Published in final edited form as:

Pharmacol Biochem Behav. 2007 November ; 88(1): 122–129.

Activation of Peripheral and Spinal Histamine H₃ Receptors Inhibits Formalin-Induced Inflammation and Nociception, Respectively

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Abstract

Pharmacological activation of histamine H₃ receptors is known to reduce the release of inflammatory peptides, thereby reducing pain and inflammation, but the site(s) and mechanism(s) of these effects are currently unknown. The present study addressed these questions by examining the effects of the H₃ agonist immpip and the H₃ antagonist thioperamide on nociceptive behaviors and swelling produced during the rat formalin test. Systemic administration of immpip (5 and 30 mg/kg, s.c.) significantly attenuated formalin-induced flinching but not licking responses during both phases. This attenuation was reversed by either systemic (15 mg/kg, i.p.) or intrathecal (20 or 50 µg) administration of thioperamide. Furthermore, immpip (30 mg/kg, s.c.) significantly inhibited formalin-induced swelling, an action which was completely reversed by systemic (15 mg/kg, i.p.), but not intrathecal (50 µg) thioperamide. Also consistent with this pattern, intrathecal immpip (50 µg) reduced flinching responses, but had no effect on formalin-induced paw swelling. The present findings suggest that activation of H₃ receptors located on peripheral and spinal terminals of deep dermal fibers attenuates formalin-induced swelling and flinching, respectively. Pharmacological stimulation of H₃ receptors could be an important therapeutic approach for many disorders related to deep dermal or inflammatory pain.

Keywords

histamine; H₃ receptor; inflammation; antinociception; analgesia; immpip; thioperamide

Introduction

There is considerable interest in histamine H₃ receptors as emerging pharmaceutical targets (Esbenshade et al. 2006). Although H₃ receptor density is highest in the brain, *in situ* hybridization studies have also found these receptors to exist in dorsal root ganglia, spinal cord and selected peripheral tissues (Pollard et al. 1993; Heron A. et al. 2001; Pilot et al. 2002). In addition, activation of peripheral H₃ receptors inhibits peptide release, suggesting a sensory

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fiber localization (Ohkubo et al. 1995; Delaunois et al. 1995; Imamura et al. 1996; Nemmar et al. 1999). Immunohistochemical studies recently confirmed the existence of H₃ receptors on sensory neurons in the dorsal root ganglia, and on sensory fibers in the skin spinal cord (Cannon et al., 2007).

Since peptidergic fibers have been implicated in nociception (Morton et al. 1989; Millan 1999; Furst 1999), the inhibition of the activity of these fibers by H₃ receptor agonists might be expected to modulate pain transmission. In fact, oral administration of the H₃ agonist pro-drug BP 2-94 to mice reduced nociceptive responses in the phenylbenzoquinone writhing and formalin tests, but failed to reduce nociceptive hot plate responding (Rouleau et al. 1997). This work suggested that H₃ agonists may attenuate some types of nociceptive transmission, but the sites and mechanisms responsible for these effects remained unclear.

Subsequently, the H₃ agonist immpip was shown to attenuate mechanical (tail pinch) responses in rats, but neither tail flick nor hot plate reflexes were affected (Cannon et al. 2003). Additional experiments using H₃ knockout mice, pharmacological antagonists, and systemic and intrathecal treatments in rats showed unequivocally that activation of spinal H₃ receptors attenuates nociceptive responses to low-intensity tail pinch stimulation (Cannon et al. 2003). More recently, a range of thermal and mechanical intensities were employed to confirm that the acute antinociceptive profile of H₃ agonists is both modality- (i.e. mechanical vs. thermal) and intensity- (low vs. high mechanical) specific (Cannon et al. 2005). It was concluded that immpip probably inhibits low-intensity mechanical nociception by activation of H₃ receptors located on the spinal terminals of A δ and possibly C high-threshold mechanoreceptors (Cannon et al. 2005). Such an antinociceptive profile is extremely unusual.

