Halothane Depresses Glutamatergic Neurotransmission to Brain Stem Inspiratory Premotor Neurons in a Decerebrate Dog Model

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Background: Inspiratory bulbospinal neurons in the caudal ventral medulla are premotor neurons that drive phrenic motoneurons and ultimately the diaphragm. Excitatory drive to these neurons is mediated by N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors and modulated by an inhibitory γ-aminobutyric acid$_A$ (GABA$_A$)ergic input. The authors investigated the effect of halothane on these synaptic mechanisms in decerebrate dogs.

Methods: Studies were performed in decerebrate, vagotomized, paralyzed, and mechanically ventilated dogs during hypercapnic hyperoxia. The effect of 1 minimum alveolar concentration (MAC) halothane on extracellularly recorded neuronal activity was measured during localized picoejection of the GABA$_A$ receptor blocker bicuculline and the glutamate agonists AMPA and NMDA. Complete blockade of the GABA$_A$ergic mechanism by bicuculline allowed differentiation between the effects of halothane on overall GABA$_A$ergic inhibition and on overall glutamatergic excitation. The neuronal responses to exogenous AMPA and NMDA were used to estimate the anesthetic effect on postsynaptic glutamatergic neurotransmission.

Results: Halothane, 1 MAC, depressed the spontaneous activity of 21 inspiratory neurons by $20.6 \pm 18.0\%$ (mean $\pm$ SD; $P = 0.012$). Overall glutamatergic excitation was depressed $15.4 \pm 20.2\%$ ($P = 0.001$), while overall GABA$_A$ergic inhibition did not change. The postsynaptic responses to exogenous AMPA and NMDA were also depressed by $18.6 \pm 35.7\%$ ($P = 0.03$) and $22.2 \pm 26.2\%$ ($P = 0.004$), respectively.

Conclusion: Halothane, 1 MAC, depressed the activity of inspiratory premotor neurons by a reduction of glutamatergic excitation. Overall inhibitory drive did not change. The postsynaptic AMPA and NMDA receptor response was significantly reduced. These findings contrast with studies in expiratory premotor neurons in which overall inhibition was significantly increased by halothane and there was no reduction in the postsynaptic glutamate receptor response.

INSPIRATORY premotor neurons of the caudal ventral respiratory group (cVRG) provide the excitatory drive to

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phrenic and inspiratory intercostal motoneurons,[1] and together with expiratory premotor neurons, their activity determines the magnitude of the respiratory tidal volume. At a corresponding level, expiratory bulbospinal neurons control the end-expiratory lung volume and the expiratory air flow rate.[2] In addition, inspiratory and expiratory premotor neurons produce phasic inhibition of expiratory and inspiratory motoneurons, respectively, in the spinal cord.[3] Systematic studies on expiratory premotor neurons in the cVRG revealed that 1 minimum alveolar concentration (MAC) halothane reduced neuronal activity by a reduction in presynaptic glutamatergic excitatory drive and an increase in overall inhibition.[4] The increase in inhibition was due to a pronounced enhancement of γ-aminobutyric acid$_A$ (GABA$_A$) receptor function.[5]
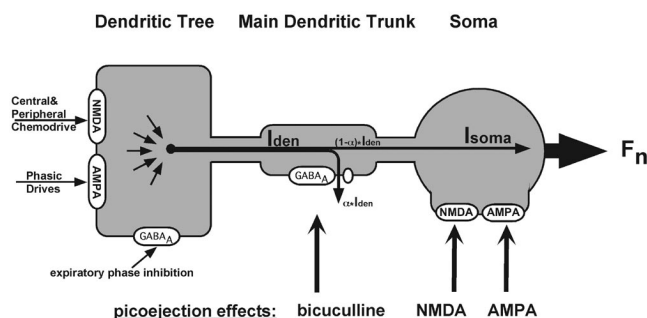
Excitatory drive to cVRG expiratory neurons originates from mainly carbon dioxide–dependent chemodrive[6] and is solely mediated by the N-methyl-D-aspartate (NMDA) glutamate receptor subtype.[7] Inspiratory neurons in the cVRG, however, receive excitatory inputs from at least two different sources (fig. 1). We have shown that tonic excitatory drive is mediated by NMDA receptors and accounts for approximately two thirds of the inspiratory neuronal activity in neuraxis-intact animals during 1 MAC halothane.[8] This drive is presumed to originate from carbon dioxide chemodrive.[1] Approximately a third of total excitatory drive to inspiratory neurons is phasic and mediated by the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) subtype of glutamate receptors.[8] It appears that this additional drive results from "self-reexcitation," *i.e.*, cVRG inspiratory neurons reciprocally and increasingly excite each other over the duration of the inspiratory phase.[1] Both inspiratory and expiratory neuronal activity is modulated by a tonic inhibitory GABA$_A$ergic input.[9] During the expiratory phase, inspiratory neurons are silent, mainly because of additional phase-specific GABA$_A$ergic inhibitory inputs (reciprocal inhibition), which are not related to the modulatory tonic GABAergic input.[9]

The current study examines the effect of halothane on neurotransmission to cVRG inspiratory neurons in an *in vivo* preparation. Complete block of GABAergic inhibition with bicuculline allowed us to compare changes in overall glutamatergic excitatory drive to the neuron between 0 and 1 MAC halothane. The complete block of GABAergic inhibition also allowed to estimate the magnitude of prevailing overall inhibition and to compare its

Fig. 1. Conceptual model illustrating the hypothetical functional location of postsynaptic receptors that influence inspiratory neuronal firing frequency. Tonic and phasic glutamatergic inputs to the dendritic tree elicit a dendritic current (Iden). On its path toward the soma, Iden is attenuated by a tonic GABA$_A$ergic input, which leads to a shunting of part of the current ($\alpha \times$ Iden, where $\alpha \leq 1$; α represents overall inhibition). The remaining current reaches the soma (Isoma = $(1-\alpha) \times$ Iden), and its magnitude determines neuronal discharge frequency (F$_n$). There is evidence for the existence of glutamate receptors on the soma and proximal dendrites. A possible function of these receptors is the mediation of behavioral inputs such as coughing, sneezing, or speech, *i.e.*, activities that also require respiratory musculature and can alter spontaneous breathing. Since picoejection is highly localized, small doses of picoejected drugs may only reach the soma and proximal dendrites of the neuron. The model assumes that the proximal receptor response to glutamate agonists is representative for the more distal glutamate receptors on the dendrites.

magnitude at 0 and 1 MAC. In addition to the effects on overall neurotransmission, we studied the effect of 1 MAC halothane on AMPA or NMDA receptor function by exogenous application of the respective agonist. Second, we performed a study to determine the resolution of our picoejection method in discriminating small changes in glutamate receptor function.

