Serotonergic Modulation of Inspiratory Hypoglossal Motoneurons in Decerebrate Dogs

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INTRODUCTION

Hypoglossal motoneurons (HMNs) innervate all tongue muscles, including the genioglossal muscle, the main protruder of the tongue, and thus contribute significantly to the maintenance of upper airway patency during inspiration (Miki et al. 1989). Partial or complete loss of inspiratory HMN (IHNM) activity during sleep (Remmers et al. 1978), during various stages of anesthesia (Eastwood et al. 2002), or during postanesthetic recovery (Dhonneur et al. 1999) can lead to upper airway obstruction with resulting hypoxia. HMNs are thought to receive inputs from several regions within the brain. In rats, serotonergic and peptidergic raphe neurons project to HMNs (Manaker and Tischler 1993; Takeuchi et al. 1983), as well as noradrenergic neurons that originate from the locus subce- realus (Aldes et al. 1992). Sleep–wake states as well as

rhythmic motor activities, including breathing, influence the magnitude of these neuronal inputs (Jacobs and Azmitia 1992; Veasey et al. 1995). In decerebrate cats, serotonin (5-HT) seems to provide substantial tonic excitatory drive to various types of HMNs (Kubin et al. 1992). Kubin et al. (1994) demonstrated in decerebrate cats during experimentally induced REM-like sleep that reduced levels of endogenous 5-HT and depression of hypoglossal activity are closely linked. Increased levels of 5-HT are released during wakefulness, when raphe neurons are active, and may increase HMN activity, whereas withdrawal of 5-HT, in particular during REM sleep when raphe neurons are inactive, may decrease HMN activity. This decreased activity is thought to contribute to the loss of muscle tone in the tongue and to compromise upper airway patency (Jacobs and Azmitia 1992).

However, the contribution of endogenous 5-HT to the physiological activity of single IHMNs has not been characterized in an in vivo preparation. In vitro studies show that activation of HMNs by 5-HT is mediated by postsynaptic neuronal 5-HT2A receptors (Fenik and Veasey 2003; Schwarzacher et al. 2002). One identified mechanism of action is a decrease in a leak K+ channel conductance (Bayliss et al. 1997; Sirois et al. 2002) that leads to increased excitability of the neuronal cell membrane.

We hypothesize that the discharge activity of IHMNs in vivo depends on endogenous 5-HT and is mediated by postsynaptic 5-HT2A receptors. We tested this hypothesis in a decerebrate dog model by characterizing the effects of exogenous 5-HT as well as endogenous 5-HT, through a selective 5-HT2A block with the antagonist ketanserin, on single IHMNs with a special focus on changes in the discharge pattern.

METHODS

Animal preparation and general methodology

This research was approved by the Medical College of Wisconsin Animal Care Committee and conformed to standards set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals. Anesthesia was induced in dogs by mask with isoflurane. They were 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% (Kazama and Ikeda 1988)). The animals were positioned in a stereo-
tactic device (model 1530, David Kopf Instruments, Tujunga, CA) with the head ventrally flexed (30°). Bilateral neck dissections were performed. 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 lateral and medial branches, desheathed, and placed onto a custom made electrode for recording. This electrode was custom made by cutting a 1-ml tuberculin syringe (Terumo Syringe, Terumo Medical, Elkton, MD) longitudinally in half, placing two 80-μm-thick teflon-coated stainless steel wires through the bottom of a trough and forming two loops of bare wire. The proximal end of the nerve was placed through the wire loops and covered with silicon gel to provide a barrier to blood and prevent drying of the nerve. The C3 phrenic nerve rootlet was desheathed and placed onto a custom made bipolar hook electrode for recording. Bilateral vagotomy was performed to achieve peripheral deafferentation from pulmonary stretch receptor input. This avoids volume feedback from the mechanical ventilation through vagal inputs to the respiratory centers. Bilateral pneumothorax was performed to minimize brain stem movement and eliminate phasic inputs from chest wall afferents. The animals were decerebrated at the midcollicular level (Tonkovic-Capin et al. 1998) and only then paralyzed with pancuronium (Baxter Healthcare, Deerfield, IL; bolus of 0.1 mg/kg, followed by 0.1 mg/kg/h). 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 to prevent brain swelling (1 mg/kg after anesthesia induction and every 6 h thereafter). A heating blanket was used to maintain esophageal temperature at 38.5 ± 1°C. Mean arterial pressure was kept at or above 100 mmHg, and 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 through the activation of the fibrinolytic system secondary to the surgical trauma (Nilsson et al. 1960; Risberg 1978), e-amino-n-caproic acid (Sigma-Aldrich, St. Louis, MO) was administered intravenously (bolus of 125 mg/kg at the beginning of surgery, followed by 15 mg/kg/h).

