Isoflurane Depresses the Response of Inspiratory Hypoglossal Motoneurons to Serotonin In Vivo

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**Background:** Endogenous serotonin (5-HT) provides important excitatory drive to inspiratory hypoglossal motoneurons (IHMNs). In vitro studies show that activation of postsynaptic 5-HT receptors decreases a leak K⁺ channel conductance and depolarizes hypoglossal motoneurons (HMs). In contrast, volatile anesthetics increase this leak K⁺ channel conductance, which causes neuronal membrane hyperpolarization and depresses HMN excitability. Clinical studies show upper airway obstruction, indicating HMN depression, even at subanesthetic concentrations. The authors hypothesized that if anesthetic activation of leak K⁺ channels caused neuronal depression in vivo, this effect could be antagonized with serotonin. In this case, the neural response to picoejection of serotonin would be greater during isoflurane than with no isoflurane.

**Methods:** Studies were performed in decerebrate, vagotomized, paralyzed, and mechanically ventilated dogs during hypercapnic hyperoxia. The authors studied the effect of approximately 0.3 minimum alveolar concentration (MAC) isoflurane on the spontaneous discharge frequency patterns of single IHMNs and on the neuronal response to picoejection of 5-HT.

**Results:** Normalized data (mean ± SD, n = 19) confirmed that 0.3 ± 0.1 MAC isoflurane markedly reduced the spontaneous peak discharge frequency by 48 ± 19% (P < 0.001) and depressed the slope of the spontaneous discharge patterns. The increase in neuronal frequency in response to 5-HT was reduced by 34 ± 22% by isoflurane (P < 0.001).

**Conclusion:** Subanesthetic concentrations of isoflurane strongly depressed canine IHMNs in vivo. The neuronal response to 5-HT was also depressed by isoflurane, suggesting that anesthetic activation of leak K⁺ channels, which is expected to result in a larger 5-HT response, was not a dominant mechanism in this depression.

HYPOGLOSSAL motoneurons (HMs) innervate all tongue muscles and thus are vital for maintenance of upper airway patency during inspiration. Partial or complete loss of inspiratory hypoglossal motoneuron (IHMN) activity during sleep, during various stages of anesthesia, or during postanesthetic recovery can lead to life-threatening upper airway obstruction. It has been long known that volatile anesthetics cause significant airway compromise at doses relevant for surgical anesthesia, but even subanesthetic concentrations, which continue to be present for some time in the postanesthesia recovery unit, can cause airway-related morbidity.

Similarly, we observed in a decerebrate canine preparation in vivo that IHMNs are significantly depressed by subanesthetic volatile anesthetic concentrations with a depression of neuronal activity of 30 ± 11% by 0.25 minimum alveolar concentration (MAC) isoflurane. In contrast, to observe a similar amount of depression in respiratory premotor neurons, threefold higher anesthetic concentrations were required. We showed that the anesthetic depression of respiratory premotor neurons is due to a depression of excitatory glutamatergic and enhancement of yaminobutyric acid type A–mediated (GABAergic) inhibitory mechanisms. The markedly increased depression of IHMNs may be in part accounted for by an anesthetic effect on the additional synapse between premotor and motoneurons. Alternatively, the more than threefold greater depression of IHMNs compared with respiratory premotor neurons suggests that a different, nonglutamatergic and non-GABAergic, anesthesia-sensitive receptor or channel mechanism may be involved.

Physiologic studies on the receptor composition of IHMNs in the canine in vivo preparation have recently shown that serotonin (5-hydroxytryptamine [5-HT]) is a potent modulator of IHMN activity through the 5-HT₂A receptor subtype. Block of serotonergic input with ketanserin led to a 68% reduction in neuronal peak frequency. Serotonergic input induced a change in gain of the neuronal firing pattern in addition to a small change in offset. This matches in vitro observations made in whole cell recordings of IHMNs in brainstem slices, which indicate that 5-HT acts in part via a barium-resistant sodium channel but mainly via a TWIK-related acid-sensitive K⁺ (TASK) channel. Block of this K⁺ leak channel with 5-HT will increase neuronal input resistance resulting in amplification of other excitatory inputs. In addition, volatile anesthetics have been shown to open the TASK channel leading to hyperpolarization of the cell, which can be antagonized by 5-HT when the membrane potential is held in a range at or above the neuronal firing threshold (≥ −60 mV).

We hypothesized that the profound depression of HMN activity by subanesthetic concentrations of isoflurane, which we observed in our in vivo preparation, was mainly due to the activation of TASK channels. If our hypothesis is correct, picoejection of 5-HT onto the
IHMN should be able to reverse the anesthetic-induced depression of the neuron. In that case, the increase in neuronal discharge frequency after picoejection of 5-HT should be larger during subanesthetic isoflurane administration than without isoflurane.

Materials and Methods
Animal Preparation and General Methodology
This research was approved by the Medical College of Wisconsin Animal Care Committee, Milwaukee, Wisconsin, and conforms to standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Anesthesia in mongrel dogs of either sex was induced by inhalation of isoflurane via mask. The dogs were then intubated with a cuffed endotracheal tube and from then on mechanically ventilated with oxygen. Isoflurane (1.3–1.8 MAC) was applied throughout the surgical procedures and only discontinued after completion of decerebration (1 MAC isoflurane in dogs = 1.4%). The animals were positioned in a stereotaxic device (model 1530; David Kopf Instruments, Tujunga, CA) with the head ventrally flexed (30°). Bilateral neck dissections were performed. Incisions were made on both sides of the upper neck area. Superficial muscles of the region were retracted, and the hypoglossal nerve was separated from its surrounding tissue. The hypoglossal nerve was exposed, cut near the bifurcation point of the lateral and medial branch, desheathed, and placed onto a custom made bipolar wire electrode for recording. The C5 phrenic nerve rootlet was cut, desheathed, and put onto a custom-made bipolar hook electrode for recording. Bilateral vagotomy was performed to achieve peripheral deafferentation from pulmonary stretch receptor input, which eliminates volume feedback from the mechanical deafferentation from pulmonary stretch receptor input, Bilateral pneumothorax was performed to minimize chest wall afferents. The animals were decerebrated at the midcollicular level and only then paralyzed (0.1 mg/kg intravenous pancuronium, followed by infusion of 0.1 mg · kg⁻¹ · h⁻¹). For single neuron recording, an occipital craniotomy was performed to expose the dorsal surface of the medulla oblongata. Dexamethasone (American Regent Laboratories, Shirley, NY) was administered intravenously before surgery to prevent brain swelling (1 mg/kg at induction and every 6 h thereafter). Esophageal temperature was maintained at normothermia for dogs (38.5° ± 1°C) with a heating pad. Mean arterial pressure was kept at or above 100 mmHg and did not differ more than 20% for the protocols between 0 and 0.3 MAC isoflurane. Protocols were performed only during steady state conditions for blood pressure. In this study, no vasopressor support was necessary to maintain stable hemodynamics. To minimize blood loss related to the activation of the fibrinolytic system secondary to the decerebration trauma, e-amino-n-caproic acid (Sigma-Aldrich Co., St. Louis, MO) was administered intravenously (bolus of 125 mg/kg at induction, followed by 15 mg · kg⁻¹ · h⁻¹).

Neuron Recording Technique, Data Collection, and Experimental Conditions
For recording, we used anatomical information from a previous study as a guide for locating IHMNs. The cholera toxin B subunit, injected into the genioglossus muscle of adult mongrel dogs, was used to retrogradely label genioglossal motoneurons, which comprise the largest subgroup of IHMNs. These IHMNs are distributed within a compact column, extending from 0.5 mm caudal to 3.5 mm rostral to obex. They are located at a mean depth of 1.24 ± 0.46 mm and centered at approximately 0.98 ± 0.12 mm lateral from the midline. Custom-made multibarrel compound glass micropipettes, consisting of a recording barrel containing a 7-μm carbon filament and three drug barrels, were used to simultaneously record extracellular neuronal action potential activity of an adequately identifiable single IHMN before and during pressure ejection of serotonin onto the neuron. Serotonin (0.5 mm) was dissolved in an artificial cerebrospinal fluid. To determine the ejected dose rates, meniscus changes were measured in the drug barrels with a 100× magnification microscope equipped with a reticle (resolution 2 nl). Single-unit extracellular neuronal activity of IHMNs, hypoglossal and phrenic nerve activities, picoejection marker pulses, end-tidal carbon dioxide concentrations, systemic blood pressure, heart rate, and airway pressure were recorded on a digital tape system (model 3000A; A. R. Vetter Co., Rebersburg, PA). End-tidal isoflurane concentration and airway concentration of inspiratory and expiratory oxygen and carbon dioxide concentrations were monitored with a POET IQ Anesthesia Gas Monitor (Criticare Systems, Inc., Waukesha, WI). These variables, or their time averages, and a rate-meter output of discharge frequency (100-ms bins) were also continuously displayed on a computerized chart recorder (Powerlab/16SP; ADInstruments, Castle Hill, Australia). On-line spike-triggered averaging was used to confirm that the recorded action potentials originated from a hypoglossal motor neuron. The presence of an axon spike potential within the hypoglossal nerve and the neuron firing in phase with the phrenic nerve activity confirmed that the recorded brainstem neuron was an IHMN. The tape-recorded data were digitized and analyzed off-line. Timing pulses at the beginning and end of neural inspiration were derived from the phrenic neurogram and were used to determine the respiratory phases. Cycle-triggered histograms (CTHs), triggered by the timing pulse at the onset of phrenic nerve activity, were used to quantify the neuronal discharge frequency.
Protocols

The protocols were performed under steady state hyperoxic hypercapnic conditions (fraction of inspired oxygen [FIO2] > 0.6, arterial oxygen tension [PaO2] > 300 mmHg, arterial carbon dioxide tension [PaCO2] 55–65 mmHg). The exact level of PaCO2 varied within those limits from animal to animal, but was kept constant within an animal once strong phasic phrenic activity was obtained; great care was taken to keep the PaO2 tightly controlled within each neuron protocol by keeping mechanical ventilation and ventilator fresh gas flows constant, by closely monitoring end-tidal carbon dioxide trends, and by intermittent blood gas sampling. Hypercapnia is required to ensure adequate phasic inspiratory hypoglossal nerve activity in decerebrate dogs during hyperoxia and anesthesia.11 Hypercapnia is necessary because some physiologic excitatory drive inputs to HMNs such as peripheral chemodrive and phasic inputs from negative pressure–sensitive upper airway receptors were abolished by hyperoxia and intubation with positive-pressure ventilation, respectively.20 A complete protocol consisted of separate picoejection periods of serotonin without isoflurane and at approximately 0.3 MAC isoflurane. Because neuron protocols can only be performed on HMNs that continue to discharge during subanesthetic isoflurane concentrations, we determined for each neuron the isoflurane concentration that caused approximately a 50% decrease of a neuron’s spontaneous peak discharge frequency (peak FN) and set this as the target anesthetic concentration for the serotonin dose–response. This constraint resulted in a mean isoflurane concentration of 0.3 ± 0.1 MAC and allowed us to maximize our data yield while minimizing the total number of animals studied. To further maximize the yield of complete neuron protocols, the order of data acquisition (0 and 0.3 MAC) was randomized, thus eliminating the need for end controls.21,22 The lack of effect of the artificial cerebrospinal fluid vehicle was periodically confirmed in separate picoejection runs.

Serotonin Dose–Response Curves

For the control period and at each dose rate, CTHs (5–15 breaths) were used to obtain mean values for the peak FN for each condition. The control peak FN was measured during the prejection control period and after recovery (FNC). Serotonin was pressure picoejected in increasing dose rates onto single neurons. Typically, dose rates were held constant for 2–5 min to obtain a quasi–steady state discharge pattern before increasing the dose rate to the next higher level.25 Dose rates were increased until no further increase in peak FN was observed, or until background activity became too intense to adequately discriminate the neuron, so as to obtain a maximal reduction in K+ conductance. Typically, picoejection durations of 10–15 min with two or three dose rates were feasible. Sufficient time was allowed for peak Fmax to return to the control level. This typically required 45 min. After complete recovery from serotonin, isoflurane was started or stopped dependent on the previous randomization, and after a minimum equilibration time of 30 min, the picoejection run with serotonin was repeated.

Data Analyses

Effects of Isoflurane on the Serotonin Dose–Response. To compare the neuronal response to serotonin under isoflurane versus no isoflurane, we fit curves through the dose–response data (fig. 1). A hyperbolic function of the form \( F = F_{\text{max}} \frac{D}{(D + K)} \) was used to account for response saturation to serotonin and used all dose–response data to estimate the response at the maximal dose rate that was achieved during both runs \( (D_{\text{max}}) \). We then interpolated \( F_N \) from both curves at \( D_{\text{max}} \). The anesthetic effect on postsynaptic 5-HT receptor function was determined as the change in net response to serotonin at \( D_{\text{max}} \).

Effects of Isoflurane on the Discharge Pattern during Serotonin Application. For the analysis of the effects of isoflurane on the discharge pattern during serotonin application, time-synchronized plots of the control discharge pattern \( (F_{\text{con}}) \) versus the discharge pattern during serotonin application \( (F_{5,\text{HT}}) \) were generated from the CTH data and used to analyze the relation between these two patterns. This method of analysis is useful in cases where modulatory effects seem to be present, because it allows estimation of the degree of modulation from the slope of these plots. This method is also independent of the time course of the discharge frequency pattern. Plots are typically linear, where a change in the regression slope indicates a change in gain and a change in the y-intercept indicates a change in tonic activity. The same analysis was used to quantify

![Fig. 1. Method to analyze the effect of isoflurane on the postsynaptic serotonin (5-HT) receptor function. A curve was fitted through the dose–response data without isoflurane (solid circles) and with isoflurane (hollow circles). The highest common serotonin dose rate (5.4 pm/min) of the two runs was designated \( D_{\text{max}} \). We then interpolated the net increase at \( D_{\text{max}} \) (arrows) without isoflurane (no iso) and with isoflurane (iso). For this neuron, the net increase was 81.2 Hz without isoflurane and 22.9 Hz with isoflurane. Y-axis: IHMN peak discharge frequency; x-axis: serotonin dose rate.](image)
changes in the control discharge pattern (without 5-HT) induced by isoflurane.

**Statistical Analysis**

To pool the data among animals, the data were normalized with respect to the control peak $F_n$ of each single neuron protocol, which was assigned a value of 100%. Normal distribution of data was confirmed using the Kolmogorov-Smirnov test. Differences in the serotonin response with and without isoflurane were tested using a multivariate analysis of variance (SuperANOVA). The changes in slope and offset were tested for statistical significance with the Student $t$ test. All values are given as mean $\pm$ SD, and $P < 0.05$ was used to indicate significant differences unless stated otherwise.

**Results**

Nineteen complete neuron protocols were obtained in nine animals. The recorded IHMNs were located from 1.0 mm caudal to 3.0 mm rostral to the obex, from 0.5 to 2.5 mm lateral from the midline, and from 1.5 to 3.5 mm below the dorsal surface. All IHMNs recorded in this study started firing with the onset of the phrenic nerve activity or shortly thereafter and exhibited an incrementing discharge pattern.

**Effects of Isoflurane on Serotonin Dose–Response**

A typical example of the 5-HT picoejection protocol used to generate IHMN neuronal dose–response data is shown in figure 2A. Stepwise increases in the 5-HT picoejection rate increased the peak $F_n$ of this neuron from approximately 40 Hz to approximately 160 Hz. At the highest ejection rate (4.9 pmol/min), 5-HT also induced activity during the normally silent expiratory phase, which can be seen in the time-expanded trace (fig. 2B). The localized nature of the picoejection is supported by the lack of effect on the peak time-averaged hypoglossal nerve activity (fig. 2A, XII). The effects of 0.3 MAC isoflurane on this neuron’s activity and response to 5-HT are shown in figure 3. When compared with no isoflurane (fig. 3A vs. fig. 3B), the peak $F_n$ of the prejection control cycles as well as the response to 5-HT were markedly attenuated. Isoflurane (0.3 MAC) also depressed the XII nerve activity by approximately 60%, whereas peak phrenic activity was only reduced by less than 10%. Interpolation from the fitted curves at the maximal dose rate ejected during both runs (3.6 pmol/min, $D_{max}$) yielded a net increase in peak $F_n$ of 93.3 Hz without isoflurane and of 38.1 Hz with isoflurane, i.e., a 59% depression of neuronal response to serotonin.

**Fig. 2.** Response of an inspiratory hypoglossal motoneuron to serotonin (5-HT) without isoflurane. (A) 5-HT caused a gradual increase in peak neuronal discharge frequency ($F_n$) with step increases in dose rate. The marker indicates the duration and dose rate of 5-HT picoejection. $F_n$: rate-meter record of inspiratory hypoglossal motoneuron discharge frequency; PNG = time-averaged phrenic nerve activity; XII = time-averaged hypoglossal nerve activity. (B) Traces show time-expanded views during the control condition and at the maximal 5-HT dose rate. NA = neuronal raw activity.

**Fig. 3.** Comparison of the inspiratory hypoglossal motoneuron responses to serotonin (5-HT) at the indicated dose rates with no isoflurane (A) and isoflurane (B). Both the control and the 5-HT–evoked neuronal activities were attenuated by isoflurane. Note the significant decline in the hypoglossal nerve activity (XII) with isoflurane, whereas the phrenic nerve activity (PNG) remains essentially unchanged. The letters a–d indicate the records for which the time-enlarged views are shown. NA = neuronal activity.
isoflurane.

The pooled data for 19 neurons showed that isoflurane at a mean concentration of 0.3 ± 0.1 MAC reduced the spontaneous peak F\textsubscript{n} from 47 ± 23 Hz to 24 ± 14 Hz, i.e., by 48 ± 19% (P < 0.001). The 5-HT-induced net increase in F\textsubscript{n} at D\textsubscript{max} was reduced from 63 ± 28 Hz to 40 ± 22 Hz, i.e., by 34 ± 22% (P < 0.001). Depression of the spontaneous peak F\textsubscript{n} was significantly greater than that of the net 5-HT response (P = 0.024). Summary data of the net 5-HT responses, normalized relative to the prejection values without isoflurane, are shown in figure 4. The net 5-HT responses were 150 ± 73% of prejection control without isoflurane and 93 ± 56% with isoflurane. Isoflurane at 0.3 MAC also significantly depressed the whole nerve activity of the hypoglossal nerve by 54 ± 17%, but not the phrenic nerve activity (−10 ± 7%).

**Effects of Isoflurane on the Discharge Pattern during Serotonin Application**

Analysis of the changes in the neuronal discharge pattern provides additional information about the nature of induced changes, where such changes may be due to a tonic shift in activity and/or due to a gain modulating effect. The effects of 5-HT on the discharge pattern with and without isoflurane were obtained from time-synchronized plots of F\textsubscript{5-HT} versus F\textsubscript{con} where the data were obtained from corresponding CTHs (e.g., figs. 5A and B, F\textsubscript{n}, same neuron shown in figs. 1 and 2). CTH data were compared only during the time period that the prejection control pattern (thick lines) was greater than 0 Hz. Linear regression analysis showed that 5-HT increased the F\textsubscript{5-HT} versus F\textsubscript{con} slopes from 1.0 (line of identity) to 1.85 (r = 0.951) and 1.80 (r = 0.957) for 0 and 0.3 MAC, respectively, indicating a 5-HT-induced gain increase with and without isoflurane (fig. 5C). This can also be seen from the increase in slope of both discharge patterns. The increase in the y-intercept indicates an additional 5-HT–induced increase in tonic activity, which in this neuron was greater at 0.3 MAC.

Summary data from 19 neurons showed that 5-HT, at maximally effective dose rates (10.2 ± 5.7 pmol/min), significantly increased the gain of the discharge pattern by similar amounts (P = 0.89) from 1.0 to 1.67 ± 0.75 and 1.69 ± 1.1 without and with 0.3 MAC isoflurane, respectively (fig. 6, slope). The corresponding 5-HT–induced increases in the offset were 33 ± 20 and 23 ± 20 Hz for no anesthesia and for 0.3 MAC isoflurane, respectively (fig. 6, y-intercept). The increase in tonic excitation was significantly less at 0.3 MAC isoflurane (P = 0.018). The average (± SD) correlation coefficient, r, for the F\textsubscript{5-HT}–F\textsubscript{con} plots was 0.819 ± 0.173, with a median value of 0.885.
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Fig. 6. Summary data (n = 19) for the effect of isoflurane on the changes in slope and y-intercept produced by maximal doses of serotonin. Without isoflurane (no ISO), serotonin increased the slope of the neuronal discharge pattern by 67 ± 75%. Under isoflurane (ISO), serotonin increased the slope by 69 ± 112%. The slopes were not different. Without isoflurane, serotonin shifted the y-intercept by 33 ± 20 Hz, and by 23 ± 20 Hz during isoflurane. The difference in y-intercepts was significant ($P < 0.001, \cdot P = 0.018$). n.s. = not significant.

Effects of Isoflurane on the Control Discharge Pattern
Analysis of the $F_{con}$ versus $F_{iso}$ plots obtained from the CTH data (fig. 7A) revealed that isoflurane depressed the IHMN activity via decreases in the slope (gain) of the plots relative to the line of identity (fig. 7B). Summary data for all 19 neurons showed that isoflurane at 0.3 ± 0.1 MAC decreased the slope by 56 ± 37% ($P < 0.001$) but did not change the offset significantly (0 ± 8 Hz; $P = 0.86$).

Discussion
This study showed a profound depression of IHMN activity by subanesthetic concentrations of isoflurane (0.3 MAC = 90 μM) in an in vivo preparation. Contrary to our hypothesis, which predicted that the absolute neuronal response to serotonin would be increased in the presence of isoflurane (fig. 8A), the neuronal response to picoejected serotonin was also strongly reduced by subanesthetic isoflurane (fig. 8B). This rules out a major contribution of hyperpolarizing $K^+$ leak channels to the isofurane induced depression. However, the reduction of the slope of the neuronal firing pattern by 0.3 MAC isoflurane suggests a strong anesthetic effect on a mechanism that provides tonic modulatory inhibition of the neuron.

To our knowledge, this is the first study to evaluate the significance of TASK channel activation on neuronal activity in vivo by a clinically relevant, subanesthetic concentration of a volatile anesthetic. Our hypothesis was based on the observation that IHMNs were signifi-

Fig. 7. Method used to analyze the effect of isoflurane on the inspiratory hypo- glossal motoneuron discharge patterns. (A) Time-synchronized, cycle-triggered histograms of neuronal discharge frequency ($F_n$) during control conditions ($F_{con}$) and during isoflurane ($F_{iso}$) were plotted against each other for the time period that the neuron was actively discharging. Regression analysis of the plot (triangles, B) revealed a linear relation with a slope of 0.34 and a y-intercept of 1 Hz. This indicates that isoflurane reduced the gain of the control discharge pattern. Such attenuation in gain is expected if the anesthetic causes an increase in tonic modulatory inhibition. LOI = line of identity; PNG = phrenic neurogram; $T_I$ = inspiratory duration.

Fig. 8. Scheme for the expected and observed effects of isoflu- rane and serotonin on the discharge frequency pattern ($F_n$) of a typical inspiratory hypoglossal motoneuron. The values for peak $F_n$ and slope in A are based on the grand average from the 19 studied neurons. (A) We expected that peak $F_n$ and slope of the control pattern with no isoflurane (CON, solid thick line) to be significantly increased by serotonin (5-HT, solid thin line; increase: upper thin arrow). We expected isoflurane (ISO, dashed thick line) to depress peak $F_n$ of the control pattern (lower thin arrow). If, as hypothesized, 5-HT completely antagonized a $K^+$ leak channel mediated, isoflurane-induced depression, the 5-HT response during isoflurane (5-HT + ISO) should have reached the same peak $F_n$ as without isoflurane (5-HT, thick arrow). (B) Isoflurane depressed the slope of the control pattern by 58% (lower thin curved arrow, ISO). 5-HT increased the slope of the discharge pattern with and without isoflurane by the same amount (fig. 6). Consequently, isoflurane also depressed the slope of the 5-HT response by 58% (upper thin curved arrow, 5-HT + ISO). Therefore, the observed 5-HT re- sponse during isoflurane was far smaller than expected (thick arrow). The results suggest that isoflurane may enhance a tonic inhibitory mechanism, e.g., a $\gamma$-aminobutyric acid type A recep- tor–mediated gain modulation that causes neuronal depression.
cantly more depressed by volatile anesthetics than respiratory premotor neurons. Immunohistologic studies showed that TASK channel messenger RNA was abundantly expressed specifically in brainstem motor nuclei including the hypoglossal motor nucleus. Therefore, the TASK channel was a target unique to HMNs that had been shown in vitro to be significantly enhanced by volatile anesthetics and blocked by serotonin. The difference between our in vitro results and the in vitro studies may be due to the difference in anesthetic concentrations. In the in vitro preparation, anesthetic effects on TASK channels were only maximal at suprachannel concentrations of halothane of approximately 0.9 mM (> 3 MAC). The anesthetic-induced current was steeply concentration dependent, with an EC50 of 0.23 mM halothane, and at 0.1 mM (approximately 0.4 rat MAC), which was the lowest halothane concentration tested in vitro, the induced conductance change was approximately 10%. We cannot rule out a small anesthetic effect on TASK channels in our preparation. The fact that the spontaneous activity was more depressed than the net 5-HT-induced response (47.4% vs. 34.4%, respectively) by isoflurane suggests that there was a small enhancement of the 5-HT response in the presence of isoflurane. To obtain an estimate of the magnitude of this effect, we used a simple compartmental model for the effects of isoflurane on both the K+ channel mechanism and other mechanisms that may be subject to effects of isoflurane, e.g., tonic GABAergic gain modulation. This model (see appendix for details) was able to delineate the amount of isoflurane effect on each of the two compartments. The results of this analysis suggest that the isoflurane effect on the K+ channel mechanism increased neuronal depression by approximately 8%, i.e., from 44.4% to 47.8%. Therefore, the dominant effect of 0.3 MAC isoflurane seems to be on mechanisms other than an increase in K+ conductance.

The significant anesthetic depression of the slope of the neuronal discharge pattern suggests an anesthetic effect on an inhibitory gain modulatory mechanism. GABA and glycine receptors have been demonstrated on HMNs in vitro, and preliminary observations from our own laboratory have shown that a bicuculline-sensitive tonic GABAergic inhibition modulates the activity of HMNs in decerebrate dogs (unpublished observation, Astrid Stucke, M.D., Research Fellow, Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin, February 2003). We have shown in the in vivo decerebrate canine preparation that 1 MAC sevoflurane and halothane enhanced overall GABAergic inhibition in brainstem respiratory premotor neurons between 10% and 20%. While presynaptic inhibitory input was reduced, GABAA receptor activity was doubled during 1 MAC volatile anesthesia. Assuming enhanced GABAergic inhibition was responsible for the selective anesthetic depression of HMNs, the difference in magnitude of effect from premotor neurons may point to a qualitatively different mechanism of GABAergic inhibition. Recently, it has been demonstrated that tonic extrasynaptic GABAergic conductances in murine hippocampal neurons are exquisitely sensitive to subanesthetic concentrations of isoflurane (83 μM or ≤ 0.3 MAC). This tonic inhibition was blocked by the GABAA antagonist bicuculline and was shown to be generated by α5 subunit–containing GABAA receptors. Immunochemistry studies have shown that α5 subunit–containing GABAA receptors are selectively distributed in the central nervous system, including neurons within the hypoglossal motor nucleus of the rat. Unlike inhibitory synaptic GABAergic currents in hippocampal neurons, which are only enhanced above 100 μM isoflurane (> 1 MAC, and EC50 of 320 μM), the extrasynaptic GABAA receptor–mediated currents doubled at an isoflurane concentration of 83 μM or approximately 0.3 MAC. Therefore, if the observed bicuculline-sensitive inhibition of HMNs in our preparation were similar in nature, profound depression of HMNs at 0.3 MAC isoflurane would occur, and the 5-HT response would be proportionately depressed by the anesthetic.

Glycine receptor function in the presence of glycine has been shown to be enhanced by volatile anesthetics in a similar fashion to the GABAA receptor function in the presence of GABA. HMNs have been shown to receive glycineric synaptic inputs that are tonically active. In spinal cord motoneurons, in vitro from 6- to 14-day-old rats, isoflurane showed opposing facilitatory and inhibitory actions on glycine release so that the absolute amount of glycineric inhibition did not increase. The importance of glycineric inhibition and its anesthetic modulation needs to be evaluated for HMNs in the future.

We have recently shown that HMNs in decerebrate dogs receive most if not all of their baseline excitatory drive via glutamatergic inputs, with AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propioinic acid) and N-methyl-D-aspartate receptor inputs contributing roughly to the same extent. In respiratory premotor neurons in our decerebrate preparation, 1 MAC anesthesia caused no or minimal depression of glutamatergic receptor function. However, in a neonatal mouse spinal cord slice, 1 MAC equivalent enflurane caused a significant (30–35%) depression of postsynaptic NMDA and AMPA glutamate receptor function in spinal motoneurons. It will be necessary to evaluate whether there is significant depression of glutamate receptor function by subanesthetic concentrations of volatile anesthetics in HMNs in vivo.

Methodologic Considerations

The advantages and limitations of the decerebrate in vivo canine preparation and the picoejection method have been extensively discussed in previous publications. To produce a significant effect of isoflu-
rane on the K⁺ leak channel and to be able to detect a significant antagonistic effect by serotonin, we applied the maximal doses of both drugs that still allowed us to record extracellular activity from these neurons. Many IHMNs discharge rhythmically under normocapnic conditions. However, because IHMNs are very sensitive to volatile anesthetics, we used moderate hypercapnia, which increases the respiratory drive to the neurons. Even so, anesthetic concentrations of 0.5 MAC often silenced these neurons so that we had to restrict our protocol to subanesthetic concentrations of isoflurane that caused approximately a 50% depression under hypercapnic conditions. Even lower concentrations of isoflurane would have been required during normocapnic conditions. However, hypercapnic hyperoxic conditions have physiologic and clinical relevance because they are typically encountered during postanesthetic recovery, where residual subanesthetic concentrations of volatile anesthetics and opioid analgesics induce respiratory depression that causes hypercapnia, which requires the use of supplemental oxygen to prevent hypoxia.

We assumed that near maximal serotonergic antagonism of any anesthetic-induced K⁺ leak channel activation was achieved when increasing dose rates of serotonin did not elicit further increase in neuronal frequency. However, maximum dose rates of serotonin tend to elicit background noise so that we limited the exercised dose rates guided by adequate neuronal recording quality. Because many dose–response plots showed evidence of saturation, we used a hyperbolic curve fit from which we interpolated the neuronal frequency at the maximal common dose rate, Dmax. Dmax was used to measure maximum effect in a dose range for which we had obtained actual data points with and without isoflurane.

Conclusions

In conclusion, we have shown that 0.3 MAC isoflurane depressed the discharge activity of IHMNs in vivo by approximately 50%. This profound depression could not be antagonized by serotonin, which ruled out a major contribution of TASK channels. Depression of the slope of the neuronal discharge pattern by subanesthetic isoflurane concentrations suggested anesthetic enhancement of a tonic modulatory inhibition of the neuron.

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References

The current study focused on the effects of isoflurane on the spontaneous discharge pattern and on the neuronal response to 5-HT. Our data do not allow conclusions on anesthetic effects on the excitatory or inhibitory mechanisms, which we therefore summarized to simplify the model (fig. 9B). The k values represent modulation factors of \( F_{\text{con}} \) (attenuation or amplification) where \( k \geq 0 \). \( F_0 \) is the \( F_{\text{con}} \) value for a given condition indicated by the subscript. Subscript “i” indicates the absence \((i = 0)\) or presence \((i = 1)\) of 5-HT and “j” indicates the absence \((j = 0)\) or presence \((j = 1)\) of isoflurane. The variable \( f_i \) represents the intermediate, interblock value for the various conditions.

The mathematical formulation of the model states:

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f_{ij} = k_0 F_{\text{con}} (1)
\]

\[
F_0 = k_0 F_{ij} (2)
\]

where \( k_0 \) depends on the condition or state:

\[
k_0 = 1 \text{ and } k_1 = 1 \text{ during control condition}
\]

\[
k_0 = k_2 \text{ during isoflurane only}
\]

\[
k_0 = k_2/k_1 \text{ during both 5-HT and isoflurane,}
\]

where \( k_1 \) and \( k_2 \) represent the modulating effects of isoflurane on the background \( K^+ \) conductance and the tonic modulation block, respectively, and where \( k_2 \) represents the amplification factor produced by 5-HT at serotonin dose rate \( D_{\text{max}} \).

\[
F_01 = k_1 k_3 F_{\text{con}} (3)
\]

\[
F_{10} = k_2 F_{\text{con}} (4)
\]

\[
F_{11} = k_1 (k_2/k_1) F_{\text{con}} (5)
\]

Rearranging these equations yields:

\[
k_1 k_3 = F_{01}/F_{\text{con}} (6)
\]

\[
k_2 = F_{10}/F_{\text{con}} (7)
\]

\[
k_1 (k_2/k_1) = F_{11}/F_{\text{con}} (8)
\]

Table 1. Algebraic Expressions of the Model Outputs for the Four Experimental Conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>S-HT (i)</th>
<th>ISO (j)</th>
<th>( f_i )</th>
<th>( f_j )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>( F_{\text{con}} )</td>
<td>( F_{\text{con}} )</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1</td>
<td>( k_0 F_{\text{con}} )</td>
<td>( k_1 k_3 F_{\text{con}} )</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>( k_0 F_{\text{con}} )</td>
<td>( k_1 F_{\text{con}} )</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>( (k_2/k_1) F_{\text{con}} )</td>
<td>( (k_0 k_2/k_1) F_{\text{con}} )</td>
</tr>
</tbody>
</table>

5-HT = serotonin; \( F_{\text{con}} \) = baseline control neuron discharge frequency in absence of isoflurane (ISO) and serotonin; \( f_i \) = intermediate, interblock value for the various conditions, where \( i \) represents absence \((i = 0)\) or presence \((i = 1)\) of serotonin and \( j \) represents absence \((j = 0)\) or presence \((j = 1)\) of isoflurane; \( F_{\text{con}} \) = neuron discharge frequency during condition 4; \( k_1 \) = modulating effect of isoflurane on the background \( K^+ \) conductance; \( k_2 \) = amplification factor produced by serotonin at dose rate \( D_{\text{max}} \); \( k_3 \) = modulating effects of isoflurane on tonic modulation block (fig. 9B).

Table 1 shows the model outputs for the four experimental conditions. For condition 4, it is assumed that 5-HT at \( D_{\text{max}} \) will antagonize and completely reverse any change in \( K^+ \) conductance caused by isoflurane and that this will produce the same maximum peak \( F_0 \) as that obtained with no isoflurane, in accordance with in vitro data. Therefore, if isoflurane attenuates \( f_i \) to \( F_{\text{con}} \), the 5-HT response will have to be increased by a factor of \( 1/k_1 \) to overcome this effect.

The following relations were used to find the k values:

\[
k_1 k_3 = F_{01}/F_{\text{con}} (3)
\]

\[
k_2 = F_{10}/F_{\text{con}} (4)
\]

\[
k_1 (k_2/k_1) = F_{11}/F_{\text{con}} (5)
\]

9. Block diagrams of a hypothetical model to allocate isoflurane (ISO) effects to component mechanisms that underlie the control of inspiratory hypoglossal motoneuron discharge activity. (A) \( F_{\text{con}} \) = spontaneous neuronal discharge frequency in the absence of isoflurane and serotonin (5-HT); \( F_{\text{con}} \) = altered \( F_{\text{con}} \) due to the effects of isoflurane alone, 5-HT alone, or both. (B) Simpler version of model with the excitatory and inhibitory mechanisms combined (right). The k values represent the magnitudes of attenuation or amplification due to the effects of isoflurane and/or 5-HT. \( F_0 \) and \( f_i \) represent the output discharge frequency of the two stages. See text for further details.

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Substituting equation 7 into equation 8 and rearranging yields:

$$k_3 = \frac{F_{11}}{F_{10}} k_1.$$  \hspace{1cm} (9)

Substituting equation 9 into equation 6 and rearranging yields:

$$k_1 = \sqrt{k_1^2} \hspace{1cm} (11)$$

$$k_3 = \frac{F_{01}}{F_{\text{con}}} k_1.$$  \hspace{1cm} (12)

The k values were calculated from the data of each neuron and pooled to obtain mean and SD values. A one-sample t test was used to determine whether the k values were significantly different from a value of 1.0, which represents no modulation. The values were $$k_1 = 0.938 \pm 0.132 \hspace{1cm}(P = 0.055), \hspace{1cm} k_2 = 2.495 \pm 0.727 \hspace{1cm}(P < 0.0001), \hspace{1cm} k_3 = 0.556 \pm 0.174 \hspace{1cm}(P < 0.0001).$$ Using the above mean values for $$k_1, \hspace{1cm} k_2, \hspace{1cm} k_3,$$ the calculated values for $$k_{00}, \hspace{1cm} f_{ij}, \hspace{1cm} \text{and } F_{ij}$$ are shown in table 2 for the four conditions.

Note that the calculated $$F_{ij}$$ values are essentially the same as those of the measured mean values.

If isoflurane had no effect on the K conductance ($$k_0 = 1$$), $$F_{\text{con}}$$ would be depressed to 55.6% ($$0.556 \times F_{\text{con}}$$). Our model, based on measured values, however, suggests that $$F_{\text{con}}$$ is actually depressed to 52.2% ($$0.938 \times 0.556 \times F_{\text{con}}$$). The difference in depression of 7.7% would be the estimated effect of isoflurane on the K conductance.

### Table 2. Calculated $$k_0, f_{ij}, k_3,$$ and $$F_{ij}$$ Values for the Four Experimental Conditions

<table>
<thead>
<tr>
<th>5-HT ($$i$$)</th>
<th>ISO ($$j$$)</th>
<th>$$k_0$$</th>
<th>$$f_i$$</th>
<th>$$k_3$$</th>
<th>$$F_{ij}$$ Calculated</th>
<th>Measured Mean $$F_n$$ Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>100.0</td>
<td>1</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>0.938</td>
<td>93.8</td>
<td>0.556</td>
<td>52.2</td>
<td>52.6</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2.495</td>
<td>249.5</td>
<td>1</td>
<td>249.5</td>
<td>249.5</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2.660</td>
<td>266.0</td>
<td>0.556</td>
<td>147.9</td>
<td>145.9</td>
</tr>
</tbody>
</table>

5-HT = serotonin; $$F_{\text{con}}$$ = baseline control neuron discharge frequency in absence of isoflurane (ISO) and serotonin; $$f_i$$ = intermediate, interblock value for the various conditions, where $$i$$ represents absence ($$i = 0$$) or presence ($$i = 1$$) of serotonin and $$j$$ represents absence ($$j = 0$$) or presence ($$j = 1$$) of isoflurane; $$F_{ij}$$ = neuron discharge frequency during condition $$ij$$; $$F_n$$ = peak discharge frequency; $$k_0$$ depends on the condition or state with $$k_0 = 1$$ during control condition, $$k_0 = k_1$$ during isoflurane only, $$k_0 = k_2$$ during serotonin only, $$k_0 = k_2/k_1$$ during both serotonin and isoflurane; $$k_1$$ = modulating effects of isoflurane on the background K conductance; $$k_3$$ = modulating effects of isoflurane on tonic modulation block (fig. 9B).