Published in final edited form as:

Anesth Analg. 2009 August ; 109(2): 641–647. doi:10.1213/ane.0b013e3181a9770f.

Rat Dorsal Horn Nociceptive-Specific Neurons are More Sensitive than Wide Dynamic Range Neurons to Depression by Immobilizing Doses of Volatile Anesthetics: An Effect Partially Reversed by the Opioid Receptor Antagonist Naloxone

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Abstract

Background—The mechanism and site of action within the spinal cord by which volatile anesthetics produce immobility are not well understood. Little work has been done directly comparing anesthetic effects on neurons with specific functional characteristics that mediate transfer of nociceptive information within the spinal cord.

Materials and Methods—Adult male rats were anesthetized and prepared for extracellular single-unit recordings from the lumbar dorsal horn. Nociceptive-specific (NS) and wide dynamic range (WDR) neurons were identified and noxious-heat evoked neuronal spike rates evaluated at 0.8 and 1.2 minimum alveolar anesthetic concentration (MAC) halothane or isoflurane. In another group, noxious-heat evoked responses from NS neurons were evaluated at 0.8, 1.2 MAC halothane, and 1.2 MAC halothane plus IV naloxone (0.1 mg/kg).

Results—Increasing halothane from 0.8 to 1.2 MAC reduced the heat-evoked neuronal responses of NS neurons (n=9) from 827 ± 122 (mean $\pm$ SE) to 343 ± 48 spikes/min ($p < 0.05$) but not WDR neurons (n=9), 617 ± 79 to 547 ± 78 spikes/min. Increasing isoflurane from 0.8 to 1.2 MAC reduced the heat-evoked neuronal response of NS neurons (n=9) from 890 ± 339 to 188 ± 97 spikes/min ($p < 0.05$), but did not alter the response of WDR neurons (n=9) in which evoked spike rate went from 576 ± 132 to 601 ± 119 spikes/min. In a separate group, the response from NS neurons went from 282 ± 60 to 74 ± 32 spikes/min ($p < 0.05$) when halothane was increased from 0.8 to 1.2 MAC. Intravenous administration naloxone increased the heat-evoked response to 155 ± 46 spikes/min ($p < 0.05$).

Conclusions—NS but not WDR neurons in the lumbar dorsal horn are depressed by peri-MAC increases of halothane and isoflurane. This depression, at least with halothane, can be partially reversed by the opioid antagonist naloxone. Given that opioid receptors are not likely involved in the mechanisms by which volatile anesthetics produce immobility, this suggests that, although the neuronal depression is of substantial magnitude and occurs concurrent to the production of immobility, it may not play a major role in the production of this anesthetic end-point.

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Conflict of Interest: None

Introduction

Immobility is an important anesthetic end-point. Clinically it is used to assess depth of anesthesia and has been the basis of potency comparisons of anesthetics for more 40 years.^{1, 2} Volatile anesthetics' action within the spinal cord is important in the production of immobility.^{3–5} Beyond this, the site or mechanism of action by which general anesthetics produce immobility is not known. Sensory afferent neurons synapse primarily within the spinal cord dorsal horn.^{6,7} Thus anesthetic action within the dorsal horn, by reducing transmission of sensory information into the motor circuits underlying movement, would be a potential mechanism by which immobility could be produced.

Minimum alveolar anesthetic concentration (MAC) is that alveolar anesthetic concentration associated with a 50% probability of movement.² Reports of the effects of anesthetic doses that just allow and just prevent movement (i.e. periMAC concentrations) on evoked responses from dorsal horn neurons provide varying results. Increasing the halothane concentration from just below to just above MAC is reported to depresses dorsal horn neurons, whereas the same isoflurane dose increase does not.^{8–11}

Neurons of the dorsal horn vary in their stimulus response properties: wide dynamic range (WDR) neurons respond to stimuli from innocuous to noxious intensities with progressively increasing frequency, whereas nociceptive-specific neurons (NS) respond only to noxious stimuli.^{12–13} Both of these classes of neurons respond to noxious stimuli and thus may be part of the neural circuitry responsible for movement in response to noxious stimulation. Studies investigating anesthetic effects on the dorsal horn typically evaluate heterogeneous populations of dorsal horn neurons. There has been little work comparing anesthetic effects on neurons with different functional characteristics, such as NS and WDR neurons. Based on unpublished observations from our laboratory, we hypothesized that NS, but not WDR neurons, would be depressed by peri-MAC increases in anesthetic concentration.

