Halothane Enhances Suppression of Spinal Sensitization by Intrathecal Morphine in the Rat Formalin Test


Background: Injection of formalin in the hindpaw of the rat induces intense C-fiber activity accompanied by brief flinching of the injected paw (phase 1) and gives rise to facilitated spinal processing characterized by renewed flinching beginning 15 min after injury and lasting 40 min or more (phase 2). In previous work, isoflurane, administered during phase 1, slightly reduced phase-2 activity, whereas the addition of intrathecal morphine dramatically inhibited phase 2, even with naloxone reversal 6 min after the formalin injection. We used a similar model to determine whether intrathecal morphine could block spinal sensitization in the absence of inhalation anesthetic.

Methods: Hot plate tests at 52°C and radiant heat-evoked hindpaw withdrawal tests were used to determine optimal doses of agonists and antagonists. The formalin test was carried out on male Sprague-Dawley rats, which were divided into five groups. A combination of naloxone 0.5 mg/kg and naltrexone 0.5 mg/kg was administered subcutaneously 6 min after the formalin injection to all animals except controls (group 1) to prevent ongoing opioid effect. Groups 1–3 received intrathecal saline, and groups 4 and 5 received intrathecal morphine 30 µg 20 min before formalin injection. Halothane was administered for 1–2 min to facilitate formalin injection for groups 1, 2, and 4. In groups 3 and 5 halothane was administered from 5 min before to 6 min after formalin injection. The number of flinches per minute was counted 1 and 5 min after formalin administration and thereafter at 5-min intervals for 1 h. The total number of flinches at 1 and 5 min was considered as phase-1 activity, and the total number of flinches during the 10–60-min interval was considered as phase 2.

Results: Phase-2 activity for groups 1 and 2 was nearly identical, demonstrating no appreciable effect of the opioid antagonist alone. Groups 3 (halothane alone) and 4 (morphine alone) exhibited a significant decrease in phase-2 activity. Group 5 (morphine plus halothane) demonstrated a profound decrease in phase-2 activity, which was significantly more profound than that of groups 3 or 4.

Conclusions: Intrathecal morphine, administered before formalin injection but antagonized before the onset of phase 2 of the formalin test, significantly suppresses sensitization of dorsal horn neurons. This suppression is significantly increased by coadministration of halothane anesthesia. (Key words: Anesthetics, inhalation: halothane. Hyperalgesia. Opioids: morphine. Spinal cord.)

IT is now well accepted that changes in neural function induced by noxious stimuli during surgery may enhance subsequent perception of pain. It has been proposed that facilitation of dorsal horn neurons is induced by high frequency barrages of C-fiber activity.1,2 A growing body of evidence suggests that administration of regional analgesia before the onset of noxious stimulation, termed preemptive analgesia, can inhibit the establishment of spinal sensitization.5–6 The role of preemptive administration of either systemic5,7 or neuraxial6 opioids in reducing spinal sensitization is not as well documented.

Subcutaneous injection of dilute formalin in the hindpaw of the rat produces a biphasic nociceptive response. Initial C-fiber activity9 is accompanied by flinching of the paw for about 5 min (phase 1), followed by cessation of activity and resumption of flinching (phase 2) beginning 15 min after injection and lasting about 40 min.10,11 The second phase depends on changes in dorsal horn cell function which occur shortly after the initial C-fiber discharge.11,12 There is evidence that phase-2 pain behavior is dependent on low levels of continued afferent input acting on sensitized dorsal horn neurons.11 In a previous study, isoflurane, administered during phase 1, slightly reduced phase-2 activity, whereas the addition of intrathecal morphine dramatically inhibited phase 2, even with naloxone reversal 6 min after the formalin injection.13 Dickenson and Sullivan12 have shown that intrathecal administration of the µ-opioid agonist Tyr- ß-AlaGlyMePheGly-ol, administered before formalin injection and reversed with naloxone several minutes...
after the formalin, profoundly suppressed phase-2 flinching in halothane-anesthetized animals. However, the ability of intrathecal opioids to suppress spinal sensitization when administered without inhalational anesthesia has not been studied. We used the formalin model to investigate the ability of spinal morphine plus the inhalational anesthetic halothane to block spinal sensitization and to determine whether intrathecal morphine could block spinal sensitization in the absence of inhalational anesthetic.

Materials and Methods

The following studies were carried out under a protocol approved by the Animal Research Facility of the Zablocki Veterans's Administration Center (Milwaukee, WI). Male Sprague-Dawley rats weighing 250–350 g were used.

Animal Preparation

Animals that received intrathecal saline or morphine were implanted with chronic intrathecal catheters introduced via an incision in the atlantooccipital membrane under halothane anesthesia as previously described by Yaksh and Rudy,14 with one modification. Catheters were introduced a distance of 11 cm because we found that intrathecal lidocaine (20 µl, 2%) produced only partial anesthesia and motor blockade of the hindlimbs when 9-cm catheters were used (unpublished data). Animals showing neurologic deficits after implantation were excluded. All testing was carried out 4–9 days after intrathecal implantation.


To determine the degree of analgesia and the adequacy and duration of reversal of opioid effects by a combination of naltroxone and naltrexone, antinociception was assessed by two tests. The 52°C hot-plate test was carried out in one group of animals. After two baseline measurements of latency to pain response (licking of hindpaw), a group (n = 5) of animals received intrathecal morphine 30 µg. Measurements were repeated 10 and 20 min later, and then animals were given 0.5 mg/kg naltroxone plus 0.5 mg/kg naltrexone subcutaneously, and additional measurements recorded after an additional 10, 25, 40, and 55 min. Cutoff time for latency to response was 60 s.

Another group of animals was studied by using the radiant heat–evoked hindpaw withdrawal test as previously described.15 A focused radiant-heat source (50-W CLX/CXR projection lamp, Ushio, Tokyo, Japan) was directed onto the hindpaw from below. Cutoff time for latency to withdrawal was 20 s. After a baseline measurement of latency to pain response (hindpaw withdrawal) a group (n = 3) of animals received intrathecal morphine 30 µg and 0.5 mg/kg naltroxone plus 0.5 mg/kg naltrexone subcutaneously at the same times as the hot-plate group and measurements of pain response were made at the same intervals.

Formalin Test

The formalin test was carried out as previously described.16 To investigate the effect of halothane anesthesia, and of halothane anesthesia plus intrathecal morphine, on spinal sensitization and to determine the effect of intrathecal morphine alone on spinal sensitization, the following paradigms, were used (table 1).

Group 1 animals (n = 5) underwent standard formalin testing. Intrathecal saline 10 µl was given 20 min before formalin. The rats were individually allowed to breathe 3% halothane until immobile (generally about 1–2 min). They were removed from the anesthetic and immediately given 50 µl subcutaneously, 5% formalin into the dorsum of the right hindpaw using a 30-G needle. Subcutaneous saline 0.3 ml was given 6 min after formalin.

Group 2 animals (n = 8) were given intrathecal saline, halothane anesthetic, and subcutaneous formalin injections as in group 1, but subcutaneous naltroxone 0.5 mg/kg plus naltrexone 0.5 mg/kg in 0.3 ml normal saline was given 6 min after formalin. This group was used as a control group for comparison with subsequent groups.

Group 3 animals (n = 5) were given intrathecal saline 20 min before formalin. Animals were anesthetized

<table>
<thead>
<tr>
<th>Table 1. Description of Treatment and Control Groups</th>
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NLX = naltroxone; NLTX = naltrexone.

Anesthesiology, V 81, No 5, Nov 1994
with 3% halothane, 10 min before formalin. When immobile, the concentration of halothane was reduced to 0.7% (0.7 minimum alveolar concentration [MAC] for rats) and maintained at that concentration for 5 min. Subcutaneous formalin was injected and anesthesia was maintained with 0.7% halothane. Subcutaneous naloxone 0.5 mg/kg plus naltrexone 0.5 mg/kg was given 6 min after the formalin, and anesthesia was immediately discontinued.

Group 4 animals (n = 6) were given intrathecal morphine 30 μg 20 min before formalin injection. The formalin test was carried out with brief halothane anesthesia as in groups 1 and 2. Six minutes after injection of formalin they were given subcutaneous naloxone 0.5 mg/kg plus naltrexone 0.5 mg/kg.

Group 5 animals (n = 7) were given intrathecal morphine 30 μg 20 min before formalin injection. These animals were anesthetized with halothane as described for group 3. After 6 min they were given subcutaneous naloxone 0.5 mg/kg plus naltrexone 0.5 mg/kg, and anesthesia was immediately discontinued.

To provide a rough assessment of analgesia at the time of formalin injection, animals in groups 4 and 5 were tested by firmly pinching the metatarsals of the hindpaw just before formalin injection and the presence or absence of a withdrawal response was determined.

After formalin injection, all animals were placed in clear plexiglass chambers for observation. Coordinated spontaneous movement was typically noted within 1 min after cessation of brief halothane and within 5 min after cessation of prolonged halothane anesthesia. Animals routinely displayed a flinching, withdrawal movement of the injected hindpaw. Flinches per minute were recorded 1 and 5 min after injection and at 5-min intervals thereafter for 1 h. The animals were then killed with 65 mg intraperitoneal pentobarbital.

Results

Nociceptive Threshold
Hot-plate Test. Animals given intrathecal morphine 30 μg had a mean baseline response latency of 21 ± 3 s. Twenty minutes after morphine injection all animals had a latency of 60 s (P < 0.001). At 10 min after subcutaneous injection of naloxone/naltrexone, mean latency was 18 ± 2, and latencies remained near baseline (P > 0.05) for the remainder of the study (table 2).

Radiant Heat-Evoked Hindpaw Withdrawal Test. Animals given intrathecal morphine 30 μg had a mean baseline response latency of 9 ± 0.3 s. Twenty minutes after morphine injection all animals had a latency of 20 ± 0.3 s (P < 0.01). At 10 min after subcutaneous injection of naloxone/naltrexone, mean latency was 12 ± 3 s, at 25 min after the subcutaneous injection mean latency was 8 ± 2 s and latencies remained near baseline (P > 0.05) for the remainder of the study (table 3).

Paw Pinch Data
All animals in groups 1–3 (intrathecal saline) demonstrated withdrawal to paw pinch before formalin injection. None of the animals in groups 4 or 5 (intrathecal morphine) withdrew to paw pinch.

Formalin Test Data
Little flinching was noted during phase 1 for animals that received intrathecal saline plus inhalational anesthesia during and for 6 min after formalin injection (group 3), and none was seen in the groups that received intrathecal opioids (groups 4 and 5). Mean total numbers of flinches in phases 1 and 2 are shown for all groups in table 4. Figure 1 demonstrates mean number of flinches per minute and mean total numbers of flinches for groups 2–5.

Phase 2 activity for groups 1 and 2 was nearly identical, demonstrating that there was no appreciable effect of the opioid antagonists alone (table 3). Animals that

Data Analysis
The total number of flinches was determined for all of the phase-2 (10–60 min) observations for each animal, and these data were compared by one-way analysis of variance (StatView II). Post hoc comparisons were done using Sheffé’s F test.

The hot-plate and hindpaw withdrawal data were analyzed using the paired Student’s t test. The mean latencies for each of the time periods were compared with the mean baseline (before intrathecal morphine) measurements using this test.

Table 2. Hotplate Test: Nociceptive Threshold

<table>
<thead>
<tr>
<th>Baseline</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>75 min</th>
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<td>21 ± 3</td>
<td>47 ± 8*</td>
<td>60 ± 0*</td>
<td>18 ± 2</td>
<td>22 ± 2</td>
<td>24 ± 5</td>
<td>26 ± 5</td>
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Values are mean ± SEM (seconds).
* Significantly different from baseline (P < 0.001).

Anesthesiology, V 81, No 5, Nov 1994
received halothane alone during phase 1 (group 3) exhibited a significant decrease in phase-2 activity when compared with group 2 ($P < 0.05$). Animals that received intrathecal morphine alone during phase 1 (group 4) exhibited a significant decrease in phase-2 activity when compared with group 2 ($P < 0.05$). Animals that received a combination of intrathecal morphine and halothane during phase 1 (group 5) demonstrated a profound decrease in phase-2 activity when compared with group 2 ($P < 0.001$). Phase-2 activity for this group was significantly less than that of groups 3 or 4 ($P < 0.05$).

### Discussion

Intrathecal morphine, which was effective only during phase 1 of the formalin test, caused significant attenuation of the hyperalgesia induced by formalin injection. This suppression was significantly enhanced by the administration of halothane during phase 1. It was not determined whether the interaction between the opioid and inhalation anesthetic is synergistic in nature. The fact that a plateau effect in the inhalation anesthetic dose response curve occurs at less than 50% suppression of phase-2 activity$^2$ precludes the use of isobolographic analysis to determine the degree of drug interaction.

Many reports have recently stressed the importance of wide–dynamic range neurons of the dorsal horn of the spinal cord on the transmission and integration of nociceptive information.$^{18}$ Dickenson and Sullivan$^{13}$ observed that injection of formalin resulted in a profound augmentation in the discharge of dorsal horn wide–dynamic range neurons in rats. The spinal administration of Tyr-o-AlaGlyMePheGly-ol before formalin injection blocked this augmentation, even with naloxone administration 2 min after the formalin. Opioids have been shown to produce analgesia if administered either before or after the painful stimulus, although a higher dose may be required to abolish pain when given after injury.$^{19}$

It is important that many of these electrophysiologic studies of C-fiber–induced spinal sensitization have been carried out in animals under a surgical plane of anesthesia, using volatile agents such as halothane$^{2,12}$ and the ability of opioid analgesic agents to block central sensitization in the absence of general anesthesia has not previously been evaluated. A recent report by Abram and Yaksh$^{15}$ showed that isoflurane, administered during phase 1, produced some reduction in phase-2 activity of the formalin test, whereas the addition of intrathecal morphine dramatically inhibited phase 2, even with naloxone reversal 6 min after the formalin injection. Our results indicate that intrathecal morphine administered alone during phase 1 produces a significant though not profound reduction in phase-2 activity, and that halothane produces a similar reduction in spinal sensitization when administered alone but significantly enhances the suppression of sensitization by intrathecal morphine.

Although the exact mechanisms underlying general anesthesia are not known, several actions of volatile anesthetics on the membrane and synaptic properties of central nervous system neurons have been demonstrated. The effects of these agents are reported to fall into two general categories-depression of excitatory transmission and enhancement of inhibitory transmission. Excitatory transmission induced by noxious stimulation has been shown to be mediated by excitatory amino acids, such as glutamate, acting at $\text{n}$-methyl-$\text{D}$-aspartate receptors.$^{10,16}$ Such excitation is enhanced by the action of substance P on the neurokinin 1 receptor.$^{20}$ The phenomenon of ‘wind-up’, the progressive increase in response of dorsal horn neurons to repetitive, brief C-fiber stimulation, appears to be mediated by $\text{n}$-methyl-$\text{D}$-aspartate receptors.$^{18}$

| Group | Phase 1 | Phase 2 | % of Control 
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<tr>
<td>1</td>
<td>$11 \pm 3$</td>
<td>$137 \pm 12$</td>
<td>$-$</td>
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<tr>
<td>2</td>
<td>$17 \pm 5$</td>
<td>$148 \pm 22$</td>
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<td>$79 \pm 20$</td>
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<tr>
<td>5</td>
<td>$0$</td>
<td>$26 \pm 13$</td>
<td>$18$</td>
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Values are mean ± SEM.

* Significantly different from group 2 ($P < 0.05$).

† Significantly different from group 2 ($P < 0.001$); significantly different from group 3 and group 4 ($P < 0.05$).
HALOTHANE-ENHANCED SPINAL MORPHINE ANTINOCICEPTION

Fig. 1. (A) Mean number of flinches per minute plotted as a function of time after injection of formalin for the group receiving intrathecal morphine or intrathecal saline and brief (1–2 min) or prolonged (from 5 min before to 6 min after formalin) halothane anesthesia. Group 2 = intrathecal saline 20 min before formalin, subcutaneous naltrexone 0.5 mg/kg plus subcutaneous naloxone 0.5 mg/kg 6 min after formalin, and brief halothane; group 3 = intrathecal saline 20 min before formalin, subcutaneous naltrexone 0.5 mg/kg plus subcutaneous naloxone 0.5 mg/kg 6 min after formalin, and prolonged halothane; group 4 = intrathecal morphine 30 μg 20 min before formalin, subcutaneous naltrexone 0.5 mg/kg plus subcutaneous naltrexone 0.5 mg/kg 6 min after formalin, and prolonged halothane; group 5 = intrathecal morphine 30 μg 20 min before formalin, subcutaneous naloxone 0.5 mg/kg plus subcutaneous naltrexone 0.5 mg/kg 6 min after formalin, and brief halothane; group 6 = intrathecal saline 20 min before formalin, subcutaneous naloxone 0.5 mg/kg plus subcutaneous naltrexone 0.5 mg/kg 6 min after formalin, and prolonged halothane. (B) Total flinches (mean ± SEM) in phase 2 for groups 2–5. *Significantly different from group 2, one-way analysis of variance (P < 0.05). §§Significantly different from group 2 (P < 0.001). §§Significantly different from groups 3 and 4 (P < 0.05).

There are now several lines of evidence for a direct effect of inhalational anesthetic agents on suppression of nociception in the spinal cord. Namiki et al. demonstrated a dose-related effect of halothane upon the graded response of wide- and dynamic range neurons in the dorsal horn neurons of the spinal cord to noxious radiant heat. After administration of halothane, the mean spontaneous and evoked discharge frequencies were significantly decreased by concentrations of 0.5, 1.0, and 1.5% halothane. Their study suggests that halothane may modify the transmission of noxious information at the spinal cord level, and thus produce analgesia. Heavner et al. also showed that excitation of dorsal horn wide- and dynamic range neurons is powerfully suppressed by high anesthetic concentrations. It has been found that in response to either a peripheral noxious stimulus or high intensity electrical stimulation of a dorsal root, the neonatal rat spinal cord generates a slow ventral root potential lasting tens of seconds. Recent evidence suggests that the slow ventral root potential corresponds in part to ventral horn neuron depolarization that is initiated by the same stimuli that evoke nociceptive sensitization and that the slow ventral root potential is mediated by substance P and glutamate. Savola et al. found that at 0.2–1.28 vol%, isoflurane reversibly depressed the slow ventral root potential. They also noted that isoflurane reversibly depressed responses to direct application of both N-methyl-D-aspartate and substance P. They concluded that these results indicate marked inhibitory effects of isoflurane on spinal neurotransmission.

Rampil et al. tested the effects of acute decerebration on the potency of isoflurane in suppression of motor activity due to noxious stimuli in rats. They found that the MAC of isoflurane was not altered by destruction of the motor cortex and that complex movements in response to nociceptive stimulation still occurred. Because MAC depends on a subject’s response to pain, these findings suggest that anesthetic-induced unresponsiveness to noxious stimuli measured by MAC testing does not depend as much on cortical or forebrain effects in the rat, but may be mediated principally in the spinal cord. In a recent study by Antognini and Schwartz, in which the circulation of goat brain was isolated and therefore could be preferentially anesthetized, it was found that isoflurane MAC was significantly higher when the brain was anesthetized in isolation from the rest of the body. This suggests that the effect of inhalation anesthetics on the spinal cord is important in the inhibition of movement in response to a painful stimulus.

Anesthesiology, V 81, No 5, Nov 1994
There are several studies which suggest that inhalational anesthetic agents play a role in blocking facilitated transmission in other parts of the central nervous system. A phenomenon similar to spinal sensitization, known as long-term potentiation, is seen in the hippocampus, and is associated with learning and memory.\textsuperscript{30} As with the spinal cord phenomenon of hyperalgesia, long-term potentiation in the hippocampus is mediated by \( \text{n}-\text{methyl-D-aspartate receptors}. \textsuperscript{31} \text{Maclver et al.} \textsuperscript{32} \) measured the effects of halothane on long-term potentiation in rat hippocampal brain slices and found that halothane reduced the probability of long-term potentiation induction. \text{Nicol} \text{and Madison} \textsuperscript{33} \) have shown that both diethyl ether and halothane hyperpolarize hippocampal and spinal neurons and that the potency of these agents in hyperpolarizing motoneurons was strongly correlated with their anesthetic potency. \text{Sugiyama et al.} \textsuperscript{34} studied the effects of halothane on neuronal transmission in the parafascicular nucleus in the guinea pig, an area known to contain a large proportion of nociceptive neurons. They found that at clinically used concentrations, halothane produced hyperpolarization as a result of enhanced potassium conductance in approximately 50\% of cells.

Whereas intrathecal opioids, administered alone, are known to modulate C-fiber transmission, inhalation anesthetics apparently work through a very different mechanism. The exact mechanism by which volatile anesthetics modify the transmission of pain in the central nervous system has yet to be elucidated. \text{Nakahiro et al.} \textsuperscript{35} \) provided evidence which suggests that inhalational agents modulate the \( \gamma \)-aminobutyric acid (GABA) receptor channel complex both in the brain and in the spinal cord. They studied the effects of halothane, isoflurane and enfurane on ionic currents induced by bath application of GABA in cultured rat dorsal root ganglion neurons, using the whole-cell patch clamp technique to record current. They found a potentiation of the GABA receptor channel response and suggested that this is the primary action of these anesthetic agents which leads to surgical anesthesia. Inhalational anesthetics at clinically relevant concentrations only, appear to inhibit disposal (uptake, release and catabolism) of synaptosomal GABA\textsuperscript{36} and this could lead to enhancement of inhibitory processes by increasing GABA concentrations at the inhibitory synapse.

GABA agonists have been found to inhibit nociceptive transmission in the spinal cord. \text{Edwards et al.} \textsuperscript{37} \) studied the effects of a benzodiazepine antagonist (flumazenil) and a GABA antagonist (bicuculline) on the analgesic effects of intrathecal midazolam, a benzodiazepine-GABA receptor complex agonist, and intrathecal fentanyl. They found that the segmental analgesia produced by midazolam but not fentanyl was antagonized by flumazenil and by bicuculline. They concluded that the segmental analgesia produced by midazolam but not fentanyl was mediated by the benzodiazepine-GABA receptor complex. \text{Niv et al.} \textsuperscript{38} \) examined the effects of intraperitoneal and intrathecal midazolam on nociception and found a sedative and hyperalgesic response to noxious stimulation in those rats that received intraperitoneal midazolam and an analgesic effect in those rats that received intrathecal drug. They proposed that the analgesic effect of midazolam stems from its action at the spinal level, whereas its sedative and hyperalgesic effects are a function of its supraspinal action.

The clinical relevance of the present study is that volatile anesthetic agents modify central sensitization of the spinal cord to pain and they significantly enhance the suppressive effect of opioid analgesics, which do not completely block spinal cord sensitization when administered alone. This conclusion is supported by human studies which demonstrate incomplete ability of opioids to block pain transmission, such as those comparing the effect of opioids on rest pain and incidental pain. \text{Ready et al.} \textsuperscript{39} \) reviewed the efficacy of epidural morphine for postoperative pain and found that the median pain scores on a 0–10 scale were 1 at rest and 4 with coughing. The lack of complete suppression of nociceptive inputs by spinal opioids helps explain the inability to provide surgical anesthesia with spinal opioids alone.

In conclusion, this study has shown that spinal opioids and inhalation anesthetics, acting independently, produce some reduction in spinal sensitization associated with noxious stimulation (subcutaneous formalin). However the combination of these interventions significantly reduces phase-2 activity. Further studies are needed to elucidate the mechanisms by which the inhalation anesthetics affect spinal nociceptive transmission.

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HALOTHANE-ENHANCED SPINAL MORPHINE ANTINOCEPCIION


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