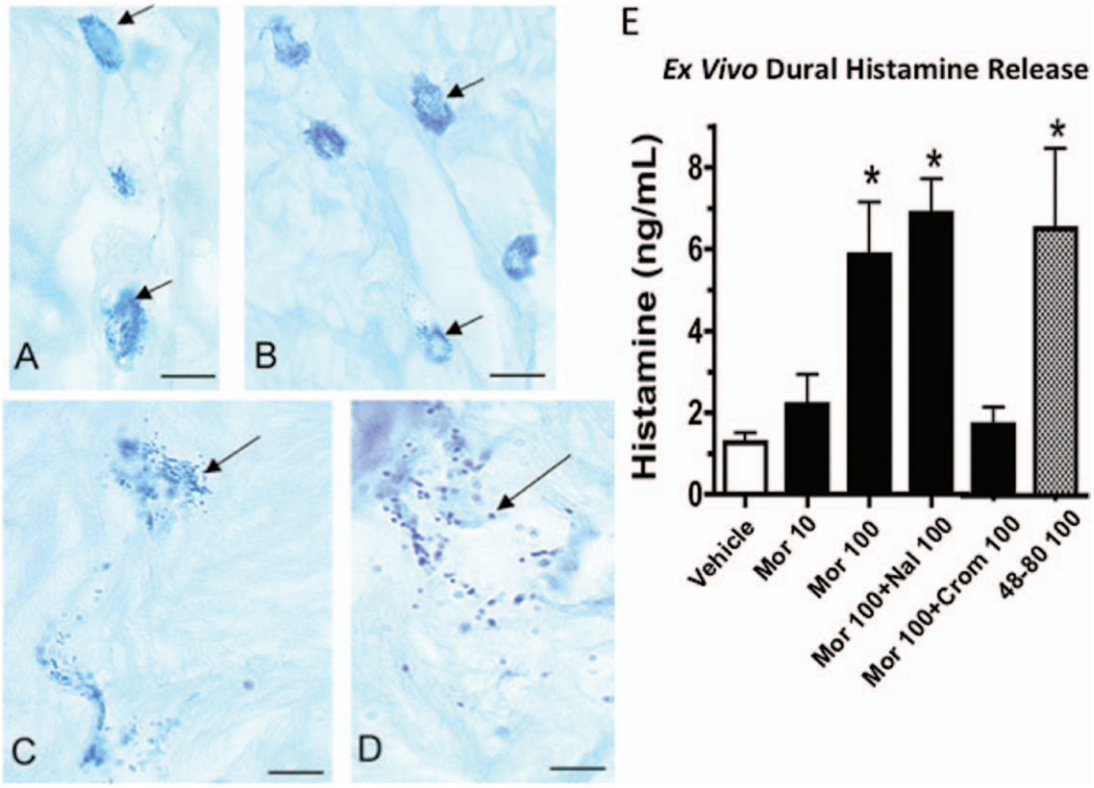


Fig. 9. Histology shows single Alcian Blue–stained mast cells (*arrows*) found in dog lumbar meninges harvested after exposure to (*A*) vehicle, (*B*) 10 μM morphine, or (*C* and *D*) 100 μM morphine (*bar* = 20 μm). (*E*) *Ex vivo* histamine release from dural fragments after treatment as indicated. Mor = morphine; Nal = naloxone; Crom = cromolyn. Doses indicated are in micromoles, indicated in legend. Each bar represents the mean ± SEM of four to eight samples. One-way ANOVA across treatments showed a significant main effect ($P < 0.0001$). *Post hoc* using Dunnett multiple comparison: * $P < 0.05$ *versus* vehicle (saline).

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:

1. 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 2 $mg \cdot ml^{-1} \cdot day^{-1}$. Consistent with this hypothesis, intradermal alfentanil had minimal effects on mast cell degranulation when delivered by continuous intrathecal infusion at the maximum tolerable dose of 20 $mg \cdot ml^{-1} \cdot day^{-1}$ and resulted in minimal dural reactions.[71] Anilinopiperidines are rarely used 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/day (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 (17 μg/day) in a patient with a history of exposure to several agents including fentanyl, baclofen, and ziconotide also developed a granuloma.[73]

Table 2. Histamine Release *Ex Vivo* from Dural Fragments by Agents

Drug*	Histamine (ng/ml)	Release (ng/ml) (mean ± SD)	*P* Value (*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	<0.01
RS-methadone	5.8, 3.2, 4.7	4.6±1.3	<0.05
Hydromorphone	6.1, 5.9, 3.8	5.3±2.3	<0.05
Fentanyl	1.1, 2.1, 2.2	1.8±0.6	>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 the Dunnett test.

