Effects of General Anesthetics on Substance P Release and c-Fos Expression in the Spinal Dorsal Horn

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ABSTRACT

Background: The authors examined *in vivo* the effects of general anesthetics on evoked substance P release (primary afferent excitability) and c-Fos expression (neuronal activation) in superficial dorsal horn.

Methods: Rats received saline, propofol (100 mg/kg), pentobarbital (50 mg/kg), isoflurane (2 minimum alveolar concentration), nitrous oxide (66%), or fentanyl (30 μg/kg). During anesthesia, rats received intraplantar 5% formalin (50 μl) to left hind paw. Ten minutes later, rats underwent transcardial perfusion with 4% paraformaldehyde. Substance P release from small primary afferents was assessed by incidence of neurokinin 1 receptor internalization in the superficial dorsal horn. In separate studies, rats were sacrificed after 2 h and c-Fos expression measured.

Results: Intraplantar formalin-induced robust neurokinin 1 receptor internalization in ipsilateral dorsal horn (ipsilateral: 54 ± 6% [mean ± SEM], contralateral: 12 ± 2%; $P < 0.05$; n = 4). Fentanyl, but not propofol, pentobarbital, isoflurane, nor nitrous oxide alone inhibited neurokinin 1 receptor internalization. However, 2 minimum alveolar concentration isoflurane + nitrous oxide reduced neurokinin 1 receptor internalization (27 ± 3%; $P < 0.05$; n = 5). All agents reduced c-Fos expression (control: 34 ± 4, fentanyl: 8 ± 2, isoflurane: 12 ± 3, nitrous oxide: 11 ± 2, isoflurane + nitrous oxide: 12 ± 1, pentobarbital: 11 ± 2, propofol: 13 ± 3; $P < 0.05$; n = 3).

Conclusion: General anesthetics at anesthetic concentrations block spinal neuron activation through a mechanism that is independent of an effect on small primary afferent peptide release. The effect of fentanyl alone and the synergistic effect of isoflurane and nitrous oxide on substance P release suggest a correlative rationale for the therapeutic use of these anesthetic protocols by blocking nociceptive afferent transmitter release and preventing the initiation of cascade, which is immediately postsynaptic to the primary afferent.

What We Already Know about This Topic

- Volatile and injectable anesthetics can reduce nociceptive signaling *via* spinal mechanisms

What This Article Tells Us That Is New

- These studies in rats suggest that although both volatile and injectable anesthetics reduce overall spinal nociceptive signaling, they unexpectedly do not alter peptide release from primary afferents; only fentanyl and the combination of isoflurane and nitrous oxide exert a presynaptic effect by blocking dorsal horn substance P release

GENERAL anesthetics are classified into inhaled (isoflurane, nitrous oxide) and intravenous agents (propofol, barbiturate). The mechanisms of their actions, although widely studied, remain controversial. As regards their membrane targets, volatile hydrocarbons, such as isoflurane are associated with interactions, which increase γ-aminobutyric acid type A ($GABA_A$)[1] and glycine receptor function[2] and/or reduce glutamate receptor function,[3,4] in addition to blocking various voltage sensitive channels, including those for calcium.[5] Injectable agents, such as propofol and barbiturates are believed to interact with the $GABA_A$ chloride ionophore.[6,7] At the system level, studies with animals having separated spinal-supraspinal perfusion have shown that the pain suppression component of the volatile anesthetic is largely mediated by a spinal action.[8] Thus, volatile and injectable anesthetics reduce nociceptive stimulus-evoked spinal activation as measured by a suppression of markers of neuronal activation, such as c-Fos.[9–11] This proposed role of anesthetics on spinal nociceptive processing raises the question of whether

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these agents in fact alter afferent input. Slice recordings have shown that isoflurane reduces monosynaptic, likely glutamatergic, excitatory postsynaptic potentials in substantia gelatinosa by a presynaptic action.[12] We sought to address this question of whether the volatile and injectable anesthetics at functionally relevant concentration/doses *in vivo*, alter afferent input by blocking their releasing function. Transient receptor potential vanilloid 1 positive (capsaicin sensitive) afferents are small, high threshold in nature, and considered to be important for the processing of nociceptive information.[13–15] Spinal μ opiates produce analgesia,[16] a finding consistent with the demonstration of the presynaptic localization of μ opiate receptors on the spinal terminals of these afferents.[17] Consistent with the fact that μ receptors are negatively coupled with voltage sensitive calcium channels that mediate terminal transmitter release, opiates and N-type calcium channel blockers reduce substance P from these peptidergic primary afferents as measured *in vivo* by extracellular concentrations[18,19] and by the use of neurokinin 1 receptor (NK1r) internalization.[20,21] These studies have shown that such dorsal horn internalization is a validated marker for the evoked release of substance P from primary afferents. Thus, in the current work, we sought to determine the effects *in vivo* of anesthetic concentrations of volatile and injectable agents on the evoked release of substance P from peptidergic primary afferents. Such work would serve to define whether or not general anesthetics at functionally defined concentrations, like other classes of agents which act on the primary afferent terminals, can block afferent terminal release from a nociceptor.