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 the midline (Brandes et al. 2004a). 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 activity of an adequately identifiable single IHMN before and during pressure ejection of 5-HT or ketanserin onto the neuron. 5-HT (0.5 mM) was dissolved in an artificial cerebrospinal fluid (aCSF) (Stuth et al. 2000). Ketanserin was first dissolved in DMSO to a concentration of 20 mM, which was subsequently diluted to 500 μM with aCSF that had previously been adjusted to a pH of ~6.0. Although freshly mixed drugs were used in these experiments, refrigerated aliquots of ketanserin have been shown to remain in solution for more than a month. The effects of the acidified aCSF vehicle on IHMNs had been found to be negligible in pilot studies (unpublished observations, 2003).

Picoejection technique

The pressure microejection system used in this study is similar to the “Picospritzer” (General Valve, Fairfield, NJ) in that each timed pressure pulse ejects a volume in the 40–400 pl range. However, by delivering repeated picospritzes of micromolar solutions of agents, typical dose rates in the picomole/minute range can be delivered. The parameter ranges used to alter dose rates were ejection pressure: 10–100 psi; pulse duration: 10–100 ms; and frequency of the ejection pressure pulses: 0.2–2 Hz. Ejected dose rate (volume/time) was measured through height changes of the meniscus in the pipette barrel with a ×100 magnification microscope equipped with a reticle (resolution ~2 nl). To obtain steady-state dose-response data, constant-rate picoejection was used, and dose rates were mainly increased through changes in ejection pulse rate. Using this approach, it is possible to produce quasi steady-state drug concentrations at given distances from the pipette tip because of the properties of diffusion. Increasing the rate of ejection increases the concentration at any given distance. The maximum achievable concentration is the barrel concentration.

Recorded variables

Single unit extracellular IHMN activity, hypoglossal and phrenic nerve activities, picoejection marker pulses, end-tidal CO2, systemic blood pressure, and airway pressure were recorded on a digital tape system (model 3000A, A. R. Vetter, Rebersburg, PA). End-tidal volatile anesthetic concentration and airway concentration of inspiratory and expiratory O2-CO2 were monitored with a POET IQ Anesthesia Gas Monitor (Criticare Systems, 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 (ADInstruments, Powerlab/16SP, Castle Hill, Australia). On-line spike-triggered averaging (STA) was used to confirm that the recorded action potentials originated from a hypoglossal motoneuron. The presence of an axon spike potential within the hypoglossal nerve activity (Fig. 1) and the neuron firing in phase with the phrenic nerve activity, confirmed that the recorded brain stem 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.

The protocols were performed under both steady-state hyperoxic and hypocapnic 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 PaCO2 tightly controlled within each

![FIG. 1. The presence of an axon spike potential in the spike-triggered average of hypoglossal (XII) nerve activity, delayed relative to a hypoglossal motoneuron (HMN) action potential confirmed that the recorded brain stem neuron was a HMN (500 sweeps/average).](Image)
neuron protocol by keeping artificial ventilation and ventilator fresh gas flows constant, by closely monitoring end-tidal CO₂ trends, and by intermittent blood gas sampling. Hypercapnia was used to increase excitatory drive to the respiratory centers, which provided robust phrenic nerve activity and inspiratory hypoglossal nerve activity under baseline conditions. This was necessary because other drive inputs to hypoglossal motor neurons 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 (Anderson et al. 1990). The experimental protocol consisted of picoejection of the agonist or antagonist at increasing incremental dose rates. After picoejection, recovery to baseline discharge frequency was awaited for every neuron. The lack of effect of the aCSF vehicle was periodically confirmed in separate runs. To avoid confusion from residual effects of serotonin on the measurement of endogenous levels of serotonin, we also performed all experiments with the antagonist ketanserin in a separate group of animals (see protocol 2). However, to show that ketanserin is a competitive antagonist of serotonin in our setup, we additionally performed a small number of agonist–antagonist studies on single neurons.

Protocol 1: effects of exogenous serotonin on the spontaneous neuronal discharge frequency

The IHMN discharge frequency \( F_n \) was measured for 10–15 respiratory cycles during a control period, at each dose rate, and after recovery. 5-HT was pressure picoejected at 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. Dose rates were increased until no further increase in peak \( F_n \) was observed but before background activity became too intense to adequately discriminate the neuron. Picoejection durations of 10–15 min with two to three different dose rates were feasible. Sufficient time was allowed for peak \( F_n \) to return to the control level. This typically required 45–60 min.