In contrast, much less is known about the modulation of inflammatory pain by H₃ agonists. Earlier studies revealed that the H₃ agonist pro-drug BP 2-94 attenuates swelling and nociceptive behaviors in some mouse models of inflammatory pain (Rouleau et al. 1997; Rouleau et al. 2000). The H₃ agonist R- α -methylhistamine was also recently shown to have systemic anti-inflammatory activity (Poveda et al., 2006). In addition, preliminary findings from our laboratory suggested that the H₃ agonist immpip attenuates formalin-induced flinching responses in rats (Cannon et al. 2005). However, the site(s) and mechanism(s) of the antinociceptive and anti-inflammatory actions of H₃ agonists following formalin remain unknown. These questions are addressed in the present study by evaluating pharmacological manipulations with the H₃ agonist immpip and the H₃ antagonist thioperamide on formalin-induced nociceptive behaviors and swelling in rats. Several potential sites of action for these drugs are considered, including spinal neurons, spinal terminals of nociceptive sensory fibers, peripheral terminals of these fibers within the skin, and the brain.

Methods and Materials

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Albany Medical College. Male Sprague-Dawley rats (300–350g, Taconic Farms, Germantown, NY) housed in groups of two or three were maintained on a 12 h light/dark cycle (lights on from 7:00 to 19:00 h). Food and water were provided *ad libitum*.

Drugs

Immpip dihydrobromide (synthesized at Vrije Universiteit, Amsterdam, Netherlands, Vollinga et al., 1994) and thioperamide maleate (Tocris, Ellisville, MO) were dissolved in saline and neutralized with 1N NaOH to pH 6.5. All doses of immpip and thioperamide are

expressed as bases. Neutral buffered formalin (10%, Sigma) was diluted in saline to produce a 2.5% formalin solution with a pH of 7.0.

Surgery

For rat intrathecal injections, the spinal subarachnoid space was chronically cannulated under general anesthesia as described previously (Yaksh et al. 1976) with modifications (Hammond, 1988). The animals were then placed in separate cages and allowed to recover for a minimum of ten days.

Formalin Test

For all experiments, animals were habituated to the formalin test environment by placing them in the test apparatus (Plexiglass chamber 15.25 in. $\times$ 15.8 in. $\times$ 9.5 in.) for one h prior to injection of formalin. Subjects were then given a subcutaneous (s.c.) injection of either immepip or saline. Thirty min later, the subjects were given an s.c. injection of 2.5% formalin (volume of 50 μ l) into the plantar surface of the left hind paw. Some subjects received an intraperitoneal (i.p.) injection of thioperamide or saline 20 min prior to formalin. Other subjects received an intrathecal (i.t.) injection of immepip, thioperamide, or saline 10 min prior to formalin injection. The time intervals used for agonist and antagonist administration were adapted from Cannon et al. (2003).

During each experiment, the number of flinches (i.e. flinching or shaking of the injected paw) was counted every min for 60 min and results were expressed as the average number of flinches per min (in 5 min periods). In addition, the total number of flinches was summed for each phase. Phase 1 was defined as the period of time beginning immediately after formalin injection and lasting 10 min. Phase 2 was defined as the time beginning 11 min post-formalin injection and ending 60 min. Digital calipers were used to measure the vertical thickness of the injected paw at the metatarsal level in all subjects (except as noted in Fig. 1). Baseline paw measurements were taken immediately prior to s.c. injections of immepip or saline (–30 min) and again just before formalin treatment (0 min). Paw measurements were repeated at the times indicated after formalin.

Statistics

Results are expressed as average number of flinches per min (grouped in 5 min periods), total number of flinches per phase, time spent licking (s), and paw swelling (mm), mean $\pm$ S.E.M. Two types of statistical analyses were performed: 1) repeated measures analysis of variance (ANOVA) and planned comparisons for analysis of the number of flinches per min or paw swelling measurements, and 2) Student's t-test for analysis of the total number of flinches per phase (Statistica, CSS, Inc., Tulsa, OK).

