Anesthesiology 2008; 108:675–83

Isoflurane Differentially Modulates Inhibitory and Excitatory Synaptic Transmission to the Solitary Tract Nucleus

James H. Peters, Ph.D., Stuart J. McDougall, Ph.D.,† David Mendelowitz, Ph.D.,‡ Dennis R. Koop, Ph.D.,§ Michael C. Andresen, Ph.D.#*

Background: **Isoflurane anesthesia produces cardiovascular and respiratory depression, although the specific mechanisms are not fully understood. Cranial visceral afferents, which innervate the heart and lungs, synapse centrally onto neurons within the medial portion of the nucleus tractus solitarius (NTS). Isoflurane modulation of afferent to NTS synaptic communication may underlie compromised cardiorespiratory reflex function.**

Methods: **Adult rat hindbrain slice preparations containing the solitary tract (ST) and NTS were used. Shocks to ST afferents evoked excitatory postsynaptic currents with low-variability (SEM <200 μs) latencies identifying neurons as second order. ST-evoked and miniature excitatory postsynaptic currents as well as miniature inhibitory postsynaptic currents were measured during isoflurane exposure. Perfusion bath samples were taken in each experiment to measure isoflurane concentrations by gas chromatography–mass spectrometry.**

Results: **Isoflurane dose-dependently increased the decay-time constant of miniature inhibitory postsynaptic currents. At greater than 300 μM isoflurane, the amplitude of miniature inhibitory postsynaptic currents was decreased, but the frequency of events remained unaffected, whereas at equivalent isoflurane concentrations, the frequency of miniature excitatory postsynaptic currents was decreased. ST-evoked excitatory postsynaptic current amplitudes decreased without altering event kinetics. Isoflurane at greater than 300 μM increased the latency to onset and rate of synaptic failures of ST-evoked excitatory postsynaptic currents.**

Conclusions: **In second-order NTS neurons, isoflurane enhances phasic inhibitory transmission *via* postsynaptic γ-aminobutyric acid type A receptors while suppressing excitatory transmission through presynaptic mechanisms. These results suggest that isoflurane acts through multiple distinct mechanisms to inhibit neurotransmission within the NTS, which would underlie suppression of homeostatic reflexes.**

ISOFLURANE is a widely used general anesthetic that is well suited for rapid induction and recovery because of its pharmacokinetic properties and relative level of safety.[1] Nonetheless, depressed cardiovascular and respiratory function are commonly reported side effects.[2,3] These effects are thought to arise through isoflurane actions within the hindbrain; however, the specific sites and mechanisms of action are not well understood.[4] General anesthetic agents primarily target γ-aminobutyric acid type A ($GABA_A$) receptors and enhance inhibitory neurotransmission. However, anesthetics have additional effects on voltage-gated ion channels, enzymes, and other ligand-gated channels.[5] This biologic diversity of target proteins makes strict predictions of anesthetic sensitivity difficult, particularly across brain regions. The subunit composition of $GABA_A$ receptors is heteromeric, and these receptors are nonuniformly expressed across the brain and change developmentally.[6] Generally, certain brain regions are thought to be responsible for particular behavioral responses and anesthetic sensitivity.[7] The behavioral endpoints of amnesia, sedation, hypnosis, and immobility generally correlate across anesthetic agents with an enhancement of $GABA_A$ and glycine receptor function plus an inhibition of glutamate-mediated excitatory neurotransmission.[6]

The mechanisms through which isoflurane anesthesia alters homeostatic regulation, including the cardiovascular and respiratory systems,[8,9] have been less studied. In general, homeostatic regulation is controlled by visceral afferents of the IX and X cranial nerves that travel centrally *via* the solitary tract (ST) and release glutamate onto neurons within the nucleus tractus solitarius (NTS). In addition to the ST, NTS neurons receive numerous inputs from various regions of the brain, and a proportion of these additional inputs form γ-aminobutyric acid–mediated (GABAergic), inhibitory synapses onto NTS neurons.[10] GABAergic signaling within the NTS is important in the coordination of cardiovascular and respiratory reflex pathways.[11–13] Clinically, isoflurane depresses the arterial baroreceptor reflex, and such changes are thought to include actions within the NTS.[4,14] To what extent GABAergic and glutamatergic signaling within NTS neurons are sensitive to the effects of isoflurane is not known. Despite considerable work in expression cell systems and cellular work that concentrated on particular neonatal neuronal populations, our understanding of anesthetic actions in native, adult neurons remains far from comprehensive.

The current work focuses on isoflurane-induced alterations in GABAergic and glutamatergic synaptic transmission at the first central neurons in these homeostatic

* Postdoctoral Fellow, † C. J. Martin Fellow, # Professor, Department of Physiology and Pharmacology, § Professor, Department of Physiology and Pharmacology, BioAnalytical Shared Resource/Pharmacokinetics Core, Oregon Health and Science University. ‡ Professor, Department of Pharmacology and Physiology, George Washington University, Washington, D.C.

Received from the Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, Oregon. Submitted for publication September 7, 2007. Accepted for publication December 4, 2007. Supported by grant Nos. HL-58760 and HL-88894 from the National Institutes of Health, Bethesda, Maryland, and Overseas Training C. J. Martin Fellowship No. 400405 from the National Health and Medical Research Council of Australia, Canberra, Australia.

Address correspondence to Dr. Peters: Department of Physiology and Pharmacology L334, Oregon Health and Science University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97239-3098. petersj.ohsu@gmail.com.

pathways. In this study, we investigated the cellular actions of isoflurane on synaptically identified, second-order NTS neurons using patch clamp electrophysiology. Bath samples were taken simultaneously with recordings to determine precise isoflurane concentrations. The results suggest that, in addition to facilitating $GABA_A$ receptor function by prolonging the decay-time constant, isoflurane depressed excitatory transmission by presynaptic actions consistent with decreased glutamate release and slowed axonal conduction without affecting postsynaptic glutamate receptor function. Taken together, these studies demonstrate that isoflurane differentially modulates presynaptic and postsynaptic processes to depress the function of second-order NTS neurons.

Materials and Methods

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at Oregon Health and Science University, Portland, Oregon, and conform to the guidelines of the National Institutes of Health publication *Guide for the Care and Use of Laboratory Animals*.

Animals

In all experiments, adult male Sprague-Dawley rats (>160 g; Charles River Laboratories, Inc., Wilmington, MA) were used. Animals were housed under 12-h light/12-h dark conditions and were fed standard pellet chow *ad libitum*.