Protocol 2: effects of the 5-HT₂A antagonist ketanserin on the spontaneous neuronal discharge frequency

For the control period and at each dose rate, the neuronal discharge activity from 10–15 respiratory cycles was averaged to obtain mean values for the peak \( F_n \) and time-averaged \( F_n \) during the inspiratory phase for each condition as in protocol 1. After establishing a stable baseline, peak \( F_n \) was measured during a pre-ejection control period \( (F_{\text{con}}) \). Then ketanserin was applied in increasing dose rates until an increase in picoejection dose rate did not result in any further decrease in peak \( F_n \). In some neurons with relatively low \( F_{\text{con}} \), the maximum effect could not be determined because it was below the discharge threshold. In these neurons, picoejection was stopped before neuronal activity was completely silenced because the presence of neuronal activity is a prerequisite to assess changes in the discharge pattern. Typically, picoejection durations of 10–15 min with three dose rates were needed. Sufficient time was allowed for peak \( F_n \) to return to the control level. This typically required 30–45 min. To obtain an accurate estimate of the endogenous serotonin level, we avoided using exogenous serotonin because of its possible long-lasting effects.

Protocol 3: effects of CO₂ on hypoglossal and phrenic nerve activities

In five dogs, the activities of hypoglossal (XII) and phrenic nerves were initially obtained during moderate hypercapnia. The tidal volume of the ventilator was then increased to lower end-tidal CO₂ (ETCO₂) to the level at which phasic inspiratory-related XII nerve activity decreased to zero. After a period of stabilization (~10 min), CO₂ gas was gradually added through a flow meter on the anesthesia machine to the inspired hyperoxic gas mixture without changing the mechanical ventilator settings. This produced graded increases in ETCO₂ to a maximum level of ~65 mmHg. The time-averaged peak XII and phrenic nerve activities were analyzed in terms of ETCO₂ to determine the “apneic” thresholds and saturation levels for both activities.

Data analysis

CTHs based on 10–15 respiratory cycles were used to quantify the peak and time-averaged neuronal discharge frequency before and during application of 5-HT or ketanserin, respectively. The time-averaged \( F_n \) was calculated as the sum of the CTH bins (50 ms each) during the inspiratory phase \( T_i \) divided by the duration of the inspiratory phase (e.g., Fig. 2A, vertical dashed lines). To compare data from animal to animal, the data were normalized with respect to the control peak \( F_n \) and time-averaged \( F_n \) of each single neuron protocol, which was assigned a value of 100%. Because the 5-HT and ketanserin picoejection protocols were performed in different groups of animals, only the control condition was common to both sets of experiments.

![Figure 2](http://jn.physiology.org/Downloadedfrom.10.23.31.on.October.20.2017)

**FIG. 2.** Analysis of neuronal data using cycle-triggered histograms (CTHs). A: peak \( F_{\text{peak}} \) and time-averaged \( F_{\text{ave}} \) neuronal discharge frequency before \( (F_{\text{con}}) \) or during 5-HT ejection \( (F_{5-HT}) \). Each graph represents the averaged values of 10–15 respiratory cycles (50-ms bins). PNG, phrenic neurrogram; \( T_i \), inspiratory duration (vertical dashed lines); \( F_{\text{ave}} \), neuronal discharge frequency. B: top: analysis period (vertical dotted lines) of CTHs. Bottom: plot of \( F_{5-HT} \) vs. \( F_{\text{con}} \). The 2 discharge patterns are linearly related. \( F_{5-HT} = \text{slope} \times F_{\text{con}} + y\text{-intercept} \) where slope (1.90) is gain and y-intercept (11.4 Hz) is offset of the pattern. LOI, line of identity. Recalculating \( F_{5-HT} \) values from \( F_{\text{con}} \) with help of the linear regression parameters yields values that closely match the original CTH (superimposed triangles in B, top).
To allow comparison between the two data sets in both experiments, we normalized to this common control condition rather than the largest value for each experiment. The control condition for the ketanserin runs was always the largest value for these experiments, but the same control condition for the serotonin runs was always the smallest value for these runs but of the same magnitude as the control for the ketanserin runs.

Time-synchronized plots of the control discharge pattern \( F_{\text{con}} \) versus the discharge pattern during drug application \( F_n \) (drug) were used to analyze the relationship between the two patterns. This method of analysis is independent of the time-course of the discharge frequency pattern. Such plots are typically linear, where a change in the regression slope indicates a change in gain and a change in intercept indicates a change in tonic activity. A plot of the control pattern against itself results in a line [line of identity (LOI)] with a slope of 1 and an intercept of 0. For statistical purposes, the slope is expressed as the percent change from a value of 1, whereas the intercept is compared with 0, and a paired \( t \) analysis is used (e.g., Fig. 2B).