Materials and Methods

Animals

Male Holtzman Sprague–Dawley rats (250–300 g; Harlan, Indianapolis, IN) were used in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, Bethesda, MD). The rats were housed in individual standard cages and maintained on a 12-h light/dark cycle (lights on at 07:00 AM). Testing was performed during the light cycle. Food and water were available *ad libitum*. All activities were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego, California.

General Anesthetics on Formalin-induced NK1r Internalization

Saline, propofol (100 mg/kg), or pentobarbital (50 mg/kg) were injected intraperitoneally. As an active control for evoked NK1r internalization, fentanyl citrate (30 μg/kg) was given intraperitoneally. For volatile anesthetic delivery, animals were placed individually in a closed plexiglass container through which oxygen or oxygen plus the anesthetic agent were delivered. Three volatile anesthetic regimens were used: (1) isoflurane (2 minimum alveolar concentration [MAC]) = 2.4% in a room air/oxygen mixture (1:1), (2) nitrous oxide (N_2O; 66 with 33% oxygen), (3) 1 MAC isoflurane combined with N_2O (66%) and oxygen (33%), or (4) 2 MAC isoflurane combined with N_2O (66%) and oxygen (33%) provided *via* an anesthetic machine (Ohmeda, Madison, WI). Fifteen minutes after intraperitoneal drug administration, or 10 min after initiation of inhaled anesthetics, rats received an intraplantar injection of formalin (5%, 50 μl) to the left hind paw.

Tissue Preparation and Immunocytochemistry

Spinal cord tissues were prepared and harvested, as described.[21] Ten minutes after formalin injection, rats were anesthetized with beuthanasia (0.5 ml, intraperitoneally), then transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate-buffered saline, pH 7.4. The lumbar spinal cord was harvested and postfixed overnight. After cryoprotection in 30% sucrose, coronal sections were made using a sliding microtome (30 μm). The spinal sections were washed with phosphate-buffered saline then incubated in a rabbit anti-NK1r polyclonal antibody overnight at room temperature. The antibody was diluted to a concentration of 1:3,000 in 0.01 M phosphate-buffered saline containing 10% normal goat serum and 0.3% Triton X-100. After rinsing in phosphate-buffered saline, spinal sections were incubated for 120 min at room temperature in a goat antirabbit secondary antibody (Alexa 488 to identify NK1 receptors) and a goat antimouse secondary antibody (Alexa 594 to identify NeuN) diluted at 1:1,000 in 0.01 M phosphate-buffered saline containing 10% goat serum and 0.3% Triton X-100. The sections were washed and mounted on glass slides and coverslipped with ProLong mounting medium.

Behavioral Effects Initiated by Formalin

Given that these animals were typically under a behaviorally disruptive dose of drug, the current studies were not aimed at systematically defining the effects of formalin-evoked flinching. However, we sought to quantify the immediate (<2 min) response to formalin injection into the hind paw by evaluating gross nociceptive behavior according to the following criteria: 0 = no response, 1 = increased muscle tone (tensing), 2 = injected hind paw withdrawal, and 3 = whole body movement.

Quantification of NK1r Internalization

NK1r internalization was quantified using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) fitted with a ×60 oil-immersion objective lens. Counting was conducted, as described previously.[20,21] The field of view was moved throughout lamina I. The focus was moved up and down intermittently through the spinal section to identify labeled neurons. Neuronal profiles that had 10 or

more endosomes in the soma and the contiguous proximal dendrites were considered to have internalized NK1 receptors. In the ispilateral and contralateral dorsal horns, the total number of NK1r-immunoreactive neurons in lamina I, with or without NK1r internalization, was counted and taken as a ratio of cells showing NK1r internalization *versus* all NK1r-positive cells and then converted into a percentage of NK1r-immunoreactive cells throughout L4–L6. The person performing the counts was blinded to the experimental treatment of each slide. Mean counts from two to five sections per spinal segment were used as representative counts for a given animal. Three to five animals per drug treatment group were used for statistical analysis (n = 3–5). Light microscopic images were taken using Magna FIRE SP (Optronics, Goleta, CA) and processed by Photoshop CS4 (Adobe, San Jose, CA).

Effect of Isoflurane and Nitrous Oxide on Exogenous Substance P

To rule out the possibility that anesthetic agents directly block the NK1r internalization mechanism, the effect of fentanyl and the combination of isoflurane and nitrous oxide on internalization induced by exogenous substance P (intrathecal injection) were examined. Rats were implanted with an intrathecal catheter for drug delivery under general anesthesia in accordance with the previous report.[22,23] Five to 7 days after the catheterization, substance P (30 nmol) was delivered intrathecally. Twenty minutes after the substance P injection, rats received intraperitoneal fentanyl or a combination of isoflurane (2 MAC) and N_2O (66%). Fifteen minutes after the initiation of anesthesia, rats were euthanized and fixed. The total number of NK1r-immunoreactive neurons in bilateral spinal lamina I, with or without NK1r internalization, was counted.