Slice Preparation

Brain stem slices were prepared from isoflurane anesthetized rats as previously described.[15] Briefly, the medulla was removed and placed in cooled artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM dextrose, and 2 mM $CaCl_2$, bubbled with 95% O_2–5% CO_2. The tissue was trimmed rostrally and caudally to yield a tissue block centered on obex. To orient the ST axons with the NTS in a common plane for cutting, the ventral surface of the brain stem block was cut. A single 250-μm-thick horizontal slice resulted that contained the ST together with the neuronal cell bodies of the medial NTS region. Slices were cut with a sapphire knife (Delaware Diamond Knives, Wilmington, DE) mounted in a vibrating microtome (VT1000S; Leica Microsystems Inc., Bannockburn, IL). Slices were secured with a fine polyethylene mesh in a custom perfusion chamber (Siskyou Design Instruments, Grants Pass, OR) and perfused with aCSF constantly bubbled with 95% O_2–5% CO_2 at 32°C and 300 mOsm.

Intracellular Recordings

The anatomical landmarks preserved in the horizontal slices allowed targeting of neurons within the medial subnucleus of the caudal NTS. Recorded neurons were located medial to the ST and within 200 μm rostral of obex. Patch electrodes were visually guided to neurons using infrared illumination and differential interference contrast optics (40× water immersion lens) on an Axioskop 2 microscope (Zeiss, Thornwood, NJ) with digital camera (Hamamatsu Photonic Systems, Bridgewater, NJ). Recording electrodes (3–4 MΩ) were filled with one of two intracellular solutions depending on the experimental protocol. For miniature inhibitory postsynaptic current (mIPSC) measurements, a high-Cl^- (54 mM, $E_{Cl} = -25$ mV) intracellular solution was used containing 10 mM NaCl, 40 mM KCl, 70 mM K-gluconate, 11 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM Na_2ATP, and 0.2 mM Na_2GTP. For stimulated excitatory postsynaptic current (EPSC) and for miniature excitatory postsynaptic current (mEPSC) measurements, a low-Cl^- (10 mM, $E_{Cl} = -69$ mV), intracellular solution contained 6 mM NaCl, 4 mM NaOH, 130 mM K-gluconate, 11 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 2 mM Na_2ATP, and 0.2 mM Na_2GTP. Intracellular solutions were pH 7.3 and 296 mOsm. All neurons were studied using voltage clamp conditions with an Axopatch 200A or MultiClamp 700B amplifier (Axon Instruments, Foster City, CA) and held at $V_H = -60$ mV using pipettes in open, whole cell patch configuration. Signals were filtered at 10 kHz and sampled at 30 kHz using p-Clamp software (version 8.2; Axon Instruments).

Synaptic Identification of Second-order Neurons

A concentric bipolar stimulating electrode (200-μm outer tip diameter; Frederick Haer Co., Bowdoinham, ME) was placed on distal portions of the visible ST rostral to the recording region. This remote placement of the stimulating electrode (1–3 mm) from recorded neurons minimized the likelihood of activation of non-ST axons or local neurons.[15] Consistent current shocks were delivered to ST every 3 s (shock duration 0.1 ms) using a Master-8 isolated programmable stimulator (A.M.P.I., Jerusalem, Israel). In some assays, responses to bursts of five shocks (20-ms intershock interval) assayed frequency-dependent amplitude depression and synaptic failures for ST-evoked EPSCs. Latency was measured as the time between the ST shock and the onset of the resulting EPSC.[15] Synaptic variability was calculated as the SEM of 30–40 ST-evoked EPSC latencies within each neuron. An SEM of less than 200 μs reliably identified direct, monosynaptic afferent contacts.[15]

Recording GABA and Glutamate Postsynaptic Currents

All drugs were administered through the aCSF bath perfusion. GABAergic neurotransmission was isolated by antagonizing ionotropic glutamate receptors (both *N*-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) with D-2-amino-5-phosphono-

valerate (AP-5, 100 μM) and 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX, 20 μM), respectively. Glutamatergic neurotransmission was isolated by administration of gabazine (SR95531, 3 μM) to block synaptic $GABA_A$ receptors. Nonspecific GABA and glycine receptor antagonists picrotoxin (100 μM; Sigma, St. Louis MO) and bicuculline (100 μM; Sigma) were administered to block all GABAergic receptor activity. Miniature events were defined by the presence of tetrodotoxin (3 μM). All drugs were purchased from Tocris (Ellisville, MO) unless otherwise noted. Isoflurane was purchased from Abbott Labs (N. Chicago, IL).

Isoflurane Sampling and Measurements

A stock of saturated isoflurane solution was produced by mixing equal volumes of isoflurane and aCSF in a sealed volumetric flask at room temperature. This saturated isoflurane stock measured approximately 12–14 mM and for experiments was then diluted to nominal final concentrations between 10 μM and 1 mM. For each exposure, isoflurane was perfused for 5 min at a flow rate of 1.5–2 ml/min. Bath samples (100 μl) were collected at four time points for each experiment; one baseline sample was taken 2 min before isoflurane, two samples were taken during isoflurane exposure (at 2 and 5 min), and one recovery sample was taken 2 min after return to bath solution. Slices were washed for 20 min after isoflurane exposure. All samples were extracted using glass Hamilton syringes and immediately added into glass vials, with heptane (500 μl) containing halothane as an internal standard, and capped. Through the use of sealed glass syringes and Teflon tubing (Small Parts Inc., Miami Lakes, FL), the perfusion system was optimized to prevent loss of isoflurane from the apparatus. Consistent amounts of isoflurane were delivered over time using this closed system such that only the bath was open to the atmosphere. Even so, the actual bath concentration of isoflurane varied from exposure to exposure, with the greatest variability occurring at the highest concentrations tested. As such, bath samples were taken for every isoflurane exposure to accurately attribute phenomena observed to the actual isoflurane concentration.

Gas chromatography–mass spectrometry was used to accurately measure isoflurane concentrations of samples taken from the bath. Analyses were based on Kharasch *et al.*[16] and were performed using a Finnigan TRACE DSQ GC/MS system (Thermo Electron Corporation, Austin, TX) configured with an AS 2000 autosampler and a split/splitless injector. Isoflurane and halothane were separated on an AT-5ms fused silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Alltech Associates, Deerfield, IL) using helium as the carrier gas at a constant flow rate of 1 ml/min. Samples (1 μl) were injected in the split mode with a split rate of either 1:100 or 1:200 depending on the concentration. The injector was maintained at 150°C, and the ion transfer line was maintained at 250°C. The Finnigan TRACE GC Ultra oven was held constant at 40°C for 3 min, and then the temperature increased at 40°C/min to 120°C. The mass spectrometer was operated in the electron impact mode with an ionization energy of 70 eV and a source temperature of 250°C. Data were acquired in the selected ion monitoring mode. The ions at m/z = 117 and 148 for isoflurane and m/z = 117 and 198 for halothane were used for quantitation. Typical retention times were 1.61 min for isoflurane and 1.93 min for halothane. Instrument control and data acquisition and analysis were accomplished using Xcalibur Version 1.4 software (Thermo Scientific, Waltham, MA). Samples were analyzed and area ratios were compared with a standard calibration curve prepared from known amounts of isoflurane. For isoflurane concentrations in the range of 100–1,000 μM, 500 μM halothane was used as the internal standard, and for concentrations in the range of 10–100 μM, 50 μM halothane was used.