Normal distribution of the data were confirmed with the Kolmogorov-Smirnov test, and data were tested for significant differences with Student’s \( t \)-test. All values are given as mean and SD, and \( P < 0.05 \) was used to indicate significant differences unless stated otherwise.

RESULTS

The IHMNs recorded in this study were found in a region from 0.25 mm caudal to 2.0 mm rostral to obex, at a mean depth of 2.75 ± 1.11 mm and centered 1.16 ± 0.31 mm lateral from the midline. All IHMNs started firing with the onset of the phrenic nerve activity or shortly thereafter and exhibited an incrementing discharge pattern.

Protocol 1: effects of exogenous serotonin on the spontaneous neuronal discharge frequency

Twenty-eight neuron protocols were obtained in 16 dogs. The mean peak \( F_{\text{con}} \) was 42 ± 21 Hz, and the mean time-averaged \( F_{\text{con}} \) 19 ± 11 Hz. Increasing dose rates of 5-HT always resulted in a gradual increase in \( F_n \) (Fig. 3). At intermediate dose rates, the neurons fired throughout the whole inspiratory phase, whereas at the maximal effective dose rate, the neurons also showed activity during the previously silent expiratory phase. Analysis of the \( F_{5-HT} \) versus \( F_{\text{con}} \) plots (e.g., Fig. 2B, bottom) indicated that 5-HT-mediated increase in inspiratory phase activity was mainly because of an increase in...
the plot slope (gain) relative to the LOI and to a smaller degree the y-intercept. This increase in gain is also reflected by increases in the CTH slopes during 5-HT application (e.g., Fig. 2B, top). However, at the highest 5-HT dose rates, tonic activity appeared during the previously silent expiratory phase, and contributed to the discharge pattern (e.g., Fig. 3, bottom right). The normalized data showed that 5-HT, at maximally effective dose rates (16 ± 24 pmol/min), increased the time-averaged $F_n$ to $340 \pm 140\%$ ($P < 0.001$; Fig. 4, left) and the peak $F_n$ to $256 \pm 79\%$ ($P < 0.001$; Fig. 4, right) relative to baseline. At maximal dose rates, the gain of the discharge patterns as measured by the slope for the $F_{5-HT}$ versus $F_{con}$ plots was increased by $61 \pm 89\%$ ($P < 0.001$; Fig. 5, left) and the offset by $38 \pm 26$ Hz ($P < 0.001$; Fig. 5, right).

**Protocol 2: effects of the 5-HT2A antagonist ketanserin on the spontaneous neuronal discharge frequency**

Twenty-one neuron protocols were obtained in nine dogs. Again, during control conditions, the IHMNs exhibited only phasic inspiratory activity, as indicated by the neuron firing in phase with the phrenic nerve. The peak $F_{con}$ was $53 \pm 20$ Hz.

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**FIG. 4.** Comparison of the effect of 5-HT and ketanserin on time-averaged $F_n$ and peak $F_n$; values are given as mean and SD. Numbers in bars indicate number of protocols in each group. At maximally effective dose rates, 5-HT increased time-averaged $F_n$ to $340 \pm 140\%$ and peak $F_n$ to $256 \pm 79\%$, whereas ketanserin decreased time-averaged $F_n$ by $80 \pm 15\%$ and peak $F_n$ by $68 \pm 15\%$. ***$P < 0.001$.**

**FIG. 5.** Effect of 5-HT and ketanserin on the slope and the y-intercept of the relationship between neuronal discharge patterns for control and maximal effective drug dose rates. 5-HT increased the slope of neuronal discharge pattern by $61 \pm 89\%$, whereas ketanserin caused a decrease of $63 \pm 24\%$. 5-HT shifted the y-intercept by $34 \pm 26$ Hz, and ketanserin reduced the y-intercept by $4 \pm 9$ Hz. Values are given as mean and SD. Numbers in bars indicate number of protocols per group. ***$P < 0.001$.**
and the time-averaged $F_{\text{con}}$ was $18 \pm 11$ Hz. Stepwise increases in ketanserin dose rates resulted in a stepwise decrease in $F_n$ (Fig. 6). At higher dose rates, a ceiling effect was noted in 13 of 21 neurons, i.e., an increase of ketanserin did not decrease $F_n$ any further. Normalization of the data showed that ketanserin at the maximally effective dose rate ($14 \pm 8$ pmol/min) decreased the time-averaged $F_n$ by $80 \pm 15\%$ and the peak $F_n$ by $68 \pm 15\%$ ($P < 0.001$; Fig. 4). The second step of the analysis was the evaluation of ketanserin-induced changes in the neuronal discharge pattern. Because a ceiling effect of the ketanserin response was not essential in determining the quality of the changes, and the results for the two neuron groups for slope and offset were not significantly different (data not shown), we pooled the data from all 21 neurons. Ketanserin reduced the gain as measured by the slope for the $F_\text{KET}$ versus $F_{\text{con}}$ plots by $63 \pm 24\%$ ($P < 0.001$; Fig. 5, left) and reduced the offset by $4 \pm 9$ Hz ($P < 0.06$; Fig. 5, right).