Materials and Methods

### Animal Preparation and General Methodology

This research was approved by the Medical College of Wisconsin Animal Care Committee (Milwaukee, Wisconsin) and conformed with 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, and the dogs were intubated with a cuffed endotracheal tube and from then on were 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%[10]). The animals were positioned in a stereotactic device (model 1530; David Kopf Instruments, Tujunga, CA) with the head ventrally flexed (30°). Bilateral neck dissections were performed. The C5 phrenic nerve rootlet was desheathed for recording, and bilateral vagotomy was performed to achieve peripheral deafferentation. This avoids interference of the artificial ventilation with the underlying central respiratory rhythm. Bilateral pneumo-

thorax was performed to minimize brain stem movement and phasic inputs from chest wall mechanoreceptors. The animals were decerebrated at the midcollicular level[11] and only then paralyzed (0.1 mg/kg pancuronium, followed by 0.1 mg · kg^{-1} · h^{-1}). An occipital craniotomy was performed to expose the dorsal surface of the medulla oblongata for single-neuron recording. Esophageal temperature was maintained at 38.5 ± 1°C. Mean arterial pressure was kept above 100 mmHg and did not differ more than 20% between 0 and 1 MAC. Blood pressure was supported as needed with infusions of phenylephrine (0.5–5 μg · kg^{-1} · min^{-1}). Protocols were only performed during steady state conditions for blood pressure.

Neuron Recording Technique, Data Collection, and Experimental Conditions

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 before and during pressure ejection of the glutamate agonists and GABA$_A$ receptor antagonist onto inspiratory neurons of the cVRG. We used the selective glutamate receptor agonists AMPA (7.5 μM; Research Biochemicals, Natick, MA) and NMDA (200 μM; Research Biochemicals) and the GABA$_A$ antagonist bicuculline methochloride (BIC, 200 μM; Research Biochemicals), which were dissolved in an artificial cerebrospinal fluid (aCSF).[4] Meniscus changes in the drug barrels were measured to determine the ejected dose rates (resolution, 2 nl). The neurons were located approximately 1.5–3 mm caudal from the obex and 2.5–4.5 mm lateral from the midline. A prior study had shown that approximately 90% of these neurons were bulbospinal,[12] *i.e.*, their soma was located in the brain stem and their axons were projected to motoneurons in the spinal cord as confirmed with antidromic stimulation techniques.[12] Single-cell inspiratory neuronal activity, phrenic nerve activity, picoejection marker pulses, airway carbon dioxide and volatile anesthetic concentrations, systemic blood pressure, and airway pressure were recorded on a digital tape system (model 3000A; A.R. Vetter Co., Rebersburg, PA). These variables or their time averages were also continuously displayed on a computerized chart recorder (Powerlab/16SP; ADInstruments, Castle Hill, Australia). Timing pulses at the beginning and end of neural inspiration were derived from the phrenic neurogram and were used to determine the respiratory phases. The tape-recorded data were digitized and analyzed off-line. Cycle-triggered histograms (CTHs), triggered at the onset of phrenic activity, were used to quantify the neuronal discharge frequency data.

The protocols were performed under hyperoxic (fraction of inspired oxygen [F$_{IO_2}$] > 0.8) and steady state hypercapnic conditions (arterial carbon dioxide tension
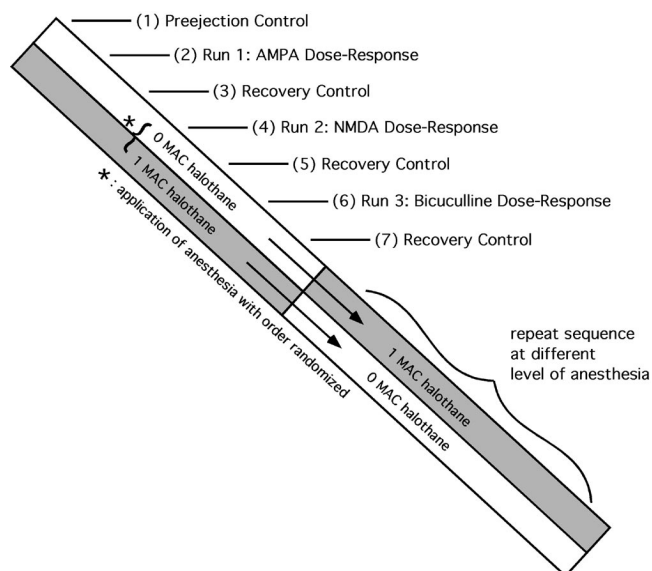
Fig. 2. Outline of the protocol. Randomization decided if the protocol began at 0 or 1 MAC halothane. The picoejection runs were performed in the order shown. Between the runs, recovery was assumed when the neuronal frequency had returned to the preejection control frequency.