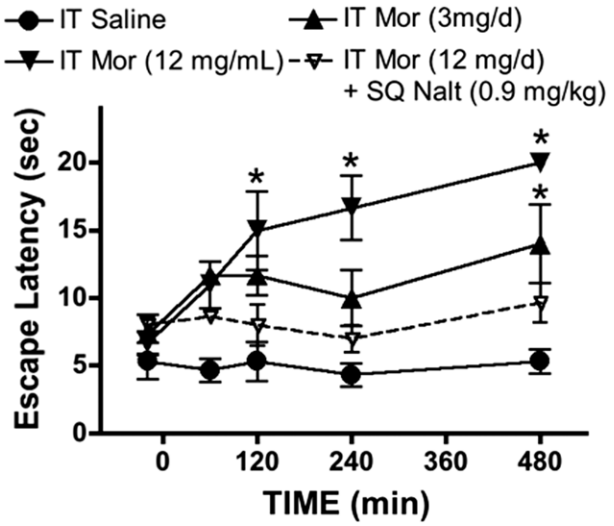


Fig. 10. Thermal escape latency (in seconds) in dogs receiving continuous infusion of the doses (in milligrams per milliliter per day) indicated of morphine alone or morphine with bolus subcutaneous delivery of naltrexone (0.9 mg/kg). Each *line* represents the mean ± SEM for four dogs. Data were analyzed with a two-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 *versus* saline were accomplished with a *post hoc* Bonferroni test: * P <0.05 *versus* saline. IT = intrathecal; Mor = morphine; Nalt = naltrexone; SQ = subcutaneous.

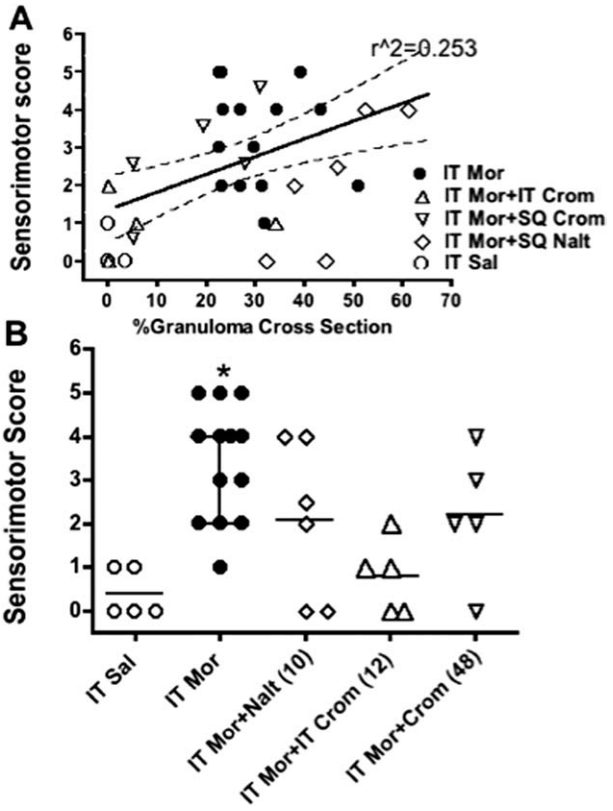


Fig. 11. (*A*) Regression line (with 95% CI) of sensorimotor scores *versus* percentage cross-sectional area of granuloma in individual dogs. Higher scores indicate increased side effects. See text. Symbols indicate treatment received by the individual dog (regression line slope was 0.047 ± 0.014 with an r^2 of 0.253). (*B*) Scattergram showing sensorimotor score plotted *versus* treatment. Kruskal-Wallis comparison across groups was significant (P = 0.0025). *Post hoc* comparison with a Dunn multiple comparison: * P < 0.05 *versus* saline. Crom = cromolyn; IT = intrathecal; Mor = morphine; Nalt = naltrexone; SAL = saline; SQ = subcutaneous.