### Ketanserin as a competitive serotonin antagonist

The ability of ketanserin to act as a competitive antagonist of exogenous 5-HT was confirmed in a small number of additional protocols by studying agonist and antagonist on the same IHMN. An example of this competitive antagonism is given in Fig. 7, where picoejection of 5-HT produced an increase in phasic activity during the inspiratory phase at low concentrations and an additional increase in activity during expiratory phase at higher concentrations (e.g., Fig. 7, left; HMN spike activity). To adequately quantify these 5-HT effects, the average discharge frequency ($\text{ave } F_n$) during each full respiratory cycle was calculated, which includes activity during both phases. A plot of $\text{ave } F_n$ (Fig. 7, left, 2nd trace) versus the estimated dose rate–dependent relative serotonin concentration (Fig. 7, left, 1st trace) is shown in Fig. 7, bottom left, for the initial run (S1). The $\text{ave } F_n$ saturated at the highest serotonin concentrations. Because only picoejection dose rate can be measured, the concentration profile is an approximated estimate based on theoretical models for diffusion from a point source, i.e., the tip of the micropipette. The concentration profile reflects the time-dependent increase that occurs when picoejection is initiated and maintained constant. The steady-state concentration is quasi-linearly related to the ejection dose rate. At high dose rates, the concentration can approach the micropipette barrel concentration, but this is also dependent on distance from the tip. After recovery from 5-HT run S1, ketanserin was picoejected on the neuron and reduced $F_n$ to zero (Fig. 7, bottom right, run K1). During this ketanserin block, a higher dose rate of 5-HT was required to overcome the competitive antagonism as seen by the right shift of the second 5-HT dose-response curve (Fig. 7, bottom left, run S2). While maintaining the peak dose rate, ketanserin was again picoejected, but a higher dose rate was required to antagonize the 5-HT effects as seen by the right shift of the second ketanserin dose-response curve (Fig. 7, bottom right, run K2). Immediately after termination of ketanserin ejection (run K2), its antagonism was reversed by yet a higher 5-HT ejection dose rate, where $\text{ave } F_n$ reached its maximum discharge rate ($\sim 20$ Hz). Picoejection of ketanserin at a yet higher dose was again required to overcome the 5-HT effect (run K3). The dose rates for runs K2 and K3 were not increased enough to produce complete reduction in $\text{ave } F_n$, because of concerns about losing the neuronal activity during these ejection maneuvers at higher ejection rates. However, once the final 5-HT ejection was terminated, the latent effects of ketanserin led to a transient silencing of the neuron, which was followed by a gradual recovery from the antagonist effects. These results show that both drugs seem to antagonize each other in a competitive manner at and even above the dose rates that were typically used in protocols 1 and 2.

### Protocol 3: effects of $CO_2$ on hypoglossal and phrenic nerve activities

To characterize the effects of decerebration, paralysis, and mechanical ventilation on the apneic thresholds of both XII and
phrenic nerve activities and to define the effects of CO₂ drive levels to produce adequate IHMN activity, the CO₂ dose-response characteristics of the XII and phrenic nerve activities were studied in five dogs. An example of the effects of increasing CO₂ levels on phrenic and XII activities are shown in Fig. 8, top, along with the corresponding plot of normalized peak activity versus ETCO₂ (Fig. 8, bottom left). Nonlinear regression, using a hyperbolic function [general form: \( Y = \frac{H}{1 + \left(\frac{X}{X_o}\right)^3} \), where \( Y \) is peak \( F_n \) and \( X \) is ETCO₂], was used to fit both activity plots and to aid in extrapolating the apneic thresholds especially for phrenic activity, because ETCO₂ was decreased only to the point where XII activity could no longer be observed. The response curve for XII activity is right-shifted and shallower, with a greater linear range than that for the phrenic activity. The apneic thresholds were 38 and 42 mmHg for the phrenic and XII activities, respectively, and an ETCO₂ of ~52 mmHg was required to produce a peak XII activity of 50% of maximum. The XII nerve activity versus ETCO₂ plots for five dogs and group mean curve are given in Fig. 8, bottom middle. In three of five animals, there was no XII activity below an ETCO₂ of 42 mmHg. The mean curves ± SE bands of both activities are given in the bottom right of Fig. 8. The XII curve was consistently right shifted and shallower than the phrenic response curve. ETCO₂ levels of 65–70 mmHg were required to produce maximal XII activity. ETCO₂ levels required for 50% of maximum ranged from 41 to 55 mmHg (Fig. 8, bottom middle).