[Pa_{CO_2}] 55–65 mmHg). The optimal level of Pa_{CO_2} was adjusted from animal to animal so that adequate phasic respiratory activity during the anesthetic state (1 MAC) was ensured. Great care was taken to keep the Pa_{CO_2} tightly controlled within each neuron protocol. One complete neuron protocol consisted of two sets of three separate picoejection runs (run 1: AMPA, run 2: NMDA, and run 3: BIC; fig. 2). One set was performed in this sequence at 0 and the other set at 1 MAC halothane (0.9%[10]). To maximize the yield of complete neuron protocols, we performed the current protocols with the order of the sets (0 and 1 MAC) randomized, which eliminated the need for end controls.[5,13] Control ejections with the vehicle aCSF, an artificial cerebrospinal fluid in which the neurotransmitters were dissolved, were performed for each experimental setup to confirm lack of vehicle effect.

Run 1: Effects of Halothane on Postsynaptic AMPA Receptors

For the control period (F_{con}) and at each dose rate, CTHs (5–20 cycles) were used to obtain values of the average peak neuronal discharge frequency (F_n) for each condition. The glutamate agonist AMPA was applied in increasing dose rates until an increase in peak F_n of at least 25 Hz was achieved. Typically, picoejection durations of 6–8 min with 2 or 3 dose rates were needed.

Statistical Analysis, Run 1. The effect of 1 MAC halothane on the postsynaptic AMPA receptor response was quantified by linear regression of F_n on dose rate since previous studies have shown the dose–response data for glutamate to be linear.[4,13] This was confirmed for inspiratory neurons. In this regression analysis, the

y-intercept was constrained to pass through the F_{con} value at the zero dose rate. Thus, any change in the slope of the regression line reflected the anesthetic-induced change in the dose–response relation. To compare the dose responses at 0 and 1 MAC, the slope values were then normalized to the slope at 0 MAC (slope$_0$), and the normalized difference was determined for each neuron [*i.e.*, Δslope = (slope$_1$ − slope$_0$)/slope$_0$]. A Wilcoxon signed-rank test was performed to test if Δslope was significantly different from no change (StatView; SAS Institute, Inc., Cary, NC). To illustrate the effect of an anesthetic-induced change in slope on F_n and to allow comparison with our previous expiratory neuron studies,[4] we also calculated the net increase in F_n at a D_{40Hz} dose rate for each dose–response relation. For this, the net increase in F_n was calculated from the 1 MAC linear regression line at the dose rate that caused a 40-Hz increase in F_n at 0 MAC.

Run 2: Effects of Halothane on Postsynaptic NMDA Receptors

After recovery from AMPA, the same picoejection run was repeated with NMDA. The analyses of the NMDA data were performed using the same procedure as described for AMPA.

Run 3: Effects of Halothane on Overall Synaptic Neurotransmission

After recovery from NMDA, the GABA$_A$ receptor antagonist BIC was picoejected until complete block of GABA$_A$ergic inhibition occurred, *i.e.*, when an increase in picoejection dose rate did not result in any further increase in F_n. Typically, picoejection durations of 5–10 min with several increasing dose rates were required. After the BIC run, complete postejection recovery was awaited, which required 30–45 min. Then, the randomized state of anesthesia was switched, and after a minimum equilibration time of 15 min, both steps of the protocol were repeated in the same fashion. State of anesthesia refers to either 1 MAC anesthesia or absence of anesthesia (0 MAC).

Statistical Analysis, Run 3. During complete GABA$_A$ergic block with BIC, F_n equals the overall excitatory drive to the neuron (F_e). Under control conditions, the prevailing GABA$_A$ergic inhibition reduces F_e to F_{con} by the inhibitory factor α, where $\alpha = (F_e - F_{con})/F_e$. To calculate the change in overall excitatory drive, the data were normalized to F_e at 0 MAC, which was assigned a value of 100%. A two-way repeated-measures analysis of variance (ANOVA) was used with main factors of anesthetic state (0 or 1 MAC) and neurotransmitter status (preejection control *vs.* maximal BIC block; SuperANOVA; Abacus Concepts, Inc., Berkeley, CA). The values for F_e and F_{con} were obtained for the 0 MAC level (F_{con0}, F_{e0}) and the 1 MAC level (F_{con1}, F_{e1}) from the experimental runs. They were then used in the

calculation of the anesthetic effect on overall excitation ΔF_e (where $\Delta F_e = [F_{e1} - F_{e0}]/F_{e0}$) and overall inhibition $\Delta\alpha$ (where $\Delta\alpha = [\alpha_1 - \alpha_0]/\alpha_0$). All results are given as mean $\pm$ SD, and $P < 0.05$ was used to indicate significant differences unless stated otherwise.

Reproducibility of the Dose–Response Curves for Glutamate Agonists

To examine the reproducibility of the picoejection technique, we performed an additional study in a series of separate animals. The glutamatergic agonist AMPA or NMDA was picoejected in at least two increasing dose rates on cVRG inspiratory or expiratory neurons at 0 MAC (run 1). After recovery to preejection control (F_{con}), the picoejection was repeated with the same drug (run 2), using the same ejection parameters (ejection pressure, pulse duration, ejection frequency), simulating regular experimental conditions. Note that cVRG expiratory neurons also have both glutamate receptor subtypes, but only NMDA receptors are endogenously active.[7]

Statistical Analysis. Dose–response plots were created from the increases in neuronal frequency following glutamate receptor agonist application for run 1 and run 2. We calculated the linear regression parameters for each plot where the y-intercept was constrained to pass through the F_{con} value. The slopes were then normalized to the slope of run 1. In addition, we determined the dose rate that caused a 40-Hz increase in F_n in run 1, *i.e.*, D_{40Hz}, and calculated the net increase in F_n at this dose rate in run 2 using the regression line. To allow for comparison of the current study with previous publications on expiratory neurons, we also calculated the net increases at the D_{40Hz} dose rate using our previous method based on interpolation between data points[4] and compared the results of these two methods. Differences in slope and net increases at D_{40Hz} between run 1 and 2 were tested using a multivariate ANOVA (SuperANOVA).