Statistical Analysis

Experimentally one brain slice from each animal was obtained, and from each brain slice a single neuron was recorded. For statistical testing, each observation was considered independent and grouped into a fixed concentration. Concentration groups were compared using unpaired, non–repeated-measure assumptions. Digitized waveforms were analyzed using an event detection and analysis program (MiniAnalysis; Synaptosoft, Decatur, GA) for all miniature synaptic currents and Clampfit 10 (Axon Instruments) for all ST-stimulated currents. To obtain accurate decay-time constants, amplitude, and baseline values for miniature synaptic currents, those events smaller then 15 pA and those with multiple peaks were excluded. All events greater than 15 pA were counted for frequency values. Decay-time constants were acquired by fitting a single exponential between the 10% and 90% peak amplitude portion of the current decay. Isoflurane concentrations for each 5-min exposure were averaged across the two samples taken (2 and 5 min) and were fixed into concentration ranges accordingly. Statistical comparisons of waveform parameters (decay-time constant, amplitude, area, and baseline) were averaged across each concentration group and compared by a one-way analysis of variance each with Bonferroni *post hoc* testing (SigmaStat, San Jose, CA). Comparisons of interevent interval within individual neurons was nonparametric and used a Kolmogorov-Smirnov test. All group data are represented as the normalized mean ± SEM with $P < 0.05$ considered statistically significant.

Results

Identification of Second-order NTS Neurons

Our studies focused on NTS neurons from the medial subnucleus (fig. 1) because this region is a common site

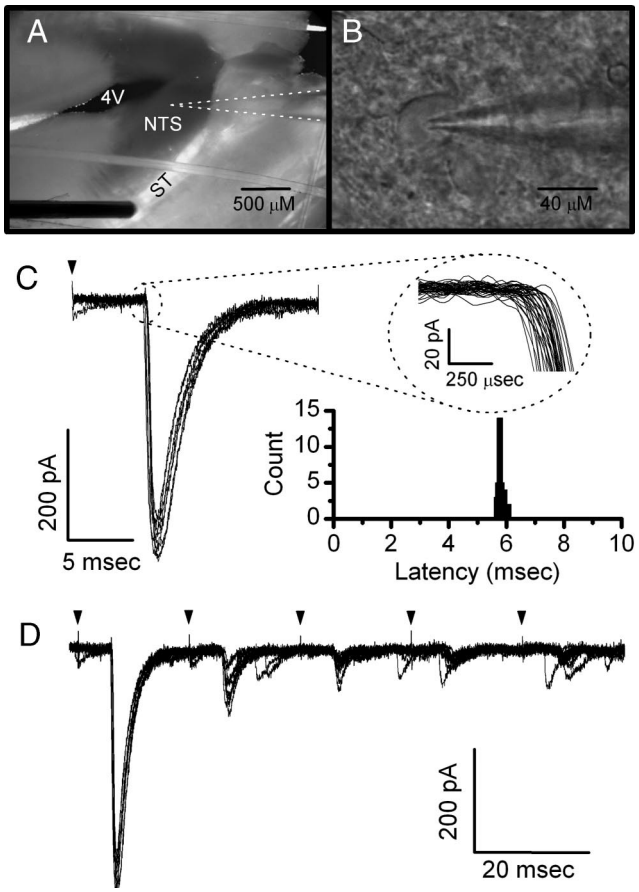

Fig. 1. Identification of a representative second-order nucleus tractus solitarius (NTS) neuron. Under bright-field illumination of the horizontal brain stem slice, the stimulating electrode was placed on the visible solitary tract (ST) at a position rostral some distance from the NTS (*A*). Using infrared illumination and differential interference contrast optics, individual neurons were located within the medial subregion and the patch electrode advanced to record from the NTS neuron (*B*). 4V = fourth ventricle. Shocks delivered to the ST evoked consistent excitatory postsynaptic currents across repeated trials (*inset*, shocks = 40) with a narrow distribution of latency values. The variability (SEM) of the latency to excitatory postsynaptic current onset was 120 μs (<200 μs) and judged to be the second order (*C*). The excitatory postsynaptic current amplitude depressed to a train of five shocks separated by 20 ms, and this frequency-dependent depression is characteristic of transmission directly from visceral afferent axons (*D*).

of cardiorespiratory afferent terminations.[11] The horizontal slice preparation retained key anatomical landmarks including the fourth ventricle and ST that guided recording electrodes to this medial region (fig. 1A) while infrared illumination and differential interference contrast optics outlined individual neurons for recording (fig. 1B). After stable intracellular recordings were established, we identified neurons that were directly contacted by primary visceral afferents by their characteristic responses to orthodromic ST activation. Electrical shocks remotely delivered to the ST recruited EPSCs that were nearly invariant in their latency, shape, and amplitude, and rarely failed (<0.1%) (fig. 1C). Latency values from repeated trials within such neurons produced narrow distributions whose synaptic variability (SEM of latency) was less than 200 μs and thus were considered to have direct ST afferent connections (second order). Peak amplitude averaged 247 ± 29 pA, and latency averaged 5.05 ± 0.45 ms (range, 2.67–7.29 ms) with an average SEM of 110 ± 20 μs in the 78 neurons included in these studies. As previously reported for second-order NTS neurons, stimulus trains of five ST shocks (20-ms interval) produced substantial frequency-dependent depression of EPSC amplitude (fig. 1D), and EPSCs were blocked by ionotropic glutamate receptor antagonists NBQX (20 μM) and AP-5 (100 μM) (data not shown). All NTS neurons included in the current study were initially characterized in this manner as second order by ST testing.

Isoflurane Enhances mIPSCs

To investigate the effects of isoflurane on phasic inhibitory events, tetrodotoxin and ionotropic glutamate receptor blockers were used to eliminate EPSCs, leaving mIPSCs isolated for study (n = 30 neurons, fig. 2A). Bath application of isoflurane prolonged the decay phase of mIPSCs without changes in amplitude or frequency (fig. 2A). Aggregate waveforms indicated prolongation of the decay phase beginning at 118 ± 8 μM isoflurane, and at very high concentrations (836 ± 80 μm), isoflurane depressed the amplitude as well as prolonged the decay phase (fig. 2B). On average, the mIPSC decay-time constant and total integrated area of the mIPSCs increased in a concentration-dependent manner (fig. 2C, top and bottom panels, n = 3–6 neurons/concentration, $P < 0.001$ and $P < 0.05$, respectively). In the most sensitive neurons, 50 μM isoflurane shifted the mIPSC time constant. The mIPSC amplitude (fig. 2C, middle panel) decreased significantly at nominally 1,000 μM isoflurane (control: 36 ± 8 pA *vs.* isoflurane: 28 ± 7 pA, n = 6 neurons, $P < 0.05$). Isoflurane did not to alter the frequency of mIPSCs (figs. 3A and B) at any concentration tested (n = 3–6 neurons/concentration, $P > 0.05$) (fig. 3C). In these adult animals, all mIPSCs were blocked by gabazine (3 μM), suggesting that glycinergic events are absent in adult medial NTS neurons (not shown). Holding currents were uniformly small and unaltered by isoflurane despite substantial Cl^- driving force ($E_{Cl} = -25$ mV; 6 ± 4, 4 ± 3, 5 ± 4, and 6 ± 1 pA observed in 50 ± 5, 118 ± 8, 310 ± 39, and 782 ± 83 μM isoflurane, respectively). These findings suggest that isoflurane acts postsynaptically to enhance inhibitory transmission chiefly by slowing the kinetics of phasic, $GABA_A$-mediated events with no discernible presynaptic actions on GABA release or postsynaptic actions on "extrasynaptic" tonic $GABA_A$ receptors.