DISCUSSION

This study shows for the first time in vivo the effect of exogenous and endogenous 5-HT on the neuronal discharge pattern of single IHMNs and confirms the significant role of 5-HT in the control of IHM neuronal activity. Our data show that both exogenous and endogenous 5-HT powerfully gain modulate the discharge patterns of IHMNs primarily through postsynaptic 5-HT₂A receptors, based on the ketanserin results. We cannot rule out a possible presynaptic effect of picoejected serotonin on the IHMN response caused by presynaptic 5-HT₁A or 5-HT₁B receptors, which are inhibitory. However,
FIG. 8. CO₂ responses of phrenic nerve and XII nerve activities in hyperoxic decerebrate dogs. Top: example of effects of CO₂, measured as end-tidal ETCO₂ (mmHg), on the moving-time averages of phrenic nerve activity (PNG, top trace) and XII nerve activity (XII, 2nd trace). Bottom left: plot of normalized peak activity vs. ETCO₂ associated with example from top traces. Nonlinear regression was used to fit both nerve activity plots and to aid in extrapolating the apneic thresholds. A hyperbolic function of the form: \( Y = \frac{(x - \phi)^3}{(x - \phi)^3 + (x_0 - \phi)^3} \) was used, where \( Y \) is peak activity, \( x \) is ETCO₂, \( \phi \) is ETCO₂-axis intercept, and \( x_0 \) is the ETCO₂ level for 50% of maximum peak activity. Response curve for XII activity is shifted to the right and shallower with a greater linear range compared with phrenic activity. Apneic thresholds are \(-38\) and \(-42\) mmHg for the phrenic and XII activities, respectively. An ETCO₂ of \(-52\) mmHg produced a peak XII activity of 50% of maximum. Bottom center: XII nerve activity vs. ETCO₂ plots for 5 dogs (thin lines) and group mean curve (thick line). In 3 of 5 animals, there was no XII activity below an ETCO₂ of \(42\) mmHg. Bottom right: mean curves \(\pm SE\) bands of both activities. XII nerve activity curve was consistently right shifted and shallower than phrenic response curve. ETCO₂ levels of 65–70 mmHg were required to produce maximal XII activity. ETCO₂ levels required for 50% of maximum ranged from 41 to 55 mmHg (center plots).

the overall response to serotonin was excitatory and may have masked a presynaptic effect. Furthermore, serotonergic modulation seems so effective that antagonism of 5-HT₂A receptors in a subgroup of neurons can completely silence neuronal activity.

Several studies have shown that 5-HT excites HMNs in vitro (Bayliss et al. 1997; Schwarzacher et al. 2002) as well as in vivo (Fenik et al. 1997; Jelev et al. 2001; Sood et al. 2003). Fenik and Veasey demonstrated in vivo injections of antagonists selective for serotonin receptor subtypes 2A, 2C, or 5 into the hypoglossal motor nucleus in rats that the 5-HT₂A receptor subtype as a powerful modulator of neuronal discharge pattern (Fig. 2B). In a similar in vivo canine preparation, we have observed two different forms of gain modulation of respiratory premotor neurons (Zuperku and McCrimmon 2002). Evidence for GABAergic gain modulation was observed during a block of GABA_A receptors by bicuculline and was attributed to shunting part of the dendrosomatic excitatory current. A second source of gain modulation was observed during a block of the small conductance Ca²⁺-activated K⁺ channels with apamin and was attributed to a change in neuronal excitability because of changes in the amplitude of the spike afterhyperpolarization. In these studies, a change in the excitatory response to picoejections of a discrete amount of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) was used to determine if a change in excitability had occurred at the soma. An increase in the AMPA response during apamin block suggested an increase in neuronal excitability (Zuperku and McCrimmon 2002). Similar studies will be required to identify the mechanism of 5-HT action in the in vivo model.

In vitro studies suggest several effects of 5-HT on neuronal membrane properties. Whole cell recordings from HMNs in a rat brain stem slice preparation indicate that 5-HT activates a barium-resistant sodium channel of relatively small amplitude, which leads to membrane depolarization (Bayliss et al. 1997). However, the main effect seems to be the G protein–mediated inhibition of a TWIK-related acid-sensitive K⁺ (TASK) channel, resulting in an increase in neuronal input resistance, which would lead to amplification of other excitatory inputs (Sirois et al. 2002). This would be consistent with the role of the 5-HT₂A receptor subtype as a powerful modulator of neuronal dis-
charge activity. Interestingly, in the preceding in vitro preparation, the volatile anesthetic halothane was shown to open the TASK channel, an effect that could be completely reversed with 5-HT (Sirois et al. 2002). It will be interesting to see if this antagonistic mechanism is of relevance in vivo where volatile anesthetics cause prominent depression of IHMNs at subanesthetic concentrations (Brandes et al. 2004b).