Results

In the main study, experiments were performed on 28 animals yielding 21 complete neuron protocols. To assess the resolution of the method, we analyzed the reproducibility of dose responses in 15 neurons from 3 animals.
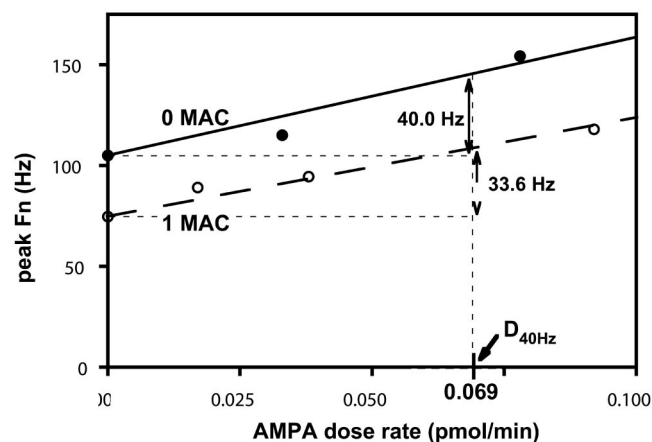
Effects of Halothane on Postsynaptic AMPA Receptors

Figure 3 shows a representative example of an inspiratory neuronal response to increasing dose rates of AMPA at 0 MAC and 1 MAC halothane, respectively. The highest picoejected dose rates for AMPA were 0.078 pmol/min at 0 MAC and 0.092 pmol/min at 1 MAC. Halothane, 1 MAC, decreased the slope of the linear regression line fitted through the dose–response plots from 582.1 Hz · pM^{-1} · min^{-1} to 488.5 Hz · pM^{-1} · min^{-1},
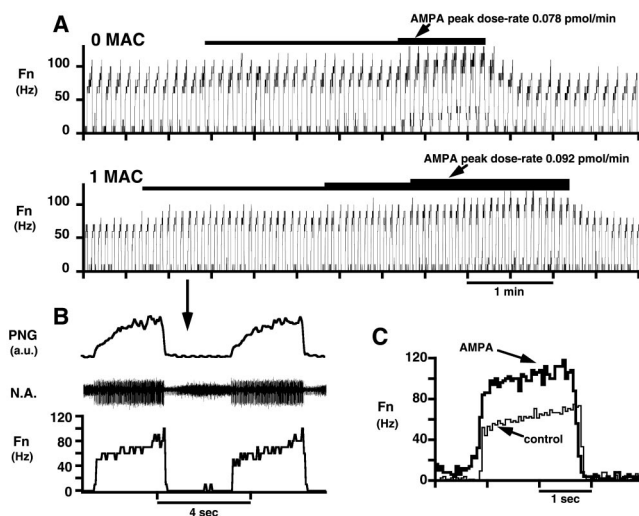
Fig. 3. (*A*) Response of an inspiratory neuron to increasing doses of AMPA at 0 and 1 MAC halothane. The picoejection response curves are shown as the rate-meter recordings of the neuronal discharge frequency F_n (in hertz). The horizontal bars indicate the picoejection duration. Maximal dose rates are given. (*B*) Time-expanded view of the neuronal rate-meter recording for 1 MAC halothane at the time indicated by the arrow. The simultaneously recorded phrenic neurogram (PNG, in arbitrary units, a.u. [*top*]) identifies the neuron as inspiratory. The neuronal raw activity (N.A.) is originally recorded as a train of action potential spikes (*middle*). (*C*) Cycle-triggered histograms of 15 respiratory cycles during preejection control (thin) and of 10 respiratory cycles at the peak AMPA dose rate (bold), both at 1 MAC halothane. The AMPA-induced net increase was approximately 40 Hz.

i.e., the AMPA receptor response was decreased by 16.1% (fig. 4). This is equivalent to a reduction of the net increase from 40 Hz at 0 MAC to 33.6 Hz at 1 MAC at the D_{40Hz} dose rate.

Fig. 4. Method used to analyze the effect of halothane on postsynaptic glutamate receptor function. The graph shows the response to picoejection of AMPA onto the neuron in fig. 2. Linear regression analysis was performed where the y-intercept was constrained to pass through control frequency (F_{con}) at the zero dose rate. To pool the data, the slope at 1 MAC halothane (dashed line) was normalized to the slope at 0 MAC (solid line). To illustrate the anesthetic effect in terms of changes in neuronal frequency, we also calculated the net increase at dose rate D_{40Hz}, *i.e.*, the dose rate that caused an increase of 40 Hz at 0 MAC.
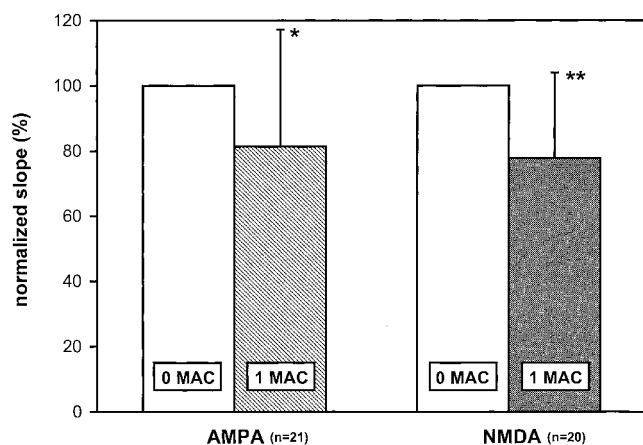
Fig. 5. Pooled summary data for the postsynaptic receptor response to AMPA and NMDA at 0 and 1 MAC halothane. The graphs show the mean slopes, derived from the linear regression lines through the dose–response values and normalized to 0 MAC. The receptor response to AMPA and NMDA is significantly depressed by the anesthetic (*$P < 0.05$, **$P < 0.01$).

The pooled normalized data from 21 neurons for AMPA-induced net increases in peak F_n are summarized in figure 5. Halothane, 1 MAC, significantly depressed the AMPA receptor response, determined by the slope of the linear regression, by $18.6 \pm 35.7\%$ ($P < 0.05$). This translates into a depression of the net increase at D_{40Hz} from 40 Hz at 0 MAC to 33.6 ± 14.3 Hz at 1 MAC.

Effects of Halothane on Postsynaptic NMDA Receptors

For the same neuron (fig. 6), the slope was 18.6 Hz · pM^{-1} · min^{-1} at 0 MAC and 15.8 Hz · pM^{-1} · min^{-1} at 1 MAC at peak dose rates of 3.6 pmol/min and 4.3 pmol/min NMDA, respectively. Thus, 1 MAC halothane decreased the NMDA receptor function in this neuron by 15.1%, which was equivalent to a reduction of the net increase from 40 Hz at 0 MAC to 34 Hz at 1 MAC at the D_{40Hz} dose rate.
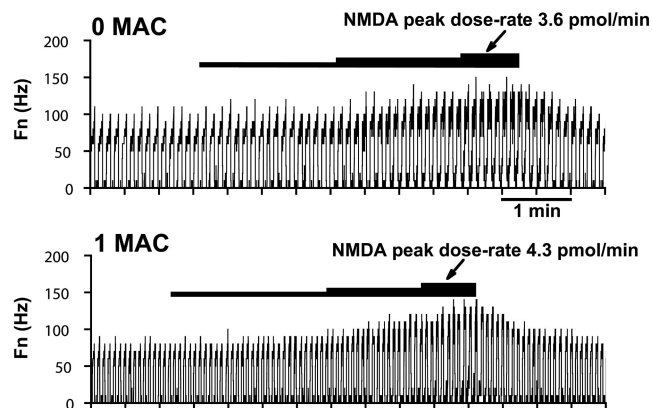

Fig. 6. Response of the neuron in figure 3 to increasing doses of NMDA at 0 and 1 MAC halothane. There is a graded increase in discharge frequency with increasing dose rates and a rapid decline in activity once the agonist application is stopped. Horizontal bars: duration of the picoejection; the peak dose rates are given.
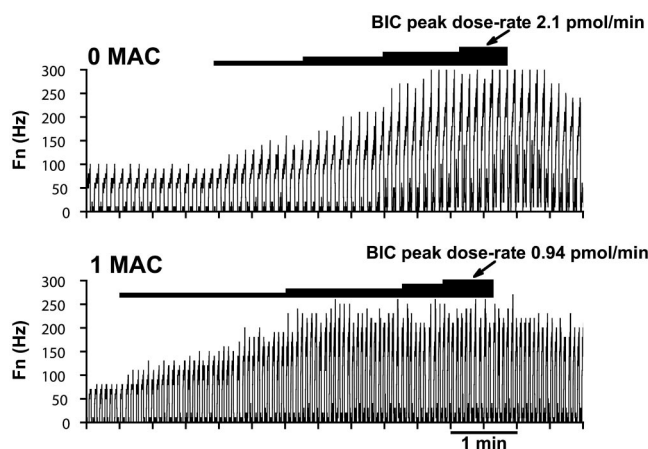

Fig. 7. Response of the neuron in figures 3 and 6 to increasing doses of the GABA_A antagonist bicuculline at 0 and 1 MAC halothane. When the increase in neuronal frequency had reached a plateau, the bicuculline dose rate was increased at least one more step to ascertain that the bicuculline effect was saturated, *i.e.*, that all GABA_A receptors were blocked.

The pooled normalized net increases in peak F_n, induced by NMDA, are summarized in figure 5. One value was identified as an outlier according to Dixon[14] and removed from further analysis. Halothane, 1 MAC, significantly depressed NMDA receptor function, determined by the slope of the linear regression, by $22.2 \pm 26.2\%$ ($n = 20$; $P < 0.01$). This is equivalent to a depression of the net increase at D_{40Hz} from 40 Hz at 0 MAC to 31.1 ± 10.5 Hz at 1 MAC halothane.

Effects of Halothane on Overall Synaptic Neurotransmission

Further analysis of the same inspiratory neuron showed that at 0 MAC, BIC increased peak F_n from 105 to 267 Hz (fig. 7). This means that under control conditions, the activity of the neuron was attenuated by tonic GABAergic inhibition to 39% of the overall excitatory drive, *i.e.*, the overall inhibitory factor α_0 was 0.61 (fig. 8, top). At 1 MAC halothane, BIC increased peak F_n from 75 to 220 Hz, yielding an α_1 value of 0.66. Thus, 1 MAC halothane enhanced overall GABAergic inhibition in this neuron by 8%. The overall excitatory drive, F_e, measured at complete BIC block of GABAergic input, was reduced by 18%, while the control frequency F_{con} was reduced by 29% by 1 MAC halothane.

The pooled data for 21 neurons show that 1 MAC halothane reduced F_{con} by $20.6 \pm 18.0\%$ ($P < 0.001$) and overall excitation by $15.4 \pm 20.2\%$ ($P < 0.01$; fig. 8, bottom). Overall inhibition α was 0.60 ± 0.1 at 0 MAC and 0.62 ± 0.1 at 1 MAC ($\Delta\alpha = +5.2 \pm 14.9\%$; $P = 0.19$).

Reproducibility of the Dose–Response Curves for Glutamate Agonists

Figure 9 shows a representative example of two consecutive dose responses to NMDA application in the
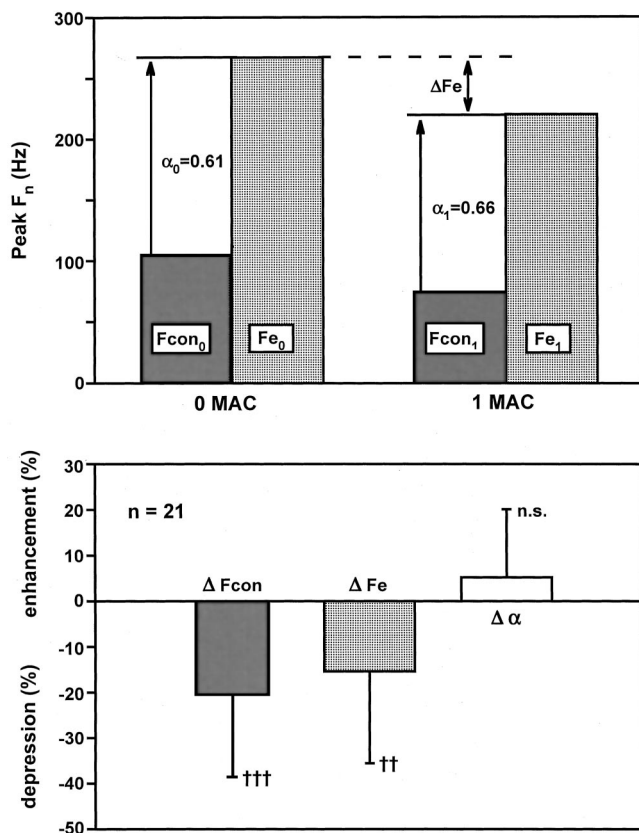
Fig. 8. (*Top*) Method used to analyze the effect of halothane on overall neurotransmission. The analysis shows the effect of halothane on the response of the neuron in figure 7 to the $GABA_A$ antagonist bicuculline. Peak neuronal frequency values were obtained from cycle-triggered histograms based on 8–10 respiratory cycles prior to picoejection of bicuculline (F_{con}) and at the maximal bicuculline response (F_e) to determine overall excitation. Halothane, 1 MAC, reduced F_{con} from 105 Hz to 75 Hz and F_e from 267 Hz to 220 Hz (ΔF_e). The magnitude of the GABAergic input to the neuron, *i.e.*, the overall inhibition (α) was calculated as $\alpha = (F_e - F_{con})/F_e$. Halothane increased α from 0.61 to 0.66. (*Bottom*) Pooled summary data. Mean changes $\pm$ SD for control neuronal frequency (ΔF_{con}), overall excitation (ΔF_e), and overall inhibition ($\Delta \alpha$) by 1 MAC halothane ($\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$, n.s. = not significant; all relative to no change).

same neuron. Linear regression analysis yielded a slope of 20.1 Hz · pM^{-1} · min^{-1} for picoejection run 1 and of 19.6 Hz · pM^{-1} · min^{-1} for run 2 (fig. 9, top). When the net increases at D_{40Hz} were calculated from the linear regression line, the net increase in run 2 was 39 Hz, *i.e.*, 2.5% lower (fig. 9, middle). When the net increase at D_{40Hz} was interpolated from data points on the original dose-response plot, the increase in run 2 was 40.6 Hz, *i.e.*, 1.5% higher than in run 1 (fig. 9, bottom).

One value was identified as an outlier[14] and removed from further analysis. The pooled data from six inspiratory and eight expiratory neurons shows that there was no significant difference in the slope of the linear regression between picoejection run 1 (100%) and run 2 (99.5 $\pm$ 27.5%; $P = 0.95$). This was equivalent to a net increase of 39.8 $\pm$ 11.0 Hz for run 2 at D_{40Hz} (individual
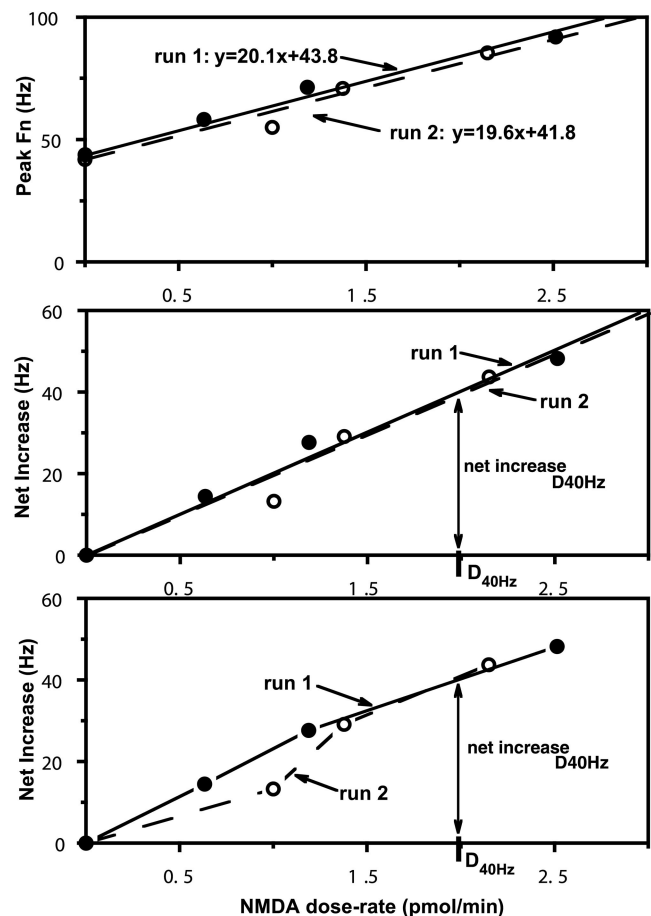

Fig. 9. Comparison of the responses of a representative neuron to two consecutive ejections of increasing NMDA dose rates (run 1 and run 2). (*Top*) A linear regression analysis of the dose–response data showed that the runs closely matched each other. (*Middle*) Interpolation at dose rate D_{40Hz}, *i.e.*, the dose rate that caused a net response of 40 Hz in the first run, yielded a net response of 39 Hz for run 2. (*Bottom*) When the D_{40Hz} values were interpolated from the original dose–response plot, which was used in previous studies, the net response at 1 MAC halothane was 40.6 Hz.

data in fig. 10). When the net increase at D_{40Hz} was interpolated between the single dose–response values, the net increase in run 2 was 37.6 $\pm$ 8.3 Hz ($P = 0.30$). There was no difference between the values calculated from the linear regression line and from those interpolated between single dose–response values. The multivariate ANOVA showed that the order of the runs did not have a significant influence on the values. Also, there was no difference in results between inspiratory and expiratory neurons.

Discussion

This *in vivo* preparation provides the first conclusive evidence that halothane depressed the activity of inspiratory premotor neurons by reduction of excitatory glutamatergic neurotransmission. Overall inhibitory neuro-
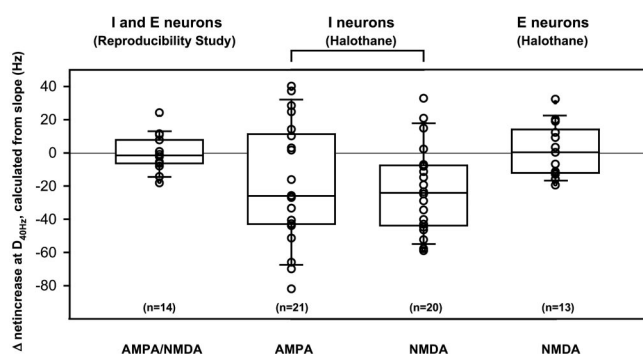
Fig. 10. Box plots summarize the distribution of the data for the reproducibility study (*left*), for protocol 1 (AMPA, *second from left*) and protocol 2 (NMDA, *second from right*) of the main study, and for the comparable study on NMDA receptors in expiratory neurons (*right*).[4] The data for expiratory neurons has been recalculated according to the updated method of analysis. Box values indicate the 25th, 50th, and 75th percentile values, and T bars indicate the 10th and 90th percentiles.

transmission remained unchanged. A significant part of the reduction of excitatory neurotransmission appeared to be mediated by anesthetic depression of the postsynaptic glutamate receptor response.

Reduction of Excitatory Drive: Presynaptic or Postsynaptic?

In related studies on canine expiratory premotor neurons, the halothane-induced depression in postsynaptic NMDA receptor response ($-3.3 \pm 38.4\%$)[4] did not reach statistical significance. Similar results were found for sevoflurane.[13] It was concluded that excitatory drive to expiratory neurons was primarily reduced at a presynaptic site. This was in agreement with data obtained in rat hippocampal slice preparations, where the magnitude of glutamatergic excitatory postsynaptic currents, elicited by stimulation of the presynaptic Schaffer collaterals, was depressed by clinical concentrations of halothane.[15] However, the kinetics of these currents and the receptor response to exogenous NMDA or AMPA were not changed.[15] In addition, paired pulse facilitation, *i.e.*, the ratio of the amplitude of the second to the first evoked excitatory postsynaptic currents, was increased by halothane,[16] which also suggested that presynaptic glutamate release was depressed by the anesthetic. Furthermore, a reduction of presynaptic glutamate release by volatile anesthetics has been demonstrated in synaptosomes[17] and in rat cortical brain slices.[18]

The decrease in AMPA and NMDA receptor response in the current study stands in contrast to our results in expiratory neurons.[4] To exclude that relevant changes in postsynaptic NMDA receptor function had been missed in expiratory neurons, we performed an additional study that assessed the resolution and accuracy of our method. Fourteen protocols from inspiratory and expiratory neurons showed that the dose responses to glutamate agonists were highly reproducible. We used linear re-

gression analysis since it is based on all available dose–response values at one anesthetic level and can thus be expected to be the better representative of the "true" dose response. The average difference between the slopes in run 1 and 2 at D_{40Hz} was $0.5 \pm 27.5\%$ Hz. With a population SD of 27.5%, based on the dose–response reproducibility study, and a type I error of 0.05, our method can discern a difference of approximately 20% with 90% power, *i.e.*, a type II error of 0.10. Part of the variability of our method may be related to the limits of discrimination of the drug ejection rate, which is determined from the meniscus changes in the drug barrel of the micropipette. In addition, there is a breath-to-breath variability in neuron activity that is intrinsic to the system (coefficient of variation, 8–10%[8]). Based on our reproducibility study, we conclude that the significant reduction of the AMPA and NMDA receptor response (-18.6 ± 35.7 and $-22.2 \pm 26.2\%$) in inspiratory neurons by halothane represents a true anesthesia-induced finding, which is not due to the variability of the method.

An anesthesia-induced reduction in the postsynaptic glutamate receptor response contrasts with many studies that show that clinical concentrations of volatile anesthetics affect the presynaptic component of excitatory neurotransmission but not postsynaptic NMDA or AMPA receptors.[15,19,20] However, Dildy-Mayfield *et al.*[21] expressed different AMPA receptor subtypes in *Xenopus* oocytes and showed that the effect of halothane depended on the respective subtype. Halothane had an inhibitory effect on the GluR3 receptor subtype and enhanced GluR6 receptor activity. Furthermore, two studies both performed in rat hippocampal slices reported anesthetic effects on postsynaptic glutamatergic neurotransmission that differed between non-NMDAergic and NMDAergic neurotransmission. Narimatsu *et al.*[22] reported that the halothane concentration necessary to depress population spikes by 50% was significantly smaller when non-NMDA receptors were selectively antagonized than when the NMDA receptors were selectively antagonized and concluded that NMDA receptors were more sensitive to halothane. Nishikawa and MacIver[16] found no difference for halothane but described that 1 MAC–equivalent isoflurane preferentially depressed field excitatory postsynaptic potentials mediated by NMDA receptors. In addition, paired pulse facilitation was increased by both anesthetic agents, which suggested that presynaptic mechanisms were also involved in the field excitatory postsynaptic potential depression.

Part of the decrease in overall excitatory drive to inspiratory neurons in our study may have occurred at a presynaptic level. However, an anesthetic-induced reduction of the postsynaptic glutamate receptor response to AMPA and NMDA that clearly exceeded the method-inherent variability was found in a sizeable subgroup of the inspiratory neurons studied (fig. 10). The discrep-

ancy between our results and studies that did not find an anesthetic effect on glutamate receptors may be due to fundamental differences between *in vitro* and *in vivo* preparations. For example, Takeda *et al.*[23] exposed decerebrate cats to short 90-s bouts of 2% halothane while recording intracellular potentials in inspiratory and expiratory bulbar neurons. They found that regardless of the type of neuron, the anesthetic could cause either membrane depolarization or hyperpolarization, typically combined with a decrease or complete cessation of neuronal firing. In addition, the direction of the anesthetic-induced change in membrane potential, produced by either halothane or intravenous thiopental, was consistent for each neuron. The authors concluded that a reduction of the synaptic input to the neuron, recognizable through an increase in membrane resistance and/or a decrease in respiratory-phase related membrane fluctuations, changed the membrane potential and excitability of the neuron. Dependent on a neuron's specific location within the network, inhibitory and excitatory inputs to the neuron vary so that an anesthetic may alter the excitability of apparently similar neurons in different ways. This "network factor" could account for some of the variability in the effect that 1 MAC halothane showed between individual neurons in our study. In our apparently homogenous population of inspiratory premotor neurons, baseline frequency (F_{con}) was strongly depressed in some neurons, while others seemed barely affected. It is possible that an anesthetic-induced change in postsynaptic neuron excitability could lead to a reduced response to glutamate agonists in the absence of a direct effect of the anesthetic on glutamate receptor function. Alternatively, the anesthetic may cause a direct depression of the NMDA and AMPA receptor function *per se*. These two issues cannot be resolved with our methodology.

Differential Anesthetic Effects on Inspiratory and Expiratory Neurons

In the *in vivo* network, inspiratory and expiratory premotor neurons form two functionally distinct, antagonistic groups. Since inspiratory premotor neurons of the cVRG directly activate phrenic motoneurons,[1] any anesthetic effect on premotor neurons will have a strong impact on the excitatory drive to the inspiratory musculature. We have previously shown that both inspiratory[24] and expiratory[7] premotor neurons can be excited by exogenous AMPA and NMDA application. However, in expiratory neurons, AMPA receptors are not endogenously activated under physiologic conditions.[7] This difference in endogenous activation of AMPA and NMDA receptors suggests that in expiratory neurons, AMPA and NMDA receptors may be localized in anatomically separate postsynaptic locations. For example, a distinct but overlapping distribution of AMPA and NMDA receptors

has been shown with immunolabeling in rat VRG neurons.[25]

Comparison of the current results with the effects of halothane in cVRG expiratory neurons suggests that the depression of the glutamate receptor response is unique for cVRG inspiratory neurons. One possible explanation is that the glutamate receptors on inspiratory and expiratory neurons belong to different subtypes with different sensitivities to halothane.[21] Another possibility is that anesthetic-induced changes are due to an indirect effect on neuronal excitability rather than on receptor function *per se*.

Halothane, 1 MAC, Does Not Change Overall Inhibitory Drive

In the current study, overall inhibition did not change from 0 to 1 MAC halothane. However, it is highly unlikely that in inspiratory premotor neurons, $GABA_A$ receptors were not affected by the anesthetic. Multiple studies consistently demonstrate an increase in $GABA_A$ receptor function with halothane.[5,26] We recently showed, for expiratory neurons *in vivo*, that 1 MAC halothane enhanced overall inhibition by 14.1 ± 17.9%,[5] which was due to a large 75% increase in $GABA_A$ receptor function, while presynaptic inhibitory drive appeared reduced.[5] A reduction in presynaptic inhibitory drive is consistent with data by Nishikawa and McIver[27] that showed in a rat hippocampal slice preparation that the inhibition of inhibitory interneurons was increased by 1 MAC halothane. We therefore hypothesize that in inspiratory neurons, $GABA_A$ receptor function is also enhanced by the anesthetic but that a concomitant depression of presynaptic inhibitory drive negates the increase in postsynaptic receptor function. As a result, overall inhibition, which can be understood as the product of presynaptic and postsynaptic inhibition,[5] did not change significantly.

Methodological Considerations

We have previously discussed the many advantages of a decerebrate preparation, including that the prevailing physiologic neurotransmitter levels remain similar to those in the intact animal and that single receptor function is not confounded by background anesthesia.[4] The importance of the latter cannot be overemphasized. Only recently, Hara and Harris[28] expressed various receptors on *Xenopus* oocytes and showed that the commonly used and supposedly innocuous background anesthetic urethane depressed AMPA and NMDA receptor currents and enhanced currents through $GABA_A$ and glycine receptors at a clinical dose (*i.e.*, ED_{50}).

Our preparation did not require a baseline anesthetic. Still, traces of the induction anesthetic isoflurane or the experimental anesthetic halothane may have been present in the brain tissue even though the end-expiratory gas analyzer did not detect them. While it is theo-

retically possible that residual subanesthetic concentrations of halothane may have contributed to the variability of the data, we can discern highly significant differences between the two anesthetic states, *e.g.*, for F_e and F_{con}. Also, there was no statistical difference between protocols that were randomized to start at 1 MAC and those that started at 0 MAC (data not shown), and in previous studies that required 0-to-1-to-0 MAC end control runs, the 0 MAC end control did not differ from the original 0 MAC run. We conclude that the equilibration time before the 0 MAC runs routinely reduced the anesthetic to a concentration at which it would have no more than a minimal effect.

In conclusion, 1 MAC halothane reduced the activity of inspiratory premotor neurons by a reduction of glutamatergic excitation. A significant part of his reduction appeared to be due to a depression of the postsynaptic receptor response to glutamate agonists. The overall GABAergic inhibitory drive to the neurons was not changed.

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