Results

Effects of immepip on formalin-induced flinching

Dose-dependent inhibition of flinching responses was observed over a range of immepip doses (Fig. 1A; repeated measures ANOVA, drug by time interaction, $P < 0.01$). The lowest dose of immepip (1 mg/kg, s.c.) produced flinching responses similar to the control responses during Phase 1 and the first half of Phase 2. However, during the second half of Phase 2, this low dose of immepip significantly reduced flinching responses (Fig. 1A). This reduction was also seen in the total number of flinches produced during Phase 2 (Fig. 1B). Larger doses of immepip (5 and 30 mg/kg) significantly attenuated formalin-induced flinching responses during Phase 1 and Phase 2 (Figs. 1A and 1B).

Effects of immepip on formalin-induced licking

In contrast to the effects on flinching, none of the three doses of immepip had a significant effect on formalin-induced licking responses (Fig. 1C). Because there appeared to be a trend toward reductions in licking after the highest dose of immepip, licking times were also summed for each animal for phase 1 and phase 2 (data not shown). Total licking times for either phase were not statistically different across the treatment groups by one-way ANOVA. Furthermore, separate t-test comparisons of either phase 1 or phase 2 total licking times from saline-treated vs immepip (30 mg/kg)-treated subjects found no differences.

Effects of immepip on formalin-induced paw swelling

Injection of formalin into the hind paw resulted in swelling as early as 10 min after injection (Fig. 1D). This swelling reached a plateau by 30 min and was maintained for up to 240 min (Fig. 1D, Figs. 2B, 2D). Non-significant reductions in average paw diameters were achieved by the lower doses of immepip, whereas the largest dose (30 mg/kg) produced highly significant reductions in swelling (Fig. 1D repeated measures ANOVA, $P<0.01$). A repeat of this experiment in the antagonist studies (Fig. 2) confirmed a large, long-lasting inhibition of swelling (Figs. 2B, 2D).

Effects of thioperamide on immepip-induced inhibition of flinching

In order to determine if the actions of immepip are mediated by H_3 receptors, the effects of the selective H_3 antagonist thioperamide were examined in conjunction with immepip treatment on both formalin-induced flinching and swelling (Fig. 2). Repeated measures ANOVAs of flinching data from combinations of saline, immepip, and thioperamide (i.p. and i.t.) treatments showed significant main effects of drug ($P<0.01$), time ($P<0.01$), and significant drug by time interaction terms ($P<0.01$). Figures 2A and 2C confirm a significant reduction in flinching responses during both phases produced by immepip. Systemic administration of thioperamide (15 mg/kg, i.p.) significantly reversed the attenuation of flinching produced by immepip during both phases (Fig. 2A). Intrathecal administration of thioperamide (50 μ g) also significantly reversed immepip-induced attenuation of flinching responses during both phases (Fig. 2C). Thioperamide alone (i.p. or i.t.) had no effect on formalin-induced flinching responses (Figs. 2A and 2C). In a parallel experiment, a lower intrathecal dose of thioperamide (20 μ g) completely abolished the inhibition of formalin-induced flinches by 5 mg/kg immepip during both phases of the formalin test (data not shown).

Effects of thioperamide on immepip-induced inhibition of paw swelling

Repeated measures ANOVAs of paw diameter data from combinations of saline, immepip, and thioperamide (i.p. and i.t.) treatments revealed significant main effects of drug ($P<0.01$), time ($P<0.01$), and drug by time interactions ($P<0.01$). Systemic (Fig. 2B), but not intrathecal (Fig. 2D), administration of thioperamide completely reversed the reduction in paw diameter produced by immepip. Thioperamide alone (administered i.p. or i.t.) had no effect on formalin-induced paw swelling (Figs. 2B and 2D). To summarize the results of Fig. 2, systemic immepip reduced both flinching and swelling following formalin treatment, effects which were both antagonized by systemic administration of thioperamide. In contrast, intrathecal thioperamide antagonized immepip's suppression of flinching but not swelling.