Quantification of c-Fos Expression

To evaluate the formalin-induced c-Fos expression in the spinal superficial dorsal horn, with different anesthetic regimens, separate groups of rats were prepared for immunocytochemistry. These rats were anesthetized, as with the NK1r internalization studies. Fifteen minutes after intraperitoneal drug administration or 10 min after initiation of inhaled anesthetics, rats received intraplantar formalin injection (5%, 50 μl) to the left hind paw. After the initial intraperitoneal injection, a second injection of fentanyl, propofol, and pentobarbital was added. Two hours after the formalin injection, rats were given beuthanasia (0.5 ml, intraperitoneally) and underwent transcardial perfusion and harvested, as previously described.[21] Free-floating sections were incubated in a rabbit anti-Fos polyclonal antibody overnight at 4°C. The antibody was diluted to a concentration of 1:5,000 in 0.01 M phosphate-buffered saline containing 10% normal goat serum and 0.3% Triton X-100. After rinsing in phosphate-buffered saline, spinal sections were incubated for 120 min at room temperature in a goat antirabbit secondary antibody (Alexa 546 to identify Fos) diluted at 1:500 in 0.01 M phosphate-buffered saline containing 10% goat serum and 0.3% Triton X-100. All sections were finally rinsed and mounted on glass slides and coverslipped with ProLong mounting medium. Fos-positive neurons in superficial dorsal horn (lamina I and II) of L4-L5 segments were counted blindly. The mean counts from L4-L5 segments of the lumbar spinal cord were used as representative counts for a given animal. Two to three sections per animal were counted. Three animals per drug treatment group were examined (n = 3).

Drug, Antibody, and Materials

Agents were purchased from the following sources: pentobarbital (Lundbeck Inc., Deerfield, IL); propofol (NOVAPLUS, Irving, TX); naloxone HCl (Sigma Chemical, St. Louis, MO); isoflurane (VETone, Meridian, ID); nitrous oxide (PRAXAIR, Danbury, CT); fentanyl citrate and beuthanasia (Merck Pharmaceuticals, Rahway, NJ). All injectable agents were administered intraperitoneally. The rabbit anti-NK1r polyclonal antibody was purchased from the Advanced Targeting Systems (San Diego, CA). The rabbit anti-Fos polyclonal antibody was purchased Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Secondary Alexa 488-conjugated antibody, Alexa 594-conjugated antibody, Alexa 546-conjugated antibody, and ProLong mounting medium were purchased from Life Technologies (Carlsbad, CA). Triton X-100 was purchased from Sigma-Aldrich (St. Louis, MO). Substance P was obtained from Peninsula Laboratories (San Carlos, CA).

Statistical Analysis

Statistical analysis was performed by Prism 5 (GraphPad, La Jolla, CA). The effects of each general anesthetic on NK1r internalization were analyzed by two-way ANOVA. To detect the differences in the presence of a significant two-way ANOVA, Bonferroni *post hoc* analysis was conducted. Behavioral differences and c-Fos expression were analyzed by one-way ANOVA, followed by Dunnet *post hoc* test. In all analyses, probability to detect the difference was set at the 5% level ($P < 0.05$).

Results

Behavioral Assessment of General Anesthetics

The effects of general anesthetics on the behavioral phenotype observed after formalin injection (5%, 50 μl) to the hind paw were assessed (table 1). Intraperitoneal propofol (100 mg/kg), pentobarbital (50 mg/kg), exposure of isoflurane (2 MAC), and combination of isoflurane and nitrous oxide produced obvious hypnotic effects and loss of the righting reflex,[24] without complications such as dyspnea. Nitrous oxide (66%) alone did not produce any observed hypnotic effects. The immediate response to formalin injection to the hind paw was evaluated on a 4-point scale (0: no response; 3: whole body movement). Combination of 2 MAC isoflurane

and nitrous oxide attenuated the movement stimulated by formalin injection to the hind paw compared with propofol, 2 MAC isoflurane, nitrous oxide and fentanyl, but displayed no difference compared with pentobarbital and 1 MAC isoflurane + nitrous oxide (behavioral score: propofol, 1.5 ± 0.3, $P < 0.05$; pentobarbital, 1.3 ± 0.5, $P > 0.05$; 2 MAC isoflurane, 1.5 ± 0.3, $P < 0.05$; 2 MAC isoflurane + nitrous oxide, 0.3 ± 0.2; 1 MAC isoflurane + nitrous oxide, 1.3 ± 0.3, $P > 0.05$; fentanyl, 1.8 ± 0.4, $P < 0.01$ *vs.* 2 MAC isoflurane + nitrous oxide, n = 4–6). Fentanyl produced an analgesic, but not a hypnotic effect on rats. Inhalation of nitrous oxide alone had neither hypnotic, nor analgesic effect on formalin-induced pain behavior (behavioral score: 3, n = 6).

Intraplantar Formalin Injection Induced NK1r Internalization

NK1r immunoreactivity was typically observed outlining the cell membrane in many superficial dorsal horn neurons (fig. 1, A–C). Significant NK1r internalization was not observed in the contralateral dorsal horn to the formalin-injected paw in L4–L6 (12 ± 2%, n = 4; fig. 1, A and D). Unilateral intraplantar injection of formalin produced robust NK1r internalization in ipsilateral dorsal horn, as evidenced by the appearance of NK1 (+) endosomes (54 ± 6%, $P < 0.001$ *vs.* contralateral, n = 4; fig. 1, B and D). This internalization was typically most evident in lamina I at the L5 and L6 levels of the lumbar spinal cord. Intraperitoneal fentanyl (30 μg/kg), as an active control, significantly inhibited formalin-induced NK1r internalization in ipsilateral dorsal horn (24 ± 8%, $P > 0.05$ *vs.* control, n = 3; fig. 1, C and D).