These examples suggest the possible role of mechanisms in addition to potential contribution of mast cells, particularly at high concentrations.

2. 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]
3. 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 used. A second *caveat* is that if the local drug target requires a low concentration relative to that required to degranulate mast cells, 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] and 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, although 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 granulomas in humans.

In conclusion, although granuloma formation is often considered to be clinically infrequent, two observations are telling: (1) there is an increasing incidence of observations/reports and in postmarketing surveillance reports describing these space-occupying masses, and (2) among clinicians

using intrathecal analgesics, 66% report a patient experiencing neurologic injury secondary to a granuloma (see Deer et al. 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. Furthermore, the canine work demonstrates a mast cell effect with *pretreatment*. We do not know whether 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 used. Further preclinical work[84,85] defining such parameters and safety is required to evaluate the risk-to-benefit ratio of this or any adjunctive therapy.

The authors particularly acknowledge their debt to Christopher M. Bernards, M.D. (Department of Anesthesiology, University of Washington, Seattle, Washington), who pointed the authors to the similarity of the meningeal phenomena to the events that occur in migraine, the aberrant effects of opiates in degranulating mast cells, and the potential role of mast cells. Sadly, Dr. Bernards passed away on January 12, 2012. The authors dedicate this article to his memory. The authors also thank Nicole A. Tozier, A.H.T. (retired), Eddie Ruhland, B.S. (San Diego), Mary Ceccolini, D.V.M. (San Diego); Dasa Cizkova, D.V.M. (Institute of Neurobiology, Slovak Academy of Sciences); and Ashley Wiese, D.V.M. (MedVet Medical & Cancer Centers, Cincinnati, Ohio), for their technical contributions to these studies.