Effects of intrathecal immepip on formalin-induced flinching and paw swelling

To confirm the spinal localization of immepip antinociception, intrathecal administration of immepip (50 μ g) reduced flinching responses during both phases of the formalin test (Fig. 3A; repeated measures ANOVA, drug by time interaction, $P<0.01$). This suppression was similar to that produced by systemic administration of immepip (Figs. 1 and 2). However, as predicted

from the findings of Fig. 2, intrathecal immepip had no effect on formalin-induced paw swelling (Fig. 3B).

Discussion

The present study offers several lines of evidence showing that activation of H₃ receptors attenuates both nociceptive behaviors and edema associated with inflammatory pain. First, the inhibition of formalin-induced flinching by the H₃ agonist immepip was dose-dependent (Fig. 1). Second, this inhibition was reversed by systemic and intrathecal administration of the H₃ antagonist thioperamide (Figs. 2A and 2C). Finally, H₃ agonist-induced attenuation of paw swelling was dose-dependent and was reversed by systemic administration of thioperamide (Figs. 1D and 2B). In addition, the present study has located the sites for both the anti-inflammatory and antinociceptive actions of this drug.

A spinal site of antinociceptive action for intrathecal immepip

The attenuation of formalin-induced flinching by intrathecal immepip (Fig. 3) makes a spinal site of action for this drug likely. In an earlier study, the same intrathecal dose of immepip as used presently (50 µg) was shown to attenuate nociceptive responses to tail pinch in rats (Cannon et al. 2003). Since systemic administration of this dose had no effect on tail pinch responses, the effect of intrathecal immepip must be a localized spinal effect (Cannon et al. 2003). Consistent with this, autoradiography experiments have revealed low but detectable levels of H₃ receptor binding sites in the superficial dorsal horn of rat spinal cords (Pollard et al. 1993).

H₃ receptors located postsynaptically on dorsal horn neurons are a possible target for the antinociceptive actions of intrathecal immepip. However, *in situ* hybridization studies of H₃ mRNA found very little to no H₃ message in dorsal horn neurons (Heron A. et al. 2001). In addition, immunohistochemical studies found little to no anti-H₃ receptor labeling on dorsal horn neurons (Cannon et al., 2007).

Within the dorsal horn, H₃ receptors could be localized on the terminals of descending bulbospinal fibers. For example, H₃ agonists were shown to reduce norepinephrine release in spinal cord slices (Celuch, 1995). However, this particular effect is not likely to account for the antinociceptive actions of intrathecal immepip, since the descending noradrenergic influence is antinociceptive, and immepip would presumably reduce this activity (Millan 2002). In contrast, there is excellent evidence that sensory neurons possess H₃ receptors. H₃ receptor mRNA was detected in large, medium, and small diameter cell bodies of rat dorsal root ganglia (Heron A. et al. 2001). Furthermore, recent immunohistochemical studies showed anti-H₃ receptor labeling on medium-sized cell bodies in dorsal root ganglia and on small-caliber, peptidergic fibers that ramified in dorsal horn laminae I, II, and V in rats and wild type mice (Cannon et al., 2007). H₃ knockout mice lack this anti-H₃ labeling, confirming the presence of authentic H₃ receptors (Cannon et al., 2007). The same mice also lack antinociceptive responses to immepip, providing a critical link between the anatomically-localized receptor and the drug response (Cannon et al. 2005). The localization of H₃ receptors on sensory neurons and fibers, and the well-established H₃-mediated inhibition of transmitter release (Ohkubo et al. 1995; Delaunois et al. 1995; Arrang et al. 1995; Imamura et al. 1996; Blandina et al. 1996; Garcia et al. 1997; Nemmar et al. 1999; Molina-Hernandez et al. 2001) strongly suggest that immepip produces antinociception by reducing sensory fiber transmitter release at spinal presynaptic sites.