Effects of General Anesthetics on NK1r Internalization

The effects of general anesthetics on formalin-induced NK1r internalization are shown in figure 2. Intraperitoneal propofol at an equianalgesic dose (100 mg/kg) did not reduce NK1r internalization in ipsilateral dorsal horn (53 ± 1%; $P > 0.05$ *vs.* control; n = 3). Similarly, intraperitoneal pentobarbital (50 mg/kg) did not reduce formalin-induced NK1r internalization (45 ± 4%; $P > 0.05$ *vs.* control; n = 3). Inhalation of isoflurane at 2 MAC did not alter formalin-induced NK1r internalization in the spinal dorsal horn (49 ± 6%; $P > 0.05$ *vs.* control; n = 3). Nitrous oxide did not reduce formalin-induced NK1r internalization (45 ± 4%; $P > 0.05$ *vs.* control; n = 3; fig. 2)

Combination of Isoflurane and Nitrous Oxide on NK1r Internalization

The combinations of isoflurane and nitrous oxide on formalin-induced NK1r internalization are shown in figure 3. The combination of 2 MAC isoflurane and N_2O (66%) significantly reduced formalin-induced NK1r internalization in ipsilateral dorsal horn compared with control rats (27 ± 3%; $P < 0.001$ *vs.* control; n = 5). Similarly, the combination of 1 MAC isoflurane and nitrous oxide reduced formalin-induced NK1r internalization in ipsilateral dorsal horn (27 ± 5%; $P < 0.001$ *vs.* control; n = 3; fig. 3). There was no difference in outcome, as determined by NK1r internalization, between these two combination paradigms.

To assess the possible role of opiate receptors in the combination of isoflurane and nitrous oxide effects on NK1r internalization in these rats, intraperitoneal naloxone (1 mg/kg) was administered 15 min before the initiation of inhalation. Ten minutes after the initiation of isoflurane (2 MAC), N_2O (66%), and O_2 (33%), rats received formalin (5%, 50 μl) injection to the left hind paw. Ten minutes after the formalin injection, rats underwent transcardial perfusion. Intraperitoneal naloxone did not diminish the effect of isoflurane and nitrous oxide on formalin-induced NK1r internalization in the ipsilateral dorsal horn (21 ± 1%; $P > 0.05$ *vs.* ipsilateral isoflurane + nitrous oxide; n = 3; fig. 3). The contralateral side of NK1r internalization was not evaluated.

Effect of Isoflurane and Nitrous Oxide on Exogenous Substance P

Intrathecal substance P (30 nmol) produced robust NK1r internalization in the bilateral spinal lamina I compared with intrathecal saline rats (saline: 14 ± 3%, n = 3; substance P:

Table 1. Behavioral Responses to Intraplantar Formalin Injection under General Anesthetics

Drug	Route	Dose	n	Behavioral Score†	Spontaneous Activity‡	Dyspnea
Propofol	Intraperitoneal	100 mg/kg	4	1.5 ± 0.3*	(–)	(–)
Pentobarbital	Intraperitoneal	50 mg/kg	4	1.3 ± 0.5	(–)	(–)
Isoflurane	Inhaled	2 MAC	6	1.5 ± 0.3*	(–)	(–)
N_2O	Inhaled	66%	6	3.0*§	(+)	(–)
Isoflurane + N_2O	Inhaled	2 MAC + 66%	6	0.3 ± 0.2	(–)	(–)
Isoflurane + N_2O	Inhaled	1 MAC + 66%	4	1.3 ± 0.3	(–)	(–)
Fentanyl	Intraperitoneal	30 μg/kg	5	1.8 ± 0.4*§	(+)	(–)

* Represent a significant difference compared with 2 MAC isoflurane + N_2O (66%), $P < 0.05$. † Behavioral score was evaluated according to four levels (0: no response, 1: muscle tone, 2: withdrawal of hind paw, and 3: whole body movement). Data are presented as mean behavioral score ± SEM. ‡ Spontaneous activity during the general anesthesia was assessed by handling, a hand clap, toe pinching, and righting reflex. § Nitrous oxide and fentanyl did not produce hypnotic effect.

MAC = minimum alveolar concentration; N_2O = nitrous oxide.