References

1. North RB, Cutchis PN, Epstein JA, Long DM: Spinal cord compression complicating subarachnoid infusion of morphine: Case report and laboratory experience. Neurosurgery 1991; 29:778–84
2. Gradert TL, Baze WB, Satterfield WC, Hildebrand KR, Johansen MJ, Hassenbusch SJ: Safety of chronic intrathecal morphine infusion in a sheep model. Anesthesiology 2003; 99:188–98
3. Yaksh TL, Horais KA, Tozier NA, Allen JW, Rathbun M, Rossi SS, Sommer C, Meschter C, Richter PJ, Hildebrand KR: Chronically infused intrathecal morphine in dogs. Anesthesiology 2003; 99:174–87
4. Michael A, Buffen E, Rauck R, Anderson W, McGirt M, Mendenhall HV: An *in vivo* canine study to assess granulomatous responses in the MedStream Programmable Infusion System™ and the SynchroMed II Infusion System®. Pain Med 2012; 13:175–84
5. Allen JW, Horais KA, Tozier NA, Wegner K, Corbeil JA, Mattrey RF, Rossi SS, Yaksh TL: Time course and role of morphine dose and concentration in intrathecal granuloma formation in dogs: A combined magnetic resonance imaging and histopathology investigation. Anesthesiology 2006; 105:581–9
6. Sabbe MB, Grafe MR, Mjanger E, Tiseo PJ, Hill HF, Yaksh TL: Spinal delivery of sufentanil, alfentanil, and morphine in dogs: Physiologic and toxicologic investigations. Anesthesiology 1994; 81:899–920
7. Yaksh TL, Hassenbusch S, Burchiel K, Hildebrand KR, Page LM, Coffey RJ: Inflammatory masses associated with intrathecal drug infusion: A review of preclinical evidence and human data. Pain Med 2002; 3:300–12
8. Coffey RJ, Burchiel K: Inflammatory mass lesions associated with intrathecal drug infusion catheters: Report and observations on 41 patients. Neurosurgery 2002; 50:78–86; discussion 86–7
9. Deer TR, Prager J, Levy R, Rathmell J, Buchser E, Burton A, Caraway D, Cousins M, De Andrés J, Diwan S, Erdek M, Grigsby E, Huntoon M, Jacobs MS, Kim P, Kumar K, Leong M, Liem L, McDowell GC II, Panchal S, Rauck R, Saulino M, Sitzman BT, Staats P, Stanton-Hicks M, Stearns L, Wallace M, Willis KD, Witt W, Yaksh T, Mekhail N: Polyanalgesic Consensus Conference–2012: Consensus on diagnosis, detection, and treatment of catheter-tip granulomas (inflammatory masses). Neuromodulation 2012; 15:483–95; discussion 496
10. Aprili D, Bandschapp O, Rochlitz C, Urwyler A, Ruppen W: Serious complications associated with external intrathecal catheters used in cancer pain patients: A systematic review and meta-analysis. Anesthesiology 2009; 111:1346–55
11. Lehmberg J, Scheiwe C, Spreer J, van Velthoven V: Late bacterial granuloma at an intrathecal drug delivery catheter. Acta Neurochir (Wien) 2006; 148:899–901; discussion 901
12. Allen JW, Horais KA, Tozier NA, Yaksh TL: Opiate pharmacology of intrathecal granulomas. Anesthesiology 2006; 105:590–8
13. Butt MT: Morphologic changes associated with intrathecal catheters for direct delivery to the central nervous system in preclinical studies. Toxicol Pathol 2011; 39:213–9
14. Sabbe MB, Grafe MR, Pfeifer BL, Mirzai TH, Yaksh TL: Toxicology of baclofen continuously infused into the spinal intrathecal space of the dog. Neurotoxicology 1993; 14:397–410
15. Yaksh TL, Grafe MR, Malkmus S, Rathbun ML, Eisenach JC: Studies on the safety of chronically administered intrathecal neostigmine methylsulfate in rats and dogs. Anesthesiology 1995; 82:412–27
16. Chiari A, Yaksh TL, Myers RR, Provencher J, Moore L, Lee CS, Eisenach JC: Preclinical toxicity screening of intrathecal adenosine in rats and dogs. Anesthesiology 1999; 91:824–32
17. Johansen MJ, Satterfield WC, Baze WB, Hildebrand KR, Gradert TL, Hassenbusch SJ: Continuous intrathecal infusion of hydromorphone: Safety in the sheep model and clinical implications. Pain Med 2004; 5:14–25
18. Theoharides TC, Donelan J, Kandere-Grzybowska K, Konstantinidou A: The role of mast cells in migraine pathophysiology. Brain Res Brain Res Rev 2005; 49:65–76
19. Artico M, Cavallotti C: Catecholaminergic and acetylcholine esterase containing nerves of cranial and spinal dura mater in humans and rodents. Microsc Res Tech 2001; 53:212–20
20. Michaloudi H, Batzios C, Chiotelli M, Grivas I, Papadopoulos GC: Mast cells populations fluctuate along the spinal dura mater of the developing rat. Brain Res 2008; 1226:8–17
21. Miller HR, Pemberton AD: Tissue-specific expression of mast cell granule serine proteinases and their role in inflammation in the lung and gut. Immunology 2002; 105:375–90
22. Kunder CA, St John AL, Abraham SN: Mast cell modulation of the vascular and lymphatic endothelium. Blood 2011; 118:5383–93
23. Ebertz JM, Hermens JM, McMillan JC, Uno H, Hirshman C, Hanifin JM: Functional differences between human cutaneous mast cells and basophils: A comparison of morphine-induced histamine release. Agents Actions 1986; 18:455–62
24. Tharp MD, Kagey-Sobotka A, Fox CC, Marone G, Lichtenstein LM, Sullivan TJ: Functional heterogeneity of human mast cells from different anatomic sites: *In vitro* responses to morphine sulfate. J Allergy Clin Immunol 1987; 79:646–53
25. Feldberg W, Paton WD: Release of histamine from skin and muscle in the cat by opium alkaloids and other histamine liberators. J Physiol (Lond) 1951; 114:490–509
26. Grosman N, Jensen SM, Johansen FF: Histamine release from isolated rat mast cells induced by opiates: Effect of sterical configuration and calcium. Agents Actions 1982; 12:417–24
27. Casale TB, Bowman S, Kaliner M: Induction of human cutaneous mast cell degranulation by opiates and endogenous opioid peptides: Evidence for opiate and nonopiate receptor participation. J Allergy Clin Immunol 1984; 73:775–81