A spinal site of antinociceptive action for systemic immepip

Due to the wide distribution of H₃ receptors throughout the central (Pollard et al., 1993) and peripheral (Cannon et al., 2007) nervous systems, systemically-administered immepip could be acting on supraspinal, peripheral, or spinal H₃ targets to attenuate formalin-induced flinching. The existence of H₃ receptors in brain stem areas (Pollard et al., 1993) contributing to the descending control of pain transmission (Millan, 2002) means that supraspinal sites should be considered carefully. The degree of spinal and brain penetration by immepip has not been explored, but systemic dosing with this drug was reported to inhibit the release of histamine in the anterior hypothalamus (Jansen et al. 1998). However, because an intrathecal dose of immepip mimics systemically-administered immepip in reducing both flinching (Fig. 1A) and nociceptive responses to mechanical pain (Cannon 2003), a spinal site of action is the simplest explanation for these antinociceptive actions of immepip.

The reversal of the effects of systemic immepip on flinching responses by intrathecal thioperamide (Fig. 2C) strongly argues that systemic immepip produces its antinociceptive effects solely at spinal H₃ receptors. This result precludes the notion that immepip reduces flinching responses by acting peripherally on an H₃-receptor-containing sensory fiber. If systemic immepip also activated peripheral H₃ receptors by reducing peripherally-initiated nociceptive transmission to attenuate flinching responses, then the afferent nociceptive signal would have never reached the spinal cord. Under these conditions, blocking spinal H₃ receptors with intrathecal thioperamide would have had no effect on the attenuation of formalin-induced flinching responses.

Different nociceptive circuits may be activated by formalin to evoke different behaviors

The selective attenuation of flinching (vs. licking) suggests that distinct nociceptive circuits may underlie these behaviors. Clarification of the nociceptors evoking these behaviors following formalin has been difficult because: 1) inflammatory peptides are expressed on multiple afferent subtypes (Ishida-Yamamoto et al. 1989; Fundin et al. 1997; Pare et al. 2001b), and 2) many studies have either not distinguished between the two behaviors (Yaksh 1997), or not made simultaneous measurements (Sawynok et al. 2003). By measuring both types of behaviors, the present work offers insight into the fibers that provoke flinching responses following formalin.

Although it is widely thought that C fibers mediate formalin-induced flinching and licking responses (Tjolsen et al. 1992; McCall et al. 1996; Yaksh 1997), the present findings require a re-examination of this hypothesis. Calcitonin gene-related peptide (CGRP) and substance P (SP), both released from afferent fibers, are known to contribute to the peripheral and spinal mechanisms of inflammatory pain (Ohkubo et al. 1990; Levine et al. 1993; Levine et al. 1999; Yaksh 1999). Since many C fibers contain these neuropeptides (Gibbins et al. 1985; Gibbins et al. 1987; Sann et al. 1998), it has been thought that both are released solely from C fibers during nociception (Millan 1999). However, it is often overlooked that some A δ fibers contain CGRP and SP (Ishida-Yamamoto et al. 1989; Fundin et al. 1997; Pare et al. 2001a), and these fibers may also participate in nociceptive transmission by releasing neuropeptides in the spinal cord. (Yaksh et al. 1980; Go et al. 1987). In addition, the attenuation of depolarization-induced release of SP from spinal afferent terminals by intrathecal morphine (Yaksh et al. 1980; Go et al. 1987) has been used to emphasize the importance of peptidergic C fibers because μ -opioid receptors are found on these fibers (Gouarderes et al. 1991; Zhang et al. 1998) and because this treatment attenuates formalin-induced flinching and licking responses (Malmberg et al. 1993). However, μ opioid receptors are expressed on a variety of nociceptive afferents, including peptidergic, thinly myelinated fibers (Gouarderes et al. 1991; Arvidsson et al. 1995; Zhang et al. 1998). An analogous argument can be made about the effects of neonatal capsaicin pretreatment on formalin-evoked flinching and licking responses

(Yaksh 1997; Peterson et al. 1997), since this treatment destroys both unmyelinated (C fibers) and some myelinated fibers (Nagy et al. 1983). Taken with the recent report that H₃ receptors are on a select population of A δ and absent on C fibers (Cannon et al., 2007), the present study suggests that C fibers do not play a critical role in mediating formalin-induced flinching responses. Instead, it is likely that H₃ receptor-containing, deep dermal, perivascular A δ fibers are primary contributors to the nociceptive flinching circuit. By the same reasoning, the failure of immepip to attenuate licking responses (Fig. 1C; (Cannon et al. 2005) argues that these H₃ receptor-containing fibers do not play an obligatory role in producing this particular behavior. C fibers are likely contributors to the stimuli which evoke formalin-induced licking responses. The differential modulation of flinching and licking behaviors following formalin has also been noted in earlier drug studies, emphasizing the possibility of distinct nociceptive circuits evoking these behaviors (Sawynok et al. 2004).