Isoflurane Inhibits ST-evoked EPSCs

In second-order NTS neurons (n = 21 neurons), ST shocks activate large EPSCs with consistent amplitudes over time (fig. 4A). Isoflurane rapidly and reversibly

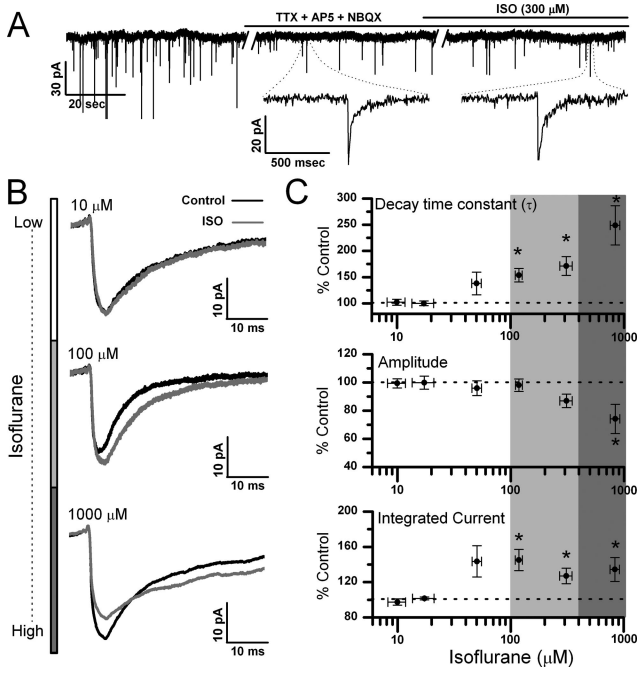

Fig. 2. Isoflurane enhances miniature inhibitory postsynaptic currents in second-order nucleus tractus solitarius neurons. Neurons were recorded under voltage clamp conditions at −60 mV. Miniature inhibitory postsynaptic currents were isolated pharmacologically using tetrodotoxin (TTX, 3 μM), 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX, 20 μM), and D(−)-2-amino-5-phosphonopentanoic acid (AP-5, 100 μM) (*A*, *left and center panels*). Neurons were then exposed to isoflurane (ISO) for 5 min (*A*, *right panel*). Events were analyzed for their decay-time constant (τ), amplitude, and integrated area. Averaged traces (>100 events) of miniature inhibitory postsynaptic current events under control conditions and in the presence of 10, 100, and 1,000 μM isoflurane from representative neurons are shown (*B*). Isoflurane dose-dependently increased the decay-time constant and integrated area of miniature inhibitory postsynaptic currents (*C*, *upper and lower panels*). Amplitude remained unchanged until the highest concentration of isoflurane (800 μM) was tested and was significantly decreased compared with control (*C*, *middle panel*). Data are shown as mean ± SEM. For all comparisons, n = 3–6 neurons/concentration, with * $P < 0.05$ *versus* control. The *grayscale panels* in *B* and *C* indicate equivalent subhypnotic (*white*), hypnotic (*light gray*), and supratherapeutic (*dark gray*) concentrations of isoflurane.

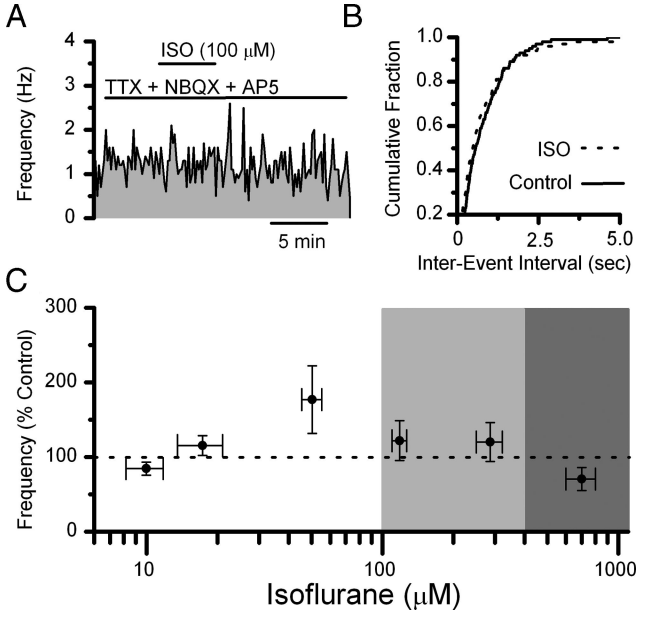

Fig. 3. Isoflurane does not change miniature inhibitory postsynaptic current frequency. Frequency data were grouped into 10-s bins and plotted over time (*A*). The interevent interval cumulative fraction curve was not changed by isoflurane (ISO) compared with control ($P > 0.05$) (*B*). Across concentrations, isoflurane did not significantly change the frequency of miniature inhibitory postsynaptic currents (n = 3–6 neurons/concentration, $P > 0.05$) (*C*). Data are shown as mean ± SEM. The *grayscale panels* in *C* indicate equivalent subhypnotic (*white*), hypnotic (*light gray*), and supratherapeutic (*dark gray*) concentrations of isoflurane. AP-5 = D(−)-2-amino-5-phosphonopentanoic acid; NBQX = 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione; TTX = tetrodotoxin.

reduced ST-evoked EPSC amplitudes and, at high concentrations, produced synaptic failures (zero amplitude) (fig. 4A). Note that full GABAergic and glycinergic blockade had no effect on ST-EPSC amplitude. On average, ST-evoked EPSC amplitude and integrated current were significantly depressed by 244 ± 35 μM isoflurane (fig. 4B and C, middle panel, n = 3–8 neurons/concentration, $P < 0.05$). Isoflurane had no effect on ST-evoked EPSC kinetics, and the decay-time constant remained unchanged at all isoflurane concentrations tested (fig. 4C, top panel, n = 3–8 neurons/concentration, $P > 0.05$). Isoflurane reversibly increased ST-evoked EPSC latency in 6 of 10 neurons tested with greater than 300 μM isoflurane (fig. 5A), indicating decreased ST axonal conduction velocity. The average change in latency was 550 ± 60 μs (n = 6 neurons), represented by a rightward shift in the latency distribution as well as a flattening of the distribution (fig. 5B). In half (5 of 10) of the neurons tested, the higher isoflurane concentrations (>300 μM) induced complete ST-evoked EPSC failures (fig. 5A). Therefore, isoflurane decreases ST-evoked EPSC amplitude and alters conduction properties of ST afferent axons.

Isoflurane Inhibits mEPSCs

To test for presynaptic and postsynaptic actions on glutamatergic transmission, mEPSCs were isolated in second-order NTS neurons (n = 27 neurons) using tetrodotoxin and combined GABA/glycine receptor antagonism. Isoflurane rapidly decreased the frequency of mEPSCs (figs. 6A and B) and shifted the frequency distribution to higher interevent intervals (fig. 6C). On average (n = 5–6 neurons/concentration, $P < 0.001$), isoflurane decreased the frequency of mEPSC events significantly from approximately 150 μM isoflurane and higher, with maximal suppression occurring at 687 μM (n = 5–6 neurons/concentration, $P < 0.05$ *vs.* control, fig. 6D). The average frequency of mEPSCs under control conditions was 11.01 ± 3.01 Hz, where 700 μM isoflurane reduced the rate by 35% to 7.46 ± 2.51 Hz. This effect of isoflurane was reversed during wash with aCSF (fig. 6B). In contrast to mIPSCs, the kinetic properties of mEPSCs were unchanged in isoflurane (fig. 7). Neither the decay-

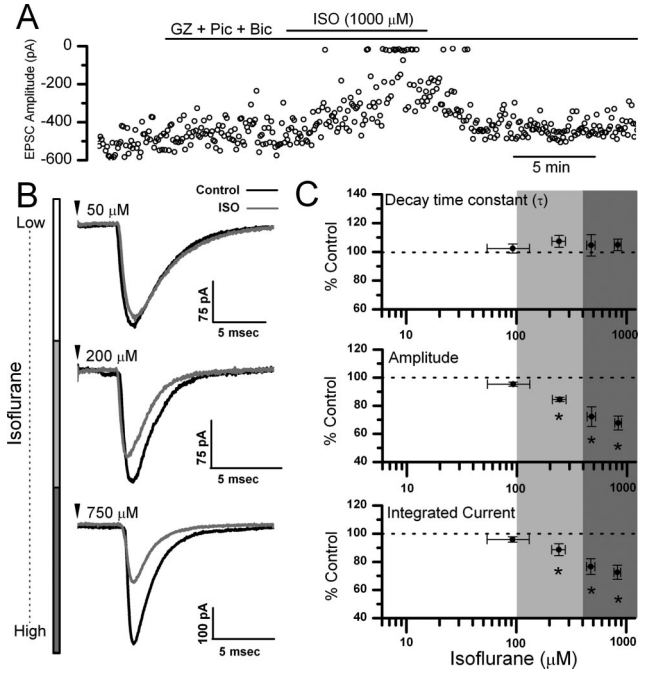

Fig. 4. Isoflurane dose-dependently inhibits solitary tract–evoked excitatory postsynaptic currents (EPSCs). EPSC amplitude is plotted over time under control conditions and in the presence of isoflurane (ISO, 1,000 μM). The data points at 0 pA are a result of synaptic failures. EPSCs were measured every 3 s in the presence of gabazine (GZ, 3 μM), picrotoxin (Pic, 100 μM), and bicuculline (Bic, 100 μM) (*A*). Averaged EPSC traces (50 sweeps) are shown from representative neurons under control conditions (*black*) and in the presence of isoflurane at 50, 200, and 750 μM (*gray*) (*B*). The *black arrows* indicate the time of solitary tract stimulation. Isoflurane dose-dependently decreased the amplitude and integrated area of EPSCs (*C*, *middle and lower panels*) while having no significant effect on the decay-time constant (*C*, *upper panel*). Data are shown as mean ± SEM. For all comparisons, n = 3–8 neurons/concentration, with * $P < 0.05$ *versus* control. The *gray-scale panels* in *B* and *C* indicate equivalent subhypnotic (*white*), hypnotic (*light gray*), and supratherapeutic (*dark gray*) concentrations of isoflurane.

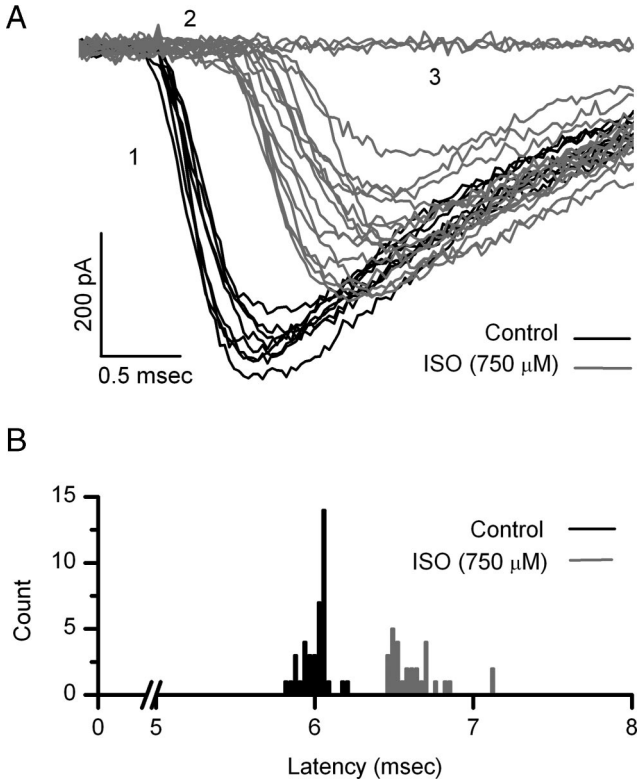

Fig. 5. Isoflurane increases the latency and variability of solitary tract–evoked excitatory postsynaptic currents (EPSCs). Raw current traces of EPSCs from one neuron under control conditions (*black*, *A1*) and in the presence of isoflurane (*gray*, *A2*). Solitary tract–evoked EPSCs were measured every 3 s. Isoflurane (ISO) produced an increased latency between the solitary tract shock and EPSC onset in 6 of 10 neurons tested (*A2*). The average change in latency was 550 ± 60 μs (n = 6 neurons). In half (5 of 10) of the neurons tested at the highest concentrations (>300 μM), at least one complete EPSC failure was also observed (*gray*, *A3*). (*B*) A corresponding frequency histogram showing the change in latency distribution under control conditions (*black*) and in the presence of isoflurane (*gray*) as represented in *A*.

time constant nor the amplitude of mEPSCs changed at any concentration of isoflurane tested (n = 5–6 neurons/concentration, $P > 0.05$, figs. 7B and C). Miniature EPSC events were blocked by ionotropic glutamate receptor antagonism (data not shown). Together, the mEPSC and ST-evoked EPSC observations suggest selective presynaptic actions of isoflurane on glutamate release, and indicate differences between the presynaptic release process for glutamate compared with GABA.

Discussion

The current studies focused on neurons within the medial subregion of the NTS, which is known to receive direct cardiorespiratory afferent innervation,[12,17] with the goal of assessing the integrated actions of isoflurane on GABA- and glutamatergic synaptic transmission. This *in vitro* slice preparation and electrophysiologic approach allow for the determination of both presynaptic and postsynaptic mechanisms of isoflurane to modulate the ST–NTS synapse in these autonomic reflex circuits of adult animals.[18,19] Isoflurane dose-dependently enhanced GABA-mediated inhibitory synaptic events while depressing glutamate-mediated excitatory neurotransmission to these NTS neurons. Our simultaneous sampling of the perfusion bath matched isoflurane concentrations to the neuronal effects. Significant IPSC enhancement occurred by 118 μM, whereas EPSC transmission depressed beginning at 166 μM or greater. The threshold concentrations for these effects were less than 1 minimum alveolar concentration for the rat (approximately 310 μM).[20] The changes in decay kinetics of mIPSCs and lack of an effect on event frequency support postsynaptic sites of isoflurane action at $GABA_A$ receptors. In contrast, decreased mEPSC frequency combined with no change in event kinetics indicates that glutamatergic transmission was depressed by presynaptic processes that included decreased synaptic glutamate release, independent of action potentials. Further, increased latency of

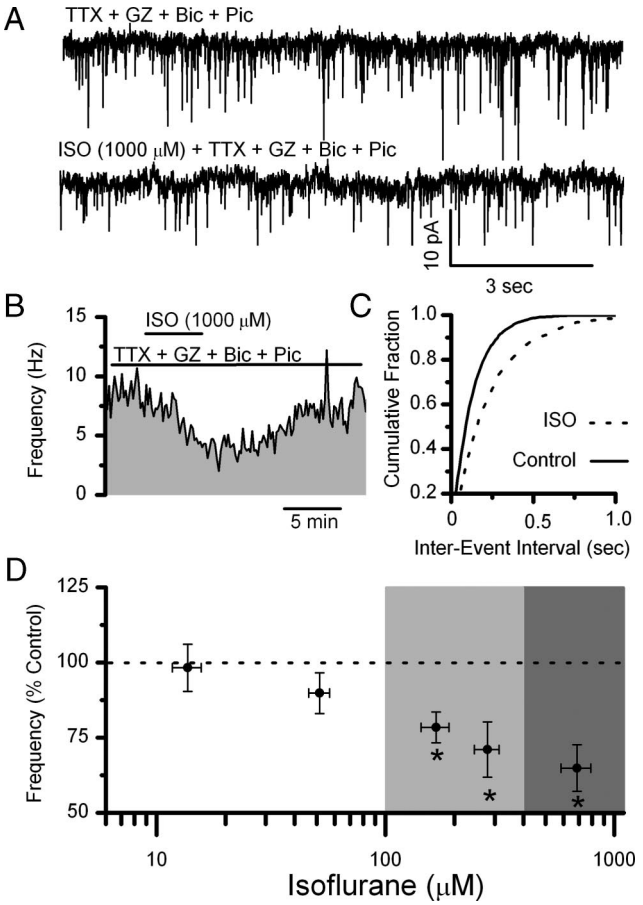

Fig. 6. Isoflurane dose-dependently decreases miniature excitatory postsynaptic current frequency. Raw current traces of miniature excitatory postsynaptic current events under control conditions (tetrodotoxin [TTX], 3 μM; gabazine [GZ], 3 μM; picrotoxin [Pic], 100 μM; and bicuculline [Bic], 100 μM) and during isoflurane (ISO, 1,000 μM) exposure (*A*). Frequency data were grouped into 10-s bins and expressed over time (*B*). The interevent interval cumulative fraction curve was shifted to the right in the presence of isoflurane ($P < 0.05$) (*C*). Data normalized to control conditions are expressed as mean ± SEM (*D*). For all comparisons, n = 5–6 neurons/concentration, with $^{*}P < 0.05$ *versus* control. The *grayscale panels* in *D* indicate equivalent subhypnotic (*white*), hypnotic (*light gray*), and supratherapeutic (*dark gray*) concentrations of isoflurane.

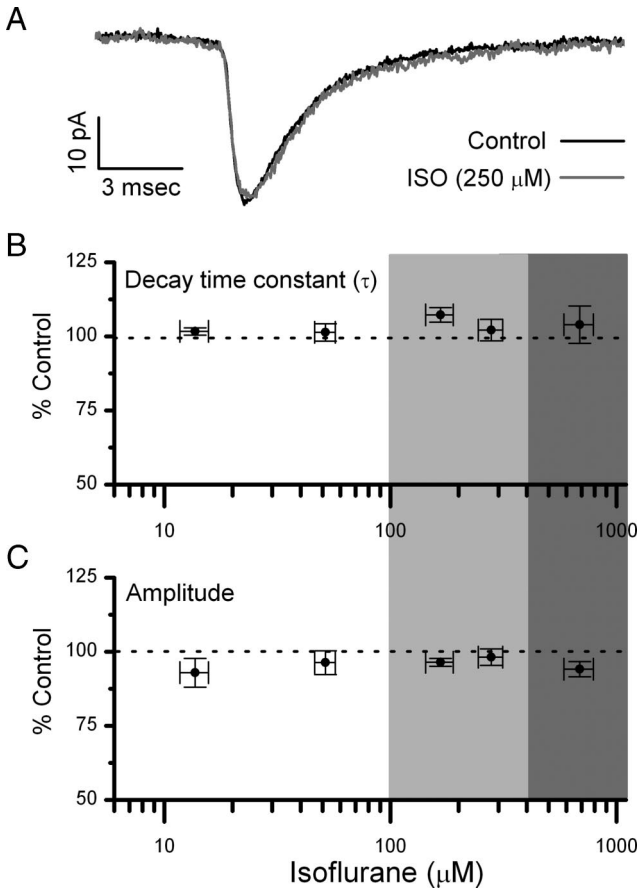

Fig. 7. Isoflurane does not change miniature excitatory postsynaptic current kinetics. Averaged traces of miniature excitatory postsynaptic current events (>100 events) under control conditions (tetrodotoxin, 3 μM; gabazine, 3 μM; picrotoxin, 100 μM; and bicuculline, 100 μM) and in the presence of isoflurane (*A*). Isoflurane (ISO) produced no significant change in either the decay-time constant or amplitude of miniature excitatory postsynaptic currents at any concentration tested (n = 5–6 neurons/concentration; $P > 0.05$); normalized data are expressed as mean ± SEM (*B* and *C*). The *grayscale panels* in *B* and *C* indicate equivalent subhypnotic (*white*), hypnotic (*light gray*), and supratherapeutic (*dark gray*) concentrations of isoflurane.

ST-evoked EPSCs and event failures suggest disrupted axonal conduction likely *via* voltage-dependent ion channels along ST afferents.[5,21] Taken together, our results indicate that isoflurane attenuates synaptic neurotransmission within the NTS *via* multiple distinct mechanisms that are within the clinically relevant concentration range.

Isoflurane Enhances Phasic GABAergic Transmission in NTS through Postsynaptic Mechanisms

As commonly reported for general anesthetic actions at $GABA_A$ receptors, we found that isoflurane significantly increased the total integrated current of phasic IPSC events through a prolongation of the event decay-time constant. This effect on the IPSC decay rate was relatively selective, considering that isoflurane did not significantly alter event amplitudes or frequency at moderate concentrations. Although somewhat variable across individual neurons, 118 μM isoflurane increased the decay-time constant of these phasic events, on average, by more than 50% and in a concentration-dependent manner. The sensitivity of mIPSCs in NTS neurons was greater than in hippocampal neurons, which show significant changes in mIPSC kinetics at 300 μM.[22] At higher isoflurane concentrations (>300 μM), the decay-time constant increased dose dependently. In contrast, the relative increase in integrated current was maximal at 118 μM isoflurane and did not increment with further increases in concentration. This difference may result from the concomitant decrease in event amplitude at high concentrations. Banks *et al.* and others report similar findings in other neurons and implicate a direct $GABA_A$ channel block.[22–24]

In contrast to the changes in kinetic parameters, isoflurane did not change the mIPSC frequency; findings consistent with selective postsynaptic actions at $GABA_A$ receptors on NTS neurons. Direct measurements of transmitter release from isolated cortical synaptosomes indicate that high concentrations of isoflurane (IC_{50} = 1,000 μM) can decrease GABA release but to a lesser extent than glutamate release.[25–27] Interestingly, our data in NTS neurons found that isoflurane selectively decreased glutamate release (see discussion below) but not GABA release. In contrast to results from neurons in the hippocampus,[28] isoflurane did not alter the holding current or activate a tonic, gabazine-sensitive current even at high concentrations. We have observed the activation of tonic $GABA_A$ currents in similar second-order NTS neurons by the intravenous anesthetic propofol.[29] This differential action of isoflurane on phasic and tonic $GABA_A$ mechanisms may reflect the particular heterogeneous expression and assembly of native GABA receptor complexes across this region. Although speculative at this time, an interesting possibility in NTS might be ρ_1 $GABA_A$ subunits, which are expressed in NTS[30] and may be resistant to isoflurane.[31]

Isoflurane Inhibits Glutamatergic Afferent Transmission through Presynaptic Mechanisms

Electrical stimulation of the ST produces large-amplitude, low-variability EPSCs which reflect visceral afferent to NTS neuronal communication.[18] Non–*N*-methyl-D-aspartate receptors mediate visceral afferent synaptic transmission in NTS.[32,33] We found that isoflurane dose-dependently decreased the amplitude and integrated current of ST-evoked EPSCs. These decreases began at 280 μM, and by 837 μM isoflurane, EPSC amplitude and integrated current were reduced by 33%—similar to findings in other neurons.[34–36] Isoflurane acted rapidly and reversed after wash. Postsynaptic modulation of glutamate receptors and/or presynaptic inhibition of vesicular glutamate release may contribute to attenuation of EPSC signaling. In NTS neurons, postsynaptic glutamate receptor kinetics were remarkably isoflurane resistant, consistent with work on expressed glutamate receptors in which isoflurane even at 1,200 μM did not alter α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor responses.[37] Considering that isoflurane decreased mEPSC frequency beginning at 166 μM and had no affect on the amplitude or kinetic parameters at any concentration tested, we conclude that inhibition of glutamatergic transmission by isoflurane resulted from presynaptic decreases in glutamate release.

Attenuation of glutamate release from presynaptic terminals may result from modulation of the synaptic bouton or altering the propagating action potential. Even when action potentials were blocked by tetrodotoxin, isoflurane still decreased the frequency of mEPSCs. This is consistent with isoflurane acting at the synaptic bouton to disrupt constitutive vesicular docking and release independent of action potentials. At calyx of Held synapses, isoflurane inhibited both the synaptic vesicular release and the propagating action potential.[36] We observed both increases in the latency to onset and failures of ST-evoked EPSCs in a majority of neurons at high concentrations of isoflurane (>300 μM). Such observations are consistent with conduction delays and block, respectively, in the ST axons leading to synaptic terminals. Such delayed arrival and/or blockade of transmission along ST afferents can occur independently of changes in the probability of glutamate release, a property of the synaptic terminals themselves.[38] Afferent axonal conduction is a voltage-dependent process that relies strongly on fast sodium and potassium channels, both of which have unique expression patterns that differ between peripheral cranial afferent neurons and central NTS neurons.[39–43] The effects of isoflurane on action potential propagation are likely a result of isoflurane interactions with these voltage-gated ion channels.[5,21] Taking these findings together, we conclude that isoflurane inhibits glutamate release both at the synaptic bouton and by attenuating action potential propagation.

Conclusions

In conclusion, isoflurane, at clinically relevant concentrations, enhanced GABA-mediated inhibitory currents and suppressed glutamate-mediated excitatory currents on NTS neurons receiving direct visceral afferent innervation. These isoflurane actions occurred *via* distinct presynaptic and postsynaptic mechanisms. These differential effects of isoflurane within the NTS may underlie depressed cardiorespiratory function.

References

1. Eger EI: Isoflurane: A review. ANESTHESIOLOGY 1981; 55:559–76
2. Eger EI: The pharmacology of isoflurane. Br J Anaesth 1984; 56 (suppl 1):71S–99S
3. Seagard JL, Elegbe E, Hopp FA, Bosnjak ZJ, von Colditz J, Kalbfleisch J, Kampine J: Effects of isoflurane on the baroreceptor reflex. ANESTHESIOLOGY 1983; 59:511–20
4. Lee JS, Andresen MC, Morrow D, Chang KSK: Isoflurane depresses baroreflex control of heart rate in decerebrate rats. ANESTHESIOLOGY 2002; 96:1214–22
5. Franks NP, Lieb WR: Molecular and cellular mechanisms of general anaesthesia. Nature 1994; 367:607–14
6. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci 2004; 5:709–20
7. Urban BW, Bleckwenn M, Barann M: Interactions of anesthetics with their targets: Non-specific, specific or both? Pharmacol Ther 2006; 111:729–70
8. Benumof JL: Respiratory physiology and respiratory function during anesthesia, Anesthesia, 5th edition. Edited by Miller RD. Philadelphia, Churchill Livingstone, 2000, pp 578–618
9. Pagel PS, Farber NE, Warltier DC: Cardiovascular pharmacology, Anesthesia, 5th edition. Edited by Miller RD. Philadelphia, Churchill Livingstone, 2000, pp 96–124
10. Jordan D, Mifflin SW, Spyer KM: Hypothalamic inhibition of neurones in the nucleus tractus solitarius of the cat is GABA mediated. J Physiol 1988; 399:389–404

11. Andresen MC, Kunze DL: Nucleus tractus solitarius: Gateway to neural circulatory control. Annu Rev Physiol 1994; 56:93–116

12. Kubin L, Alheid GF, Zuperku EJ, McCrimmon DR: Central pathways of pulmonary and lower airway vagal afferents. J Appl Physiol 2006; 101:618–27

13. Kubo T, Kihara M: Evidence for gamma-aminobutyric acid receptor-mediated modulation of the aortic baroreceptor reflex in the nucleus tractus solitarii of the rat. Neurosci Lett 1988; 89:156–60

14. Muzi M, Ebert TJ: A comparison of baroreflex sensitivity during isoflurane and desflurane anesthesia in humans. ANESTHESIOLOGY 1995; 82:919–25

15. Doyle MW, Andresen MC: Reliability of monosynaptic transmission in brain stem neurons *in vitro*. J Neurophysiol 2001; 85:2213–23

16. Kharasch ED, Thummel KE, Mautz D, Bosse S: Clinical enflurane metabolism by cytochrome P450 2E1. Clin Pharmacol Ther 1994; 55:434–40

17. Andresen MC, Doyle MW, Jin Y-H, Bailey TW: Cellular mechanisms of baroreceptor integration at the nucleus tractus solitarius. Ann N Y Acad Sci 2001; 940:132–41

18. Andresen MC, Doyle MW, Bailey TW, Jin Y-H: Differentiation of autonomic reflex control begins with cellular mechanisms at the first synapse within the nucleus tractus solitarius. Braz J Med Biol Res 2004; 37:549–58

19. Doyle MW, Bailey TW, Jin Y-H, Appleyard SM, Low MJ, Andresen MC: Strategies for cellular identification in nucleus tractus solitarius slices. J Neurosci Methods 2004; 37:37–48

20. Franks NP, Lieb WR: Temperature dependence of the potency of volatile general anesthetics: Implications for *in vitro* experiments. ANESTHESIOLOGY 1996; 84:716–20

21. Ouyang W, Wang G, Hemmings HC Jr: Isoflurane and propofol inhibit voltage-gated sodium channels in isolated rat neurohypophysial nerve terminals. Mol Pharmacol 2003; 64:373–81

22. Banks MI, Pearce RA: Dual actions of volatile anesthetics on $GABA_A$ IPSCs: Dissociation of blocking and prolonging effects. ANESTHESIOLOGY 1999; 90:120–34

23. Hapfelmeier G, Haseneder R, Eder M, Adelsberger H, Kochs E, Rammes G, Zieglgansberger W: Isoflurane slows inactivation kinetics of rat recombinant $\alpha1\beta2\gamma2$L GABA(A) receptors: Enhancement of GABAergic transmission despite an open-channel block. Neurosci Lett 2001; 307:97–100

24. Verbny YI, Merriam EB, Banks MI: Modulation of γ-aminobutyric acid type A receptor–mediated spontaneous inhibitory postsynaptic currents in auditory cortex by midazolam and isoflurane. ANESTHESIOLOGY 2005; 102:962–9

25. Westphalen RI, Hemmings HC Jr: Selective depression by general anesthetics of glutamate *versus* GABA release from isolated cortical nerve terminals. J Pharmacol Exp Ther 2003; 304:1188–96

26. Westphalen RI, Hemmings HC Jr: Volatile anesthetic effects on glutamate *versus* GABA release from isolated rat cortical nerve terminals: Basal release. J Pharmacol Exp Ther 2006; 316:208–15

27. Westphalen RI, Hemmings HC Jr: Volatile anesthetic effects on glutamate *versus* GABA release from isolated rat cortical nerve terminals: 4-Aminopyridine-evoked release. J Pharmacol Exp Ther 2006; 316:216–23

28. Caraiscos VB, Newell JG, You T, Elliott EM, Rosahl TW, Wafford KA, MacDonald JF, Orser BA: Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. J Neurosci 2004; 24:8454–8

29. McDougall SJ, Bailey TW, Mendelowitz D, Andresen MC: Propofol enhances both phasic and tonic GABAergic transmission at second-order neurons within the cardiorespiratory region of the solitary tract nucleus (NTS). Soc Neurosci Abstr 2007; 196.7

30. Milligan CJ, Buckley NJ, Garret M, Deuchars J, Deuchars SA: Evidence for inhibition mediated by coassembly of GABAA and GABAC receptor subunits in native central neurons. J Neurosci 2004; 24:9241–50

31. Harrison NL, Kugler JL, Jones MV, Greenblatt EP, Pritchett DB: Positive modulation of human gamma-aminobutyric acid type A and glycine receptors by the inhalation anesthetic isoflurane. Mol Pharmacol 1993; 44:628–32

32. Andresen MC, Yang M: Non-NMDA receptors mediate sensory afferent synaptic transmission in medial nucleus tractus solitarius. Am J Physiol 1990; 259:H1307–11

33. Jin Y-H, Bailey TW, Doyle MW, Li BY, Chang KSK, Schild JH, Mendelowitz D, Andresen MC: Ketamine differentially blocks sensory afferent synaptic transmission in medial nucleus tractus solitarius (mNTS). ANESTHESIOLOGY 2003; 98: 121–32

34. MacIver MB, Mikulec AA, Amagasu SM, Monroe FA: Volatile anesthetics depress glutamate transmission *via* presynaptic actions. ANESTHESIOLOGY 1996; 85:823–34

35. Ranft A, Kurz J, Deuringer M, Haseneder R, Dodt HU, Zieglgansberger W, Kochs E, Eder M, Hapfelmeier G: Isoflurane modulates glutamatergic and GABAergic neurotransmission in the amygdala. Eur J Neurosci 2004; 20:1276–80

36. Wu XS, Sun JY, Evers AS, Crowder M, Wu LG: Isoflurane inhibits transmitter release and the presynaptic action potential. ANESTHESIOLOGY 2004; 100: 663–70

37. Plested AJ, Wildman SS, Lieb WR, Franks NP: Determinants of the sensitivity of AMPA receptors to xenon. ANESTHESIOLOGY 2004; 100:347–58

38. Bailey TW, Jin Y-H, Doyle MW, Smith SM, Andresen MC: Vasopressin inhibits glutamate release *via* two distinct modes in the brainstem. J Neurosci 2006; 26:6131–42

39. Glazebrook PA, Ramirez AN, Schild JH, Shieh CC, Doan TN, Wible BA, Kunze DL: Potassium channels Kv1.1, Kv1.2 and Kv1.6 influence excitability of rat visceral sensory neurons. J Physiol 2002; 541:467–82

40. Schild JH, Khushalani S, Clark JW, Andresen MC, Kunze DL, Yang M: An ionic current model for neurons in the rat medial nucleus tractus solitarius receiving sensory afferent input. J