Gain modulation by 5-HT as well as norepinephrine (NE) has been previously observed in vitro using square-wave currents applied to HMsNs in brain stem slice preparations (e.g., Berger et al. 1992; Parkis et al. 1995). However, in a study where phrenic motoneurons were injected with a more physiological synaptic input current, that typically exhibits membrane potential oscillations, gain modulation was not observed with the bath application of NE (Parkis et al. 2003). The oscillatory potentials, which presumably arise from synchronous presynaptic inputs, are able to synchronize the spike discharge pattern of the HMNs. Bath application of NE did not increase the discharge frequency, but increased the duration of the discharge. It was suggested, that at the level of the motoneuron and muscle behavior, the functional consequences of the oscillations result in an increase in efficiency and protect against motor unit fatigue (Parkis et al. 2003). However, our in vivo data clearly show that 5-HT increases IHMN peak $F_n$ and ketanserin decreases it. This finding is similar to those of Fenik et al. (1997) in decerebrate cats where methysergide was the antagonist. Thus it seems that the in vitro high-frequency oscillation (HFO) hypothesis may not directly apply to the in vivo findings.

**Methodological considerations**

**DECEREBRATION.** We have discussed the advantages and limitations of the decerebrate preparation in previous publications (Dogas et al. 1998; Krolo et al. 1999, 2000; Stucke et al. 2002; Stuth et al. 2000). The major advantage is that decerebration allows investigation of neurotransmission without the confounding effects of anesthetics on ligand gated receptors (Hara and Harris 2002) and other ion channels of the neuronal membrane (Sirois et al. 2000, 2002). This seems especially important in IHMNs, which are already significantly depressed by subanesthetic concentrations of volatile anesthetics (Brandes et al. 2004b).

**EFFECTS OF CHEMODRIVE.** The mid-collicular decerebration may have possibly modified the chemodrive inputs to the IHMNs, resulting in a drive level that is different from that of awake animals. A previous study comparing the responses of decerebrate dogs with anesthetized dogs has shown a left shift in the phrenic nerve ventilatory response and apneic threshold (24.7 vs. 37.6 mmHg) to carbon dioxide with decerebration (Nielsen et al. 1986). We have examined the phrenic and hypoglossal nerve responses to carbon dioxide in five dogs in our decerebrate setup (Fig. 8) and extrapolated the thresholds for phrenic and hypoglossal neural apnea to ~32 and 37 mmHg, respectively. Thus the phrenic apneic threshold is very similar to that of sleeping unanesthetized dogs, which have apneic thresholds shifted ~5 mmHg left of the PaCO$_2$ (~37 mmHg) of normocapnic breathing (Nakayama et al. 2002). However, despite a possible left shift in the CO$_2$ response curve with decerebration, Fig. 8 shows that brisk phasic inspiratory hypoglossal nerve activity is rarely observed at normocapnia. In three of five animals there was no XII nerve activity below an ETCO$_2$ of 42 mmHg, and the half-maximum level of peak activity occurs at ~52 mmHg. In addition, the XII nerve activity seems to increase approximately linearly with increasing carbon dioxide concentrations in the 42–55 mmHg ETCO$_2$ range. Therefore we are constrained to study phasic inspiratory hypoglossal motor neuron activity during increased central chemodrive conditions, which we obtained with hyperoxic hypercapnia.

The functional deafferentation of peripheral chemoreceptor inputs with hyperoxia may have contributed to the smaller left shift of the CO$_2$ response curves and reflects the central CO$_2$ apneic threshold in our decerebrate preparation. A similar apneic threshold of 35.3 ± 5.6 mmHg was observed for phrenic nerve activity during hyperoxia in decerebrate cats (Iscoe et al. 1998).

Even though these studies were performed during hypercapnia, it should be noted that during anesthetic recovery, hypercapnia in this range is common, and that during sleep many obstructive sleep apnea patients operate in this range. Thus there is clinical and pathophysiological relevance to this level of hypercapnia.