Site of anti-inflammatory action of H₃ agonists

Activation of peripheral H₃ receptors can reduce the release of inflammatory peptides (Ohkubo et al. 1995; Delaunois et al. 1995; Imamura et al. 1996; Nemmar et al. 1999), consistent with the reported anti-inflammatory activity of BP 2-94, an H₃ receptor pro-drug (Rouleau et al. 1997; Rouleau et al. 2000). However, the site of anti-inflammatory action of such H₃-acting drugs was unknown. The present results, which revealed that immepip-induced attenuation of paw swelling was completely reversed by systemic (but not intrathecal) administration of thioperamide (Figs. 2B and 2D), solidify an anti-inflammatory role for peripheral H₃ receptors. Activation of H₃ receptors on peptidergic, A δ perivascular fibers of the deep dermis is likely to inhibit peripheral neuropeptide release, thereby reducing inflammation.

It is curious as to how activation of peripheral H₃ receptors might attenuate inflammation but not inhibit nociceptive flinching responses. Activation of other peripheral G_i/G_o-linked receptors, such as μ -opioid receptors, attenuates both nociceptive transmission and inflammation (Raja et al. 1999). The explanation for this difference may lie in the respective signaling mechanisms associated with these receptors. Whereas both μ -opioid and H₃ receptors attenuate the release of neuropeptides from peripheral terminals presumably via the inhibition of N- and P/Q-type Ca⁺² channels associated with exocytosis (Twycross 1999; Brown et al. 2001; Roberson et al. 2002; Cesselin et al. 2002), μ -opioid receptors also inhibit nociceptive transmission via activation of G-protein coupled, inwardly-rectifying K⁺ channels (GIRKs) (Twycross 1999; Cesselin et al. 2002). H₃ receptors are not associated with this latter activity (Brown et al. 2001).

Significance of histamine and its receptors during inflammation and pain

Endogenous histamine plays an important pro-nociceptive role during inflammatory pain (Millan 1999; Basbaum et al. 2000). Injection of formalin results in an initial burst of nociceptor activity, and the release of several chemical messengers, including histamine (Tjolsen et al. 1992; Parada et al. 2001). Pharmacological studies (Parada et al. 2001) and experiments with histamine receptor knockout mice (Owen et al. 1980a; Owen et al. 1980b; Mobarakeh et al. 2000) show a critical pro-nociceptive role for the H₁ receptor in both phases of responses following formalin, although H₂ receptors may also be significant (Owen et al. 1980a; Owen et al. 1980b).

Since histamine is released during inflammatory pain, it seems possible that H₃ receptors might be activated during the formalin test. Thus, endogenous histamine would be expected to produce pro-nociceptive actions at H₁ receptors, but antinociceptive actions at H₃ receptors. If so, then the H₃ antagonist thioperamide should have produced either pro-nociceptive or pro-inflammatory effects during the formalin test. However, thioperamide alone had no effect on

nociceptive behaviors or inflammation (Fig. 2), suggesting that endogenous histamine is not activating peripheral or spinal H₃ receptors during inflammatory pain.