Depression of the neuronal response to noxious stimuli by anesthetic doses that suppress movement, although coincident, is not proof of relevance to anesthetic site of action. In order to investigate the relevance of neuronal depression to the production of immobility by inhaled anesthetics, we asked whether systemic administration of the opioid receptor antagonist naloxone, a drug that has been shown not to affect MAC, would reverse this depression.^{14–16}

Materials and Methods

Animals

Twenty-eight adult male Sprague-Dawley rats weighing 545 ± 39 gm (mean $\pm$ SD) were used in these experiments with the approval of the Animal Care and Use Committee of the University of California, Davis. Rats were housed in a temperature-controlled room (set to 21°C) with 12 hour light: dark cycle and received food and water *ad libitum*.

Surgical Preparation

For anesthetic induction, rats were placed into a chamber into which isoflurane (5%) or halothane (4%) in 100% oxygen (1 liter/min) was delivered. When the righting reflex was lost, rats were removed from the chamber and anesthetic delivery continued via facemask at concentrations sufficient to prevent movement in response to toe pinch. A rectal temperature probe was placed and a heating pad used to maintain body temperature at 38 ± 1 °C. A tracheostomy tube was placed and mechanical ventilation commenced (CIV101, Columbus Instruments, Columbus, OH). Respiratory gases were sampled via a cannula placed into the anesthetic circuit level with the proximal end of the tracheostomy tube. A calibrated gas analyzer (Rascal II, Datex-Ohmeda, Helsinki, Finland) was used to monitor anesthetic

concentrations and partial pressure of carbon dioxide (PCO₂). Mechanical ventilation was adjusted to maintain an expired PCO₂ of 35 ± 5 mmHg. One carotid artery was cannulated with PE50 tubing to enable direct arterial blood pressure measurement (PB240, Puritan Bennett Corporation, Pleasanton, CA). Unilateral carotid ligation in the rat has been shown not to result in cerebral ischemia.¹⁷ The jugular vein was cannulated with PE50 tubing for delivery of drugs and fluids. Intravenous lactated Ringer's solution or an artificial colloid solution (Hextend, BioTime Inc, Berkley, CA) was delivered as required for maintenance of mean arterial blood pressure above 60mmHg.

A midline incision was made over the dorsum and the paraspinous musculature dissected free from the spinous processes and dorsal aspects of T12 to L2. Dorsal laminectomies were performed at T13 and L1 to expose the lumbar intumescence. The dorsolateral surfaces of T12 and L2 were freed from connective tissue and muscle to enable placement of vertebral clamps. After surgical preparation the MAC of either halothane or isoflurane was determined in each animal. After equilibration (15 min for isoflurane or 20 min for halothane), a 12-inch hemostat was applied across the tail to the first ratchet lock and oscillated at approximately 1 Hz for 60 seconds. If the animal moved (either lifted its head or grossly moved any limb), the anesthetic concentration was increased by 10–20%. If the animal did not move, the anesthetic concentration was decreased by 10–20%. After adjustment of the anesthetic concentration, equilibration was allowed (15 min for isoflurane or 20 min for halothane) and the animal retested. This process was continued until 2 successive concentrations were found, one that permitted and one that prevented movement. The arithmetic mean of those 2 concentrations was deemed MAC for that anesthetic in that animal.

After MAC determination, the rat was placed into a stereotaxic frame (D. Kopf Instruments, Tujunga, CA) and secured using vertebral clamps and ear bars. The dura was removed from the exposed lumbar cord segments and covered with a thin layer of warm transparent agar. During neuronal recording, pancuronium (0.5mg/kg/hour) was administered.

Neuron Recording and Characterization

A 15 M Ω tungsten microelectrode (FHC, Bowdoinham, ME) was advanced into the spinal cord dorsal horn in 5 μ m increments using a hydraulic drive (D. Kopf Instruments, Tujunga, CA). Action potentials were amplified (P511, Grass, Braintree, MA), band-pass filtered between 300 and 3000 Hz, displayed and recorded with a PowerLab interface and Chart5 software (ADInstruments, Colorado Springs, CO).

An electric stimulus (1 ms 0.5Hz), applied via needle electrodes placed subcutaneously in the plantar aspect of the hindpaw, was used to search for single units. After a single unit was isolated, mechanical stimuli were used to characterize the neuron and map its receptive field. Units responding to both non-noxious (light touch, von Frey hair < 2 g bending force) and, with increasing intensity, to noxious (von Frey hair > 4.0 g bending force, pinch) stimuli were classified as WDR neurons. Those units responding only to noxious stimuli were classified as NS neurons. Care was taken to prevent tissue damage before characterization of neuron. Receptive fields for the single units were mapped using touch for WDR and pinch for NS neurons.

Once the single unit had been isolated, characterized and mapped, responses to noxious thermal stimulation were recorded. The receptive field on the hindpaw was centered over a Peltier thermode and heated to 54°C for 10 sec at a rate of 10°C.sec⁻¹ from an adapting temperature of 35°C.¹⁸

Depth readings from the hydraulic microdrive were used to record neuronal depth from the spinal cord surface. In animals in which 2 neurons were studied, neurons with overlapping

receptive fields that were clearly differentiable based upon spike waveform were recorded concurrently, or in halothane and isoflurane groups one neuron was isolated from each side of the spinal cord.

Experimental Protocol: Halothane and Isoflurane Groups

Animals were anesthetized with either halothane or isoflurane. Nine neurons of each type, NS and WDR, were studied for each anesthetic (36 neurons in total). Neurons were initially isolated and characterized at 0.8 MAC. The neuronal response to heat was evaluated at 0.8 and 1.2 MAC in random order. A stable expired anesthetic concentration was maintained for 15 min for isoflurane and 20 min for halothane between changes in anesthetic dose.

At each anesthetic concentration, the heat-evoked neuronal response was evaluated at least 3 times with 5 minutes between stimuli. The number of spikes in the 30 seconds before stimulus application was counted to establish spontaneous neuronal activity rate. Heat-evoked neuronal response was evaluated by counting the number of spikes in the 60 seconds from the onset of the thermal stimulus. Spike rate per minute over and above spontaneous activity for each test at the same anesthetic and dose were averaged for each neuron.

Experimental Protocol: Naloxone Group

Animals were anesthetized with halothane and NS neurons isolated and characterized at 0.8 MAC. The neuronal response to heat was evaluated initially at 0.8 then at 1.2 MAC with 20 minutes of stable expired anesthetic concentration maintained between changes in anesthetic dose. At each anesthetic dose the heat-evoked neuronal response was evaluated as described above. One minute after final evaluation at 1.2 MAC naloxone 0.1 mg/kg was administered IV. The neuronal response to heat was evaluated 4 minutes after naloxone administration, and every 5 minutes thereafter, for at least 3 evaluations.

Data Analysis

Data are expressed as mean $\pm$ SE unless otherwise stated. Data were log transformed where required to meet normality assessed by the Kolmogorov and Smirnov method. Paired Student's t-tests were used to compare the effect of increasing anesthetic concentrations on heat-evoked spike rates within groups. Within the naloxone group, repeated measures ANOVA with Tukey *post hoc* tests were used to evaluate effect of treatment on evoked neuronal responses. Recording depths between the groups were compared using ANOVA. Significance level was set at $p < 0.05$.

Results

Halothane MAC in this study was 1.2 ± 0.1 (mean $\pm$ SD) % atm ($n = 16$). Isoflurane MAC was 1.4 ± 0.1 (mean $\pm$ SD) % atm ($n = 12$). Increasing halothane from 0.8 to 1.2 MAC reduced the mean heat-evoked neuronal response of NS neurons ($n = 9$) by $55 \pm 7\%$ from 760 ± 127 to 310 ± 51 spikes/min ($p < 0.001$). Although the magnitude of change varied, the evoked response from all NS neurons evaluated was reduced by increasing halothane dose from 0.8 to 1.2 MAC (Figure 1A).

Increasing the halothane dose overall had no significant effect on heat-evoked responses from WDR neurons ($n = 9$) which were 532 ± 92 and 448 ± 77 spikes/min at 0.8 and 1.2 MAC, respectively. The effect of increasing halothane dose had more variable effects on WDR neurons with responses from some neurons being depressed, whereas from others they were facilitated (Figure 1B).

Increasing the isoflurane dose from 0.8 to 1.2 MAC reduced the mean heat-evoked response from NS neurons ($n = 9$) by $73 \pm 8\%$, from 878 ± 342 to 186 ± 97 spikes/min ($p < 0.05$). The responses from all NS neurons evaluated were depressed at 1.2 MAC (Figure 2A). Responses from the WDR neurons overall were increased by $19 \pm 15\%$ when the isoflurane dose was increased from 0.8 to 1.2 MAC. The mean heat-evoked response rates were 559 ± 135 and 594 ± 117 spikes/min at 0.8 and 1.2 MAC, respectively. This effect was not significant and the direction of change for individual neuronal responses variable (Figure 2B). Example extracellular single unit recordings from a NS and WDR neuron at 0.8 and 1.2 MAC isoflurane are shown in Figure 3.

In the naloxone group, the heat-evoked responses of NS neurons ($n = 6$) were reduced by $73 \pm 10\%$ from 282 ± 60 to 74 ± 32 spikes/min when the halothane dose was increased from 0.8 to 1.2 MAC ($p < 0.05$) (Figure 4). Heat-evoked responses at 1.2 MAC halothane, after IV administration of 0.1 mg/kg naloxone, were increased to 155 ± 46 spikes/min ($p < 0.05$) (Figure 4). This was, on average, a return to $56 \pm 12\%$ of the response seen at 0.8 MAC. In all NS neurons tested in this manner, increasing the halothane dose from 0.8 to 1.2 MAC reduced responses (Figure 4) and administration of naloxone increased responses (Figure 4).

Neuronal recording depth did not differ among treatment groups or between neuronal classes. Recording depth averaged $385 \pm 34 \mu\text{m}$ and ranged from 60 to 907 μm .

Discussion

Heat-evoked responses from NS neurons of the rat lumbar dorsal horn were depressed by increasing halothane or isoflurane dose from 0.8 to 1.2 MAC. All NS neurons studied were depressed to some degree by this increase in anesthetic dose. The average magnitude of this change was substantial, representing a 55 to 73% depression from 0.8 to 1.2 MAC for halothane and isoflurane, respectively. The effect of the same periMAC halothane or isoflurane dose increase on evoked responses from WDR neurons was variable: some neurons were depressed, whereas others were facilitated such that the overall response was not significantly altered.

Previous reports describing the effects of inhaled anesthetics on spinal cord dorsal horn neurons have yielded varying results. Evoked responses of dorsal horn neurons to noxious stimuli have reportedly been depressed by periMAC dose increases of halothane and isoflurane.^{8–11,19} However, further studies failed to confirm isoflurane-induced depression of dorsal horn neurons and suggest periMAC increases of isoflurane dose may facilitate neuronal response to noxious stimulation.^{10,11} These experiments vary with respect to the type, duration, and intensity of noxious stimuli applied as well as the types of neurons studied. Neuronal response to submaximal noxious stimuli may be more easily depressed than responses to the high intensity stimuli used in the present study. Most reports describe periMAC dose increases of volatile anesthetics as having variable effects on individual neurons, that is, that there is no consistent direction of change in response.

WDR neurons tend to be the dominant neuronal type in most studies of the dorsal horn as they are more numerous.^{13,20,21} There have been no studies directly comparing the effects of anesthetics on NS versus WDR neurons. Techniques frequently used to search for and characterize dorsal horn neurons often involve repeated mechanical stimuli. This may damage tissues and result in inflammation in the area of the neuron's receptive field. One of the aforementioned studies describes obvious inflammation of the hindpaw as a consequence of the search stimuli.⁸ Peripheral inflammation can alter the response characteristics of dorsal horn neurons. Inflammatory lesions created in the distal limb have been shown to reduce the threshold of NS neurons to non-noxious levels within minutes and the change may persist for more than 90 minutes.^{22,23} In the presence of peripheral inflammation, sensitized NS neurons

could be mistakenly characterized as WDR neurons. This phenomenon may account for the varied results seen in effects on dorsal horn neurons, many of which were characterized as WDR but may have actually been a mixture of NS and WDR.

Effort was made in this study to avoid application of any tissue-damaging stimulus to the distal limb until after a single unit had been isolated via low frequency electrical stimulation. In this way we aimed to correctly characterize neurons according to their stimulus response properties. We selected stimulus duration and frequency that were insufficient to create wind-up and minimized the likelihood of activation of C-fibers from deep tissues.

Recent work suggests that anesthetics act in the ventral spinal cord to produce immobility and that anesthetic action in the dorsal horn is not critical.²⁴ The present data are consistent with this scenario. Ventrally situated central pattern generators are emerging as a likely key target where anesthetics act to produce immobility.²⁵ However, the latter study did suggest that volatile anesthetic effects in the dorsal horn make a small (10–20%) contribution to their immobilizing effects. The present study suggests that this small contribution may be manifested mainly by a selective NS neuron depression in the dorsal horn.

We cannot exclude the possibility that anesthetic action on supraspinal structures affects WDR and NS neurons. In the rostroventral medulla, peri-MAC increases in isoflurane have been found to increase the activity of OFF cells and depress that of ON cells, overall tending to a greater inhibition at just above MAC.²⁶ We are unaware of any data addressing whether this descending modulation acts differently on WDR and NS neurons. Diffuse noxious inhibitory control (DNIC) may have varying effects on different categories of dorsal horn neurons; however, isoflurane has been found to reduce, rather than enhance, DNIC at peri-MAC concentrations.²⁷

Halothane-induced depression of NS neuronal responses to noxious heat were reversed by systemic administration of the opioid antagonist naloxone. You *et al* observed that depression of dorsal horn WDR neurons by halothane was completely reversed by naloxone, whereas depression of simultaneously recorded motor units were only partially reversed.²⁸ The reversal of halothane induced neuronal depression by naloxone confirms the findings of our study in a different class of dorsal horn neuron. The depressant effects of halothane in the aforementioned study were, however, seen at anesthetic doses 3–4 times those required to prevent movement in response to supramaximal stimuli in their spinalized animals.

A number of lines of evidence suggest that opioidergic actions are not important in the mechanism of anesthetic-induced immobility. Although potent μ opioid receptor agonists have been shown to reduce MAC in many species, in some species, such as the horse, they do not consistently produce MAC-sparing effects.^{29–33} In addition, a number of studies have shown that the opioid receptor antagonist naloxone does not alter MAC.^{14–16,34,35} Halothane does not increase the concentration of endogenous opioid peptides in the cerebrospinal fluid nor alter ligand binding to opioid receptors in cultured cell lines.^{36,37} In contrast, Dahan *et al* reported sevoflurane MAC to be 20% greater in μ opioid receptor knockout mice compared to wild types and naloxone increased sevoflurane MAC by 18% in wild type mice but had no effect on knockouts.³⁸ There are problems inherent to studying knockout animals. Attribution of an effect only to the intended genetic manipulation is complicated by the altered expression levels of multitudes of additional genes that occur as a compensatory mechanism for the original mutation.³⁹ The naloxone-induced MAC increase in the Dahan *et al.* study is more difficult to discount, although their numbers were small, the experimenters not blinded and the coefficient of variation in their study (up to 15%) close to their detected change of 18%. Another group repeated the study with larger numbers, using saline controls and were blinded to treatment.¹⁵ They failed to confirm the findings of the original study.

Taken together these reports overwhelmingly suggest that opioidergic systems are not involved in the production of immobility by volatile anesthetics. Although volatile anesthetic depression of NS neurons might explain the small fraction of dorsal horn involvement in immobility, it is not surprising that a partial reversal of this small effect by naloxone would result in undetectable changes in MAC. Further studies will be necessary to tell us if this anesthetic effect is associated with another spinally mediated effect of volatile anesthetics.

In summary, the present study describes a distinct difference in the effect of periMAC dose increases of halothane and isoflurane on evoked neuronal responses in NS versus WDR rat dorsal horn neurons. Noxious heat-evoked responses from NS neurons were consistently and substantially depressed, whereas those from WDR neurons were unaffected. The fact that this depression can be reversed, at least in part, by an opioid receptor antagonist when evidence suggests that opioidergic systems do not mediate anesthetic induced immobility, suggests that this effect of the volatile anesthetics and immobility may not be causally related.

Acknowledgments

NIH Grant GM 61283 (to JFA) and GM 78167 (to SLJ) supported this work.

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Figure 1.

Increasing halothane dose from 0.8 to 1.2 MAC depressed noxious heat-evoked neuronal responses from nociceptive-specific (NS) neurons by $55 \pm 7\%$ (mean $\pm$ SE) ($*p < 0.05$) (A) but had no effect on response from wide dynamic range (WDR) neurons (B) in the rat dorsal horn. Both population (solid squares, mean $\pm$ SE) and individual data are plotted. Lines join the responses from individual neurons at the 2 anesthetic doses. The periMAC increase in anesthetic dose consistently depressed responses from NS neurons while having a variable effect on those from WDR neurons.

Figure 2.

Increasing the isoflurane dose from 0.8 to 1.2 MAC depressed noxious heat-evoked neuronal responses from nociceptive-specific (NS) neurons in the rat dorsal horn by $73 \pm 8\%$ (mean $\pm$ SE) ($*p < 0.05$) (A). The same isoflurane dose increase had no overall effect on the response from dorsal horn wide dynamic range (WDR) neurons (B). Population (solid squares, mean $\pm$ SE) and individual data are plotted. Lines join the responses from individual neurons at the 2 anesthetic doses. The periMAC increase in anesthetic dose consistently depressed NS neuronal response while variably affecting the responses from WDR neurons.

Figure 3.

Examples of typical extracellular single unit recordings from a nociceptive-specific (NS) neuron and a wide dynamic range (WDR) neuron in the rat dorsal horn. A noxious thermal stimulus (54°C for 10 sec indicated by black bar between tracings) was applied to the receptive field of the neuron on the plantar aspect of the hindpaw and the neuronal responses recorded at 0.8 and 1.2 MAC isoflurane.

Figure 4.

Increasing the halothane dose from 0.8 to 1.2 MAC depressed noxious heat-evoked neuronal responses from nociceptive-specific (NS) neurons in the rat dorsal horn by $73 \pm 10\%$ (mean $\pm$ SE) ($*p < 0.05$). Administration of naloxone (0.1mg/kg IV) reversed this depression ($**p < 0.05$). Population (solid squares, mean $\pm$ SE) and individual data are plotted. Lines join the responses from individual neurons for the three treatments.