28. Levy JH, Brister NW, Shearin A, Ziegler J, Hug CC Jr, Adelson DM, Walker BF: Wheal and flare responses to opioids in humans. Anesthesiology 1989; 70:756–60
29. Blunk JA, Schmelz M, Zeck S, Skov P, Likar R, Koppert W: Opioid-induced mast cell activation and vascular responses is not mediated by mu-opioid receptors: An *in vivo* microdialysis study in human skin. Anesth Analg 2004; 98:364
30. Barke KE, Hough LB: Opiates, mast cells and histamine release. Life Sci 1993; 53:1391–9
31. Baldo BA, Pham NH: Histamine-releasing and allergenic properties of opioid analgesic drugs: Resolving the two. Anaesth Intensive Care 2012; 40:216–35
32. Yaksh TL, Provencher JC, Rathbun ML, Myers RR, Powell H, Richter P, Kohn FR: Safety assessment of encapsulated morphine delivered epidurally in a sustained-release multivesicular liposome preparation in dogs. Drug Deliv 2000; 7:27–36
33. Yaksh TL, Rathbun ML, Dragani JC, Malkmus S, Bourdeau AR, Richter P, Powell H, Myers RR, Lebel CP: Kinetic and safety studies on intrathecally infused recombinant-methionyl human brain-derived neurotrophic factor in dogs. Fundam Appl Toxicol 1997; 38:89–100
34. Wegner K, Horais KA, Tozier NA, Rathbun ML, Shtaerman Y, Yaksh TL: Development of a canine nociceptive thermal escape model. J Neurosci Methods 2008; 168:88–97
35. Rubinstein I, Nadel JA, Graf PD, Caughey GH: Mast cell chymase potentiates histamine-induced wheal formation in the skin of ragweed-allergic dogs. J Clin Invest 1990; 86:555–9
36. Becker AB, Chung KF, McDonald DM, Lazarus SC, Frick OL, Gold WM: Mast cell heterogeneity in dog skin. Anat Rec 1985; 213:477–80, 530–1
37. Langguth P, Khan PJ, Garrett ER: Pharmacokinetics of morphine and its surrogates: XI. Effect of simultaneously administered naltrexone and morphine on the pharmacokinetics and pharmacodynamics of each in the dog. Biopharm Drug Dispos 1990; 11:419–44
38. Garrett ER, el-Koussi A el-D: Pharmacokinetics of morphine and its surrogates V: Naltrexone and naltrexone conjugate pharmacokinetics in the dog as a function of dose. J Pharm Sci 1985; 74:50–6
39. Divin MF, Holden Ko MC, Traynor JR: Comparison of the opioid receptor antagonist properties of naltrexone and 6 beta-naltrexol in morphine-naïve and morphine-dependent mice. Eur J Pharmacol 2008; 583:48–55
40. Xanthos DN, Gaderer S, Drdla R, Nuro E, Abramova A, Ellmeier W, Sandkühler J: Central nervous system mast cells in peripheral inflammatory nociception. Mol Pain 2011; 7:42
41. Sayed BA, Christy A, Quirion MR, Brown MA: The master switch: The role of mast cells in autoimmunity and tolerance. Annu Rev Immunol 2008; 26:705–39
42. Levy D: Migraine pain, meningeal inflammation, and mast cells. Curr Pain Headache Rep 2009; 13:237–40
43. Messlinger K, Fischer MJ, Lennerz JK: Neuropeptide effects in the trigeminal system: Pathophysiology and clinical relevance in migraine. Keio J Med 2011; 60:82–9
44. Metcalfe DD, Baram D, Mekori YA: Mast cells. Physiol Rev 1997; 77:1033–79
45. He S, Walls AF: Human mast cell tryptase: A stimulus of microvascular leakage and mast cell activation. Eur J Pharmacol 1997; 328:89–97
46. Vergnolle N: Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. J Immunol 1999; 163:5064–9
47. Sayed BA, Christy AL, Walker ME, Brown MA: Meningeal mast cells affect early T cell central nervous system infiltration and blood-brain barrier integrity through TNF: A role for neutrophil recruitment? J Immunol 2010; 184:6891–900
48. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg ML: Mast cells as early responders in the regulation of acute blood-brain barrier changes after cerebral ischemia and hemorrhage. J Cereb Blood Flow Metab 2010; 30:689–702
49. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM: Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells. J Immunol 1987; 138:2611–5
50. Itoh Y, Sendo T, Oishi R: Physiology and pathophysiology of proteinase-activated receptors (PARs): Role of tryptase/PAR-2 in vascular endothelial barrier function. J Pharmacol Sci 2005; 97:14–9
51. Hermens JM, Ebertz JM, Hanifin JM, Hirshman CA: Comparison of histamine release in human skin mast cells induced by morphine, fentanyl, and oxymorphone. Anesthesiology 1985; 62:124–9
52. Lagunoff D, Martin TW, Read G: Agents that release histamine from mast cells. Annu Rev Pharmacol Toxicol 1983; 23:331–51
53. Kosterlitz HW, Waterfield AA: In vitro models in the study of structure-activity relationships of narcotic analgesics. Annu Rev Pharmacol 1975; 15:29–47
54. Catala M: Embryonic and fetal development of structures associated with the cerebro-spinal fluid in man and other species. Part I: The ventricular system, meninges and choroid plexuses. Arch Anat Cytol Pathol 1998; 46:153–69
55. O'Rahilly R, Müller F: The meninges in human development. J Neuropathol Exp Neurol 1986; 45:588–608
56. Pawankar R, Yamagishi S, Takizawa R, Yagi T: Mast cell-IgE- and mast cell-structural cell interactions in allergic airway disease. Curr Drug Targets Inflamm Allergy 2003; 2:303–12
57. Krishnaswamy G, Ajitawi O, Chi DS: The human mast cell: An overview. Methods Mol Biol 2006; 315:13–34
58. Mousli M, Hugli TE, Landry Y, Bronner C: Peptidergic pathway in human skin and rat peritoneal mast cell activation. Immunopharmacology 1994; 27:1–11
59. Ferry X, Eichwald V, Daeffler L, Landry Y: Activation of beta-gamma subunits of G(i2) and G(i3) proteins by basic secretagogues induces exocytosis through phospholipase Cbeta and arachidonate release through phospholipase Cgamma in mast cells. J Immunol 2001; 167:4805–13
60. Shefler I, Zavaro O, Raz T, Baram D, Sagi-Eisenberg R: Inhibition of basic secretagogue-induced signaling in mast cells by cell permeable G alpha i-derived peptides. Int Arch Allergy Immunol 2008; 145:131–40
61. Klinker JF, Seifert R: Morphine and muscle relaxants are receptor-independent G-protein activators and cromolyn is an inhibitor of stimulated G-protein activity. Inflamm Res 1997; 46:46–50
62. Hutchinson MR, Lewis SS, Coats BD, Rezvani N, Zhang Y, Wieseler JL, Somogyi AA, Yin H, Maier SF, Rice KC, Watkins LR: Possible involvement of toll-like receptor 4/myeloid differentiation factor-2 activity of opioid inactive isomers causes spinal proinflammation and related behavioral consequences. Neuroscience 2010; 167:880–93
63. Hutchinson MR, Loram LC, Zhang Y, Shridhar M, Rezvani N, Berkelhammer D, Phipps S, Foster PS, Landgraf K, Falke JJ, Rice KC, Maier SF, Yin H, Watkins LR: Evidence that tricyclic small molecules may possess toll-like receptor and myeloid differentiation protein 2 activity. Neuroscience 2010; 168:551–63
64. Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR: Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun 2010; 24:83–95
65. Iwamura C, Nakayama T: Toll-like receptors in the respiratory system: Their roles in inflammation. Curr Allergy Asthma Rep 2008; 8:7–13
66. Nie X, Cai G, Zhang W, Wang H, Wu B, Li Q, Shen Q: Lipopolysaccharide mediated mast cells induce IL-10

producing regulatory T cells through the ICOSL/ICOS axis. Clin Immunol 2012; 142:269–79

67. Murphy S, Kelly HW: Cromolyn sodium: A review of mechanisms and clinical use in asthma. Drug Intell Clin Pharm 1987; 21(1 Pt 1):22–35
68. Altounyan RE: Review of clinical activity and mode of action of sodium cromoglycate. Clin Allergy 1980; 10(Suppl): 481–9
69. Alton EW, Norris AA: Chloride transport and the actions of nedocromil sodium and cromolyn sodium in asthma. J Allergy Clin Immunol 1996; 98(5 Pt 2):S102–5; discussion S105–6
70. Crossman DC, Dashwood MR, Taylor GW, Wellings R, Fuller RW: Sodium cromoglycate: Evidence of tachykinin antagonist activity in the human skin. J Appl Physiol 1993; 75:167–72
71. Yaksh TL, Steinauer JJ, Williams SL, Malkmus SA: Alfentanil: Correlations between absence of effect upon subcutaneous mast cells and absence of granuloma formation after intrathecal infusion in the dog. Neuromodulation 2012. doi: 10.1111/j.1525-1403.2012.00534.x
72. Zacest AC, Carlson JD, Nemecek A, Burchiel KJ: Surgical management of spinal catheter granulomas: Operative nuances and review of the surgical literature. Neurosurgery 2009; 65:1161–4; discussion 1164–5
73. Gupta A, Martindale T, Christo PJ: Intrathecal catheter granuloma associated with continuous sufentanil infusion. Pain Med 2010; 11:847–52
74. Janssen LJ, Wattie J, Betti PA: Effects of cromolyn and nedocromil on ion currents in canine tracheal smooth muscle. Eur Respir J 1998; 12:50–6
75. Eady RP: The pharmacology of nedocromil sodium. Eur J Respir Dis Suppl 1986; 147:112–9
76. Leung KB, Flint KC, Brostoff J, Hudspith BN, Johnson NM, Pearce FL: A comparison of nedocromil sodium and sodium cromoglycate on human lung mast cells obtained by bronchoalveolar lavage and by dispersion of lung fragments. Eur J Respir Dis Suppl 1986; 147:223–6
77. Pearce FL, Al-Laith M, Bosman L, Brostoff J, Cunniffe TM, Flint KC, Hudspith BN, Jaffar ZH, Johnson NM, Kassessinoff TA: Effects of sodium cromoglycate and nedocromil sodium on histamine secretion from mast cells from various locations. Drugs 1989; 37(Suppl 1):37–43; discussion 69–77
78. Siraganian RP, de Castro RO, Barbu EA, Zhang J: Mast cell signaling: The role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. FEBS Lett 2010; 584:4933–40
79. Yamamoto N, Takeshita K, Shichijo M, Kokubo T, Sato M, Nakashima K, Ishimori M, Nagai H, Li YF, Yura T, Bacon KB: The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-yl-amino]nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents. J Pharmacol Exp Ther 2003; 306:1174–81
80. Bowersox SS, Singh T, Nadasdi L, Zukowska-Grojec Z, Valentino K, Hoffman BB: Cardiovascular effects of omega-conopeptides in conscious rats: Mechanisms of action. J Cardiovasc Pharmacol 1992; 20:756–64
81. Skov MJ, Beck JC, de Kater AW, Shopp GM: Nonclinical safety of ziconotide: An intrathecal analgesic of a new pharmaceutical class. Int J Toxicol 2007; 26:411–21
82. Yaksh TL, de Kater A, Dean R, Best BM, Miljanich GP: Pharmacokinetic analysis of Ziconotide (SNX-111), an intrathecal N-type calcium channel blocking analgesic, delivered by bolus and infusion in the dog. Neuromodulation 2012; 15:508–19
83. Hoederath P, Gautschi OP, Land M, Hildebrandt G, Fournier JY: Formation of two consecutive intrathecal catheter tip granulomas within nine months. Cent Eur Neurosurg 2010; 71:39–42
84. Yaksh TL: Spinal delivery and assessment of drug safety, Fundamental Neuropathology for Pathologists and Toxicologists: Principles and Techniques. Edited by Bolon B, Butt MT. Hoboken, Wiley & Sons, 2011, pp 452–62
85. Walker SM, Yaksh TL: Neuraxial analgesia in neonates and infants: A review of clinical and preclinical strategies for the development of safety and efficacy data. Anesth Analg 2012; 115:638–62