Given that histamine has higher affinity for the H₃ receptor for than the H₁ receptor (Hill et al. 1997), it is curious that the histamine released by formalin treatment seems to act on H₁ receptors, but not act on H₃ receptors. One potential explanation may be a difference in the localization of H₁ and H₃ receptors in the skin and spinal cord. Although no immunohistochemical studies have been performed with H₁ receptor antibodies, *in situ* hybridization studies in guinea pig dorsal root ganglia have revealed that H₁ receptor mRNA is found in small-sized cell bodies that are non-peptidergic and express the isolectin IB4 (Kashiba et al. 1999). In contrast, H₃ receptors are located on small-caliber, deep dermal, peptidergic, perivascular Aδ fibers (Cannon et al., 2007). Thus, H₁ and H₃ receptors appear to be located on separate subsets of small-caliber nociceptive fibers. It may be possible that histamine is released in the vicinity of H₁ receptor-containing fibers during inflammatory pain, whereas H₃ receptor-containing fibers may be located out of the range of the released histamine. Further studies are necessary to confirm or refute this hypothesis.

Acknowledgements

This work was supported by grants from the National Institute on Drug Abuse (DA-03816, DA-015915, and DA-07307). We would like to thank Dr. Frank Rice (Albany Medical College, Albany, NY) for valuable discussions.

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Figure 1. Effects of the H₃ agonist immepip on formalin-induced flinching responses, licking responses, and paw swelling

Rats received immepip (1, 5, or 30 mg/kg, s.c.) or saline vehicle, followed 30 min later by intraplantar formalin. A: Number of flinches per min was recorded and grouped into 5 min periods (ordinate, mean $\pm$ S.E.M.) for the times after formalin shown (abscissa, min). B: The total number of flinches per min was summed for each phase (ordinate, mean $\pm$ S.E.M.). Phase 1 was established as 0-10 min, whereas Phase 2 was established as 11-60 min. C: Time spent licking (ordinate, s, mean $\pm$ S.E.M.) is shown in 5 min intervals (abscissa, min). D: Paw diameter (ordinate, mm, mean $\pm$ S.E.M.) was recorded at the times shown (abscissa, min) after formalin injection. Data are pooled from two separate experiments: a) saline and immepip (5 mg/kg) treatments (n=6) in which paw volume was not measured (data in Figs 1A-1C), and b) all four treatment groups in which behavior and paw volume was measured (data in all 4 figures, n=5). *, ** P < 0.05, P < 0.01 vs. saline at same time interval, respectively.

Figure 2. Effects of the H₃ antagonist thioperamide on immepip-induced attenuation of flinching responses (A, C) and paw swelling (B, D) after formalin treatment

Rats received immepip (imm, 30 mg/kg, s.c.) or saline vehicle (sal, s.c.). *A, B*: Effects of systemic thioperamide were studied. Ten min after immepip, thioperamide (thio, 15 mg/kg, i.p.) or saline (sal) were injected, followed by intraplantar formalin 20 min later. *C, D*: Effects of intrathecal thioperamide were studied. Twenty min after immepip, intrathecal thioperamide (50 µg) or saline (sal) were given, followed by formalin 10 min later. Numbers of flinches per min, grouped in 5 min periods (*A, C*, ordinate, mean ± S.E.M.), and paw diameters (*B, D*, ordinate, mm, mean ± S.E.M.) were recorded at the times shown after formalin (abscissa, min). No significant differences were found in flinching responses or paw diameters between the following groups, thus permitting the groups to be pooled and identified as in parentheses: 1) s.c. saline/i.p. saline vs. s.c. saline/i.t. saline (s.c. sal/sal, open circles), 2) s.c. saline/i.p. thioperamide vs. s.c. saline/i.t. thioperamide (s.c. sal/thio, open squares), and 3) s.c. immepip/i.p. saline vs. s.c. immepip/i.t. saline (s.c. imm/sal, closed circles). *, ** $P < 0.05$, $P < 0.01$ vs. sal/sal at same time interval, respectively. +, ++ $P < 0.05$, $P < 0.01$ vs. imm/sal at same time interval, respectively.

Figure 3. Effects of intrathecal immepip on formalin-induced flinching responses (A) and paw swelling (B)
Rats received saline (sal, s.c.), followed 20 min later by an intrathecal injection of saline (sal) or immepip (imm, 50 μ g). Ten min later, subjects received intraplantar formalin. The experiment proceeded and data are shown as in Fig. 2. *, ** $P < 0.05$, $P < 0.01$ vs. sal/sal at same time interval, respectively.