**Influence of other factors**

Several factors may have contributed to the magnitude of the endogenous serotonin levels we estimated, where ketanserin reduced peak IHMN activity by ~68%. While serotonergic pathways seem to be functional in decerebrate preparations (Sakai et al. 2000), interruption of descending supracollicular pathways may abolish central inhibitory regulation of raphe serotonergic neurons and may thus have increased the level of the endogenous serotonergic input to the IHMNs that we observed. The increase in spinal levels of serotonin after decerebration seems to be the main cause of muscle rigidity (Sakai et al. 2000). The elevated level of ETCO$_2$ we used may be a significant contributor to the endogenous serotonin level, because it seems that medullary raphe serotonergic neurons may function as central chemosensors (Nattie et al. 2004; Richerson 2004; Severson et al. 2003; Wang et al. 2001). In addition, the state of the preparation can strongly influence the endogenous level of the serotonergic input to the IHMNs. These states include anesthesia, decerebration, quiet wakefulness, REM sleep, non-REM sleep (Dreshaj et al. 1998; Horner 2001; Kubin et al. 1998; Sood et al. 2005), and stage of development. In very young animals, exogenous serotonin inhibits IHMNs through the presynaptic inhibitory autoreceptors (e.g., 5-HT$_{1B}$ receptors) of the serotonergic neurons (Singer et al. 1996). There is also evidence suggesting that vagotomy can increase raphe activity through a reduction in vagal afferent-mediated inhibition of the raphe neurons (Sood et al. 2005).

In these acute experiments, we did not observe any qualitative differences on central respiratory activity by adding dexamethasone to our surgical preparation. Dexamethasone may alter neuromodulator function by affecting catecholamine biosynthesis through tyrosine hydroxylase, a rate-limiting enzyme in catecholamine biosynthesis. However, Joseph et al. (1998) showed in rats that, although acute administration of 1 mg/kg dexamethasone stimulated tyrosine hydroxylase activity in the
carotid body and thereby reduced the hypoxic ventilatory response, no effects from acute administration where seen in brain stem catecholamine areas. Only chronic administration over 10 days affected central tyrosine hydroxylase activity. Because the peripheral chemoreceptors are already functionally denervated in our preparation, we would expect minimal effects of acute dexamethasone administration.

**Picoejection**

The picoejection method allows highly localized drug ejection onto the recorded neuron, which is prerequisite for unequivocal interpretation of the data. Concentrations are lower than with microiontophoresis, reducing the probability of non-specific effects caused by high local concentrations. In addition, monitoring of ejected drug volumes by direct monitoring of the meniscus of the drug barrel allows the calculation of dose-response curves for the applied substances. The limitations of this method have been previously delineated (Dogas et al. 1998; Krolo et al. 1999, 2000; Stucke et al. 2002; Stuth et al. 2000). While it cannot be excluded that the picoejection response of the recorded neurons may be influenced by additional drug effects on presynaptic neurons, the importance of this contamination seems small for the following reasons. 1) The qualitative character of the neuronal response was consistent for all neurons studied. 2) The response was always consistent with the expected postsynaptic effects of the drug. For example, 5-HT always increased neuronal discharge frequency, whereas an activation of presynaptic 5-HT$_{1B}$ receptors could have caused an inhibition of the neuron (Singer et al. 1996). Similarly, ketanserin always decreased neuronal discharge frequency. 3) The picoejected doses did not change hypoglossal nerve activity. Concentration of a picoejected drug decreases rapidly with distance from the electrode tip. A theoretical analysis of the diffusion of a drug from a constant point source shows that the concentration decreases inversely (1/distance) with the distance from the source (Stone 1985).

**Selectivity of ketanserin**

While ketanserin has also been shown to antagonize adrenergic $\alpha_1$ receptors, it seems to do so at much higher concentrations than those required to antagonize 5-HT$_2$ receptors. For example, the IC$_{50}$ at 5-HT$_2$ receptors was found to be 0.65 nM, whereas at $\alpha_1$ receptors, the IC$_{50}$ was 30.0 nM or 46.5 times higher in isolated perfused rat tail arteries (Marwood 1994). In these studies, the picoejection dose rates were always started at low levels and gradually increased until a maximal effect was observed. This happened over a narrow dose rate range as indicated by the data of Fig. 7. Thus it is highly likely that the ketanserin effects were mainly due to antagonism of 5-HT. Furthermore, we were able to show that ketanserin functioned as a classical competitive antagonist as suggested by the parallel shifts in the log dose-response curves for both 5-HT and ketanserin of Fig. 7.

Notably, our in vivo experiments show that endogenous 5-HT is a powerful modulator of physiological IHMN activity. This effect seems to be mediated primarily by postsynaptic 5-HT$_{2A}$ receptors and consists of gain modulation of the neuronal discharge pattern via an increase in membrane resistance together with a relative depolarization. An increase in membrane resistance increases the excitability of the neuron to synaptic inputs, while a relative depolarization moves the membrane potential closer to the firing threshold and thus increases excitation of the neuron.

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