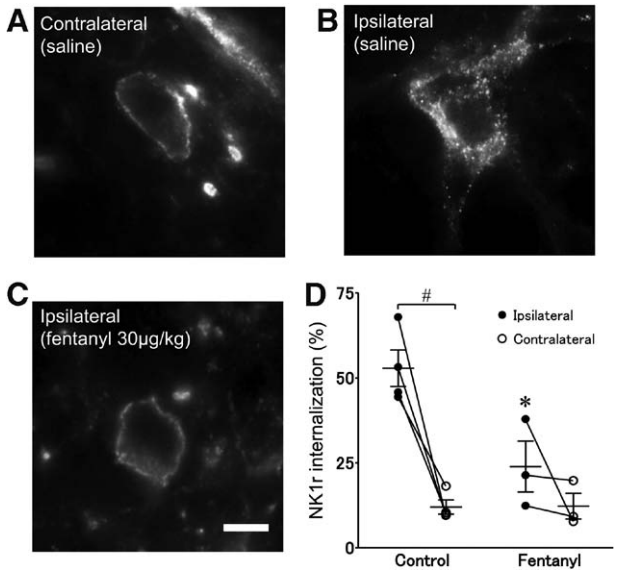

Fig. 1. Effects of intraplantar formalin injection on neurokinin 1 receptor (NK1r) internalization. (*A–C*) Representative light microscopic images of NK1r internalization induced by unilateral formalin injection into the hind paw. (*A*) Image of NK1r-immunoreactive neuron in the contralateral spinal lamina I from a rat administered intraperitoneal saline. (*B*) Image of formalin-induced NK1r internalization in the ipsilateral spinal lamina I from a rat administered intraperitoneal saline. Note the lack of a homogeneous cell membrane and presence of NK1-containing endosomes internalizing into the cytoplasm compared with contralateral neuron. (*C*) Intraperitoneal fentanyl (30 μg/kg, intraperitoneal) blocked formalin-induced NK1r internalization. Images are taken at ×60. *Scale bar* is 10 μm. (*D*) Scattergram presents the percent of NK1r (+) neurons showing internalization after unilateral intraplantar formalin in the ipsilateral and contralateral lumbar (L4–L6) dorsal horn of each animal as a function of receiving pretreatment with vehicle or fentanyl (30 μg, intraperitoneal). Unilateral injection of formalin produced a robust ipsilateral NK1r internalization in lumbar lamina I compared with the contralateral side ($P < 0.05$). Intraperitoneal fentanyl significantly reduced the formalin-induced lumbar dorsal horn NK1r internalization as compared with vehicle ($P < 0.05$). #Represents a significant difference between ipsilateral and contralateral spinal dorsal horn. *Represents a significant difference between control and treated animal, $P < 0.05$.

72 ± 6%; $P < 0.0001$; n = 4; fig. 4). Intraperitoneal fentanyl did not block the internalization induced by exogenous substance P (63 ± 3%; $P > 0.05$ *vs.* intrathecal substance P; n = 3). Similarly, the combination of isoflurane and nitrous oxide did not block the internalization induced by exogenous substance P (62 ± 6%; $P > 0.05$ *vs.* intrathecal substance P; n = 3).

Effects of General Anesthetics on c-Fos Expression

Unilateral injection of formalin to the hind paw produced significant enhancement in the number of c-Fos expressing neurons in the ipsilateral superficial dorsal horn in L4-L5 compared with the contralateral side (ipsilateral: 34 ± 4, contralateral: 5 ± 2; $P < 0.0001$; n = 3; fig. 5, A and B).

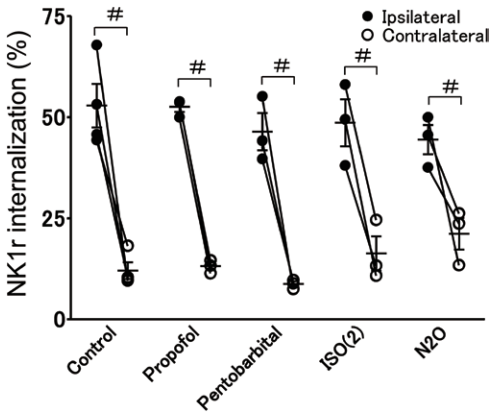

Fig. 2. Effects of general anesthetics on formalin-induced neurokinin 1 receptor (NK1r) internalization. Scattergram presents the percent of NK1r (+) neurons showing internalization after unilateral intraplantar formalin in the ipsilateral and contralateral lumbar (L4–L6) dorsal horn of each animal as a function of receiving pretreatment with vehicle or intraperitoneal propofol (100 mg/kg), pentobarbital (50 mg/kg), or isoflurane at 2 minimum alveolar concentration (MAC) alone or N_2O (66%). As indicated, none of these treatments altered the NK1r internalization in the ipsilateral lamina I compared with saline-treated animals. #Represents a significant difference between ipsilateral and contralateral spinal dorsal horn. ISO (2) = 2 MAC of isoflurane; N_2O = nitrous oxide.

Intraperitoneal fentanyl (30 μg/kg) suppressed the c-Fos expression in the ipsilateral superficial dorsal horn (fentanyl: 8 ± 2; $P < 0.0001$; n = 3). Isoflurane (1 and 2 MAC), N_2O (66%), the combination of isoflurane (2 MAC) + N_2O (66%), pentobarbital (50 mg/kg), and propofol (100 mg/kg) all significantly reduced c-Fos expression in the superficial dorsal horn (isoflurane 1 MAC: 15 ± 2, $P < 0.001$, n = 3; 2 MAC: 12 ± 3, $P < 0.001$, n = 3; N_2O: 11 ± 2, $P < 0$ .001, n = 3; isoflurane + nitrous oxide: 12 ± 1, $P < 0.001$, n = 3; pentobarbital: 11 ± 2, $P < 0.001$, n = 3; and propofol: 13 ± 3, $P < 0.001$, *vs.* control, n = 3; fig. 5C).

Discussion

We examined the effect of general anesthetics on primary afferent release *in vivo* by assessing the spinal release of substance P. This model was chosen for several reasons. (1) Dorsal horn substance P is largely contained and released from small nociceptive primary afferents. (2) This release can be defined *in vivo* by examining the internalization of the NK1r. Agents acting upon primary afferent terminals such as μ opioids will prevent terminal substance P release. N-type, but not L- or T-type, channel blockers will prevent evoked release from the primary afferent.[21] Other transmitters, such as glutamate, although of interest, are ubiquitously distributed in most excitatory dorsal horn neurons. Hence, changes in spinal glutamate release might occur from both afferents and interneurons and would not permit assessments of changes in afferent terminal function.

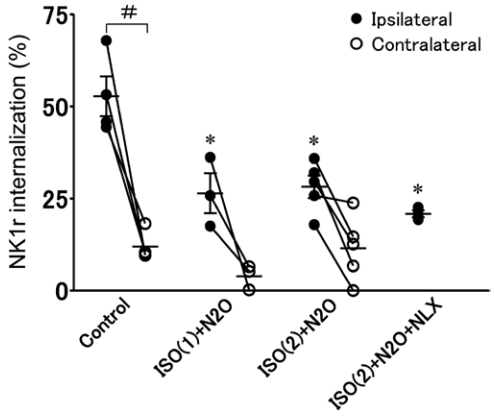

Fig. 3. Synergistic effect of 1 or 2 minimum alveolar concentration (MAC) of isoflurane and nitrous oxide on formalin-induced neurokinin 1 receptor (NK1r) internalization. Scattergram presents the percent of NK1r (+) neurons showing internalization after unilateral intraplantar formalin in the ipsilateral and contralateral lumbar (L4–L6) dorsal horn of each animal as a function of receiving pretreatment with a combination of isoflurane at 1 or 2 MAC with N_2O (66%). As indicated, either combination significantly reduced NK1r internalization in the ipsilateral dorsal horn compared with saline-treated animals ($P < 0.05$). The inhibition of NK1r internalization produced by isoflurane (2 MAC) and nitrous oxide (66%) was not reversed by intraperitoneal naloxone (1 mg/kg). Contralateral NK1r internalization in naloxone-treated rats was not evaluated. #Represents a significant difference between ipsilateral and contralateral spinal dorsal horn. *Represents a significant difference between control and treated animal, $P < 0.05$. ISO (1) = 1 MAC isoflurane; ISO (2) = 2 MAC isoflurane; N_2O = nitrous oxide; NLX = naloxone.

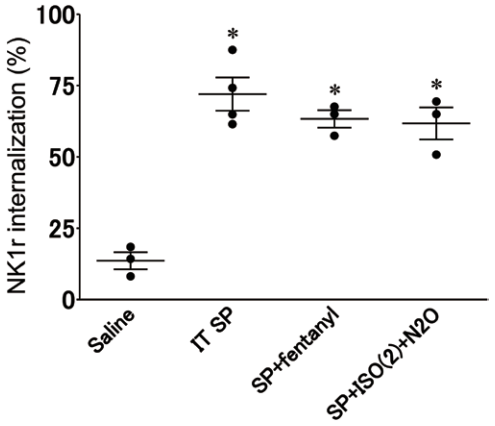

Fig. 4. The effects of fentanyl and combination of isoflurane and nitrous oxide on the neurokinin 1 receptor (NK1r) internalization induced by exogenous substance P. Scattergram presents the percent of NK1r (+) neurons showing internalization in the ipsilateral and contralateral lumbar (L4–L6) dorsal horn of each animal as a function of receiving intrathecal substance P (30 nmol) alone or with fentanyl or isoflurane, at 2 minimum alveolar concentration (MAC) with N_2O (66%). As indicated, intrathecal substance P produced a robust bilateral NK1r internalization in lumbar lamina I NK1 (+) neurons as compared with intrathecal saline. Neither intraperitoneal fentanyl (30 μg/kg), nor a combination of isoflurane (2 MAC) and nitrous oxide (66%) altered the intrathecal substance P evoked NK1r internalization. *Represents a significant difference between control and treated animals, $P < 0.05$. ISO (2) = 2 MAC isoflurane; N_2O = nitrous oxide; SP = substance P.

Substance P Release and NK1r Internalization

Previous work has shown that the paw stimulation evoked internalization of the dorsal horn NK1 receptor reflects upon the release of substance P from the transient receptor potential vanilloid 1 expressing primary afferent terminal.[20,25,26] The NK1r is a G protein coupled receptor, which internalizes when occupied by an agonist, with the degree of internalization reflecting the extracellular substance P release from primary afferents.[20,27] This *in vivo* release is attenuated by μ opiate receptors (*e.g.*, morphine and fentanyl) and is mediated by N-type voltage-sensitive calcium channels, as evidenced by the inhibitory effects of intrathecal ziconotide.[21] Confirmation that any block of internalization is mediated by changes in presynaptic release, as opposed to a direct effect upon the internalization process, is supported by the observation that treatments blocking evoked internalization (fentanyl, isoflurane + nitrous oxide) did *not* block internalization evoked by direct activation of NK1 receptors by intrathecal injection of substance P.

Primary Afferent Transmitter Release and General Anesthetic Action

As expected, based on previous work,[20] systemic fentanyl, at a behaviorally efficacious dose, resulted in block of substance P release. Unexpectedly, none of the anesthetics administered alone had any effect upon release. In the absence of an effect upon afferent terminal peptide release, it is interesting to consider the hypothesized mechanisms of general anesthetic action.

$GABA_A$ Facilitation. Injectable anesthetics, such as propofol and pentobarbital have been reported to regulate membrane excitability by an increase in Cl^- conductance mediated by $GABA_A$ receptors.[6,7] Mutations of these ionophores diminish anesthetic potency of propofol.[28] Isoflurane has similarly been shown to augment activation of the $GABA_A$ receptor ionophore.[29] However, $GABA_A$ receptor agonists, muscimol have no effect upon primary afferent peptide release.[30] Although $GABA_A$ receptor ionophores regulate large afferent excitability,[12] such effects on small afferents have not been identified.

Potassium Channels. Isoflurane has been shown to enhance the functionality of various potassium channels including the two-P-domain K^+ channels, TASK (TWIK-related acid-sensitive K^+ channels) and TREK-1 (TWIK-related K^+ channels), actions which hyperpolarize the membrane.[31] In the absence of an effect upon substance P release, these mechanisms must not serve to block small afferent terminal release at the concentrations defined to be anesthetic *in vivo*.

Calcium Channels. Several inhaled agents, including isoflurane and halothane, have been reported to block N-type[32,33] and T-type[34] calcium channel function. We

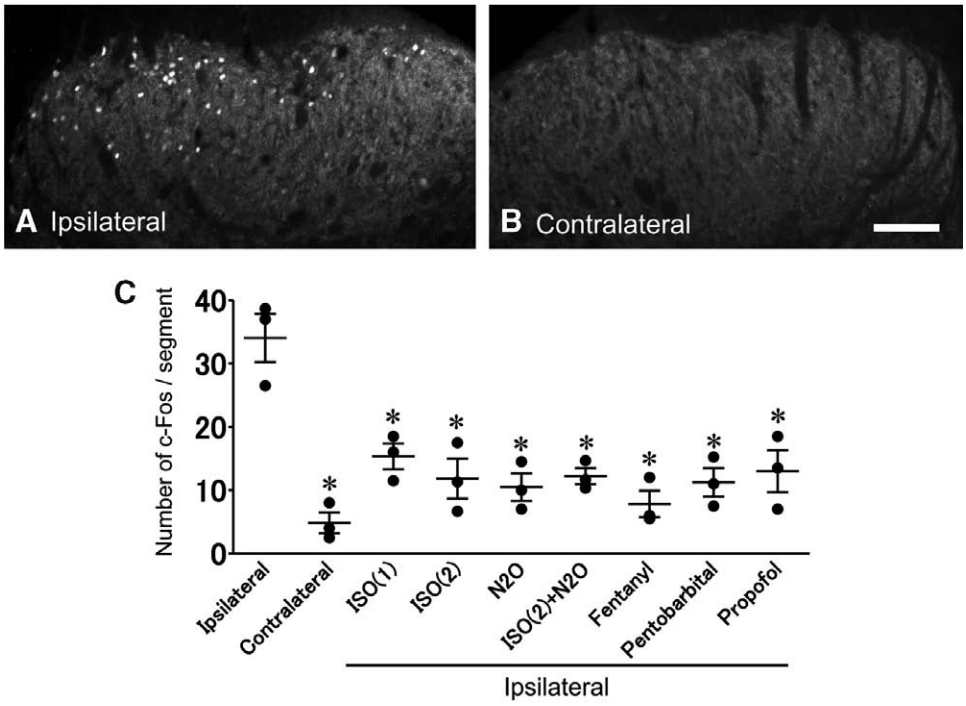

Fig. 5. Effect of general anesthetics on c-Fos expression in the spinal dorsal horn. (*A* and *B*) Light microscopic images of immune-stained c-Fos expression in the superficial dorsal horn (L4-L5). (*A*) Intraplantar formalin injection (5%, 50 μl) significantly increased c-Fos expression in the ipsilateral spinal dorsal horn compared with (*B*) contralateral side. (*C*) Scattergram presents the number of c-Fos (+) cells in the ipsilateral and contralateral lumbar (L4-L5) dorsal horn of each animal after intraplantar formalin as a function of receiving isoflurane at 1 or 2 minimum alveolar concentration (MAC), N_2O (66%), isoflurane at 2 MAC with N_2O (66%), intraperitoneal fentanyl (30 μg/kg), pentobarbital (50 mg/kg), or propofol (100 mg/kg). As indicated, each agent reliably inhibited formalin-induced c-Fos expression in the superficial dorsal horn. *Represents a significant difference between control and treated animal, $P < 0.05$. Magnification ×10. *Scale bar* is 100 μm. ISO (1) = 1 MAC isoflurane; ISO (2) = 2 MAC isoflurane; N_2O = nitrous oxide.

showed that intrathecal ziconotide, a blocker of N-type calcium channels, and opiates, which block opening of N-type calcium channels, also blocked dorsal horn substance P release.[21] Despite this covariance, the current study demonstrated that systemic propofol, barbiturates, isoflurane, or nitrous oxide did not block afferent-evoked substance P release, indicating that at anesthetic concentrations, these agents do not regulate spinal N-type channel function *in vivo*.

Nitrous Oxide and Its Synergistic Interaction with Isoflurane

Neither nitrous oxide at the maximum usable concentrations, nor 2 MAC isoflurane blocked substance P release. Unexpectedly, isoflurane (1 and 2 MAC) and N_2O (66%) together reduced evoked substance P release. The mechanism of this interaction is unknown. Although previous work has suggested that nitrous oxide may lead to opiate receptor activation,[35] a high dose of naloxone showed no effect upon the suppressed release. The synergistic interaction observed here parallels the behavioral literature. Although some reports refer to the fact that volatiles such as halothane and isoflurane may antagonize nitrous oxide analgesia,[36] we saw no evidence of such a negative effect on substance P release of c-Fos activation here, and nitrous oxide is MAC-sparing, when used with volatile anesthetics.[37–39]

General Anesthetics and Evoked c-Fos Expression

Spinal expression of c-Fos is enhanced in the ipsilateral dorsal horn after unilateral nociceptive stimulation.[40,41] This increase reflects postsynaptic excitation of dorsal horn neurons mediated by primary afferent input (monosynaptic) or through the large dorsal horn interneuronal pools of glutamatergic neurons with excitatory linkages mediated by a variety of glutamate receptors, including those of the *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid subtypes.[42,43] Our work showed that although none of the general anesthetics at efficacious concentrations had any effect upon primary afferent release, all resulted in a pronounced suppression of evoked spinal c-Fos expression. These results indicate that the anesthetic effects are mediated either by mechanisms involving nonpeptidergic afferent input that is anesthetic sensitive, or their actions are postsynaptic to the primary afferent. These findings are similar to those reported for propofol, but not pentobarbital, given preinjury/stimulation.[9] Isoflurane, but not halothane, administered at concentrations that suppress reflex movement

(1 MAC) diminish c-Fos expression.[10] High concentrations of isoflurane (1.5 MAC) depressed c-Fos expression in spinal lamina II, whereas fentanyl reduced expression in lamina V.[11] Results with nitrous oxide have been controversial. Although some have reported little or no effect of nitrous oxide,[44,45] or even an increase,[46] many studies, including the current one, demonstrate that nitrous oxide alone reduced dorsal horn c-Fos expression.[11,47,48] Thus, in the current work, nitrous oxide prevented c-Fos expression in superficial dorsal horn (lamina I-II). Hagihira *et al.*[48] reported that nitrous oxide reduced c-Fos in the neck of dorsal horn (lamina V–X), but not in the superficial layers (laminae I-II). Interestingly, these authors speculated that this differential effect suggested that nitrous oxide did not have any effects on neurons directly driven by afferent input. There are several caveats to the effect of nitrous oxide. First, many cells showing increased c-Fos with nitrous oxide were reported to be GABAergic neurons.[49] Second, if there were any inhibition by nitrous oxide of GABAergic interneurons, this would itself confound any suppressant effects of nitrous oxide on cellular c-Fos expression in neurons postsynaptic to the GABA interneuron.

Overall, the lack of effect of the anesthetics on c-Fos reported here, in conjunction with the literature discussed in the preceding paragraph, emphasize the likelihood that the anesthetic actions mediated at the spinal cord level are directed at targets postsynaptic to the primary afferent. Consistent with this postsynaptic action *in vivo* propofol depresses slow ventral root potential, otherwise evoked by local injection of substance P, suggesting a site of action on systems postsynaptic to the substance P releasing primary afferent.[50] Other studies have also supported indirectly the likelihood of a postsynaptic effect.[12,51,52]

Significance of Current Observations

Previous work in a variety of models has supported the assertion that the ability of general anesthetics to alter the organized behavioral response or the response of dorsal horn neurons to a noxious stimulus is mediated at the level of the spinal dorsal horn.[53–57] The current work demonstrates that general anesthetics at concentrations which yield an anesthetic state failed to block release from a peptidergic, typically high threshold, sensory afferent. In contrast, all agents at these concentrations resulted in suppression of evoked c-Fos expression. These results, thus, suggest that although these anesthetics reduce excitation of neurons, which displayed c-Fos expression (many of which are believed to be spinofugal projection neurons),[58] this effect does not result from the block of the small afferent-evoked excitation. We note that failure of these anesthetics to block substance P release suggests that even in the presence of MAC anesthesia, the second-order neurons is still exposed to the activation mediated by of excitatory receptors. Considerable work has shown that such small afferent input can lead to changes in second-order neuron functions, which underlie facilitated states. Thus, in previous work, we have shown that after intraplantar formalin model, isoflurane delivered only during the early phase 1 flinching does not alter phase 2 flinching. In contrast, early treatment with a μ opiate during only the first phase significantly reduces the magnitude of the second phase.[59,60] Continuing in this vein, it is important to note that the electrophysiological phenomena of spinal "wind up" requiring extreme surgical interventions (*e.g.*, dissection and laminectomy) is typically examined under general anesthetics.[61–64] We accordingly hypothesize that in the face of many prototypical general injectable and volatile anesthetics, small afferent traffic continues to result in an activation of second-order neuron and that it is the activation of neuron or interneuron, which is suppressed by anesthetics. Under these conditions, the second order neuron is subject to the initiation of facilitation cascades that lead to persistent changes in spinal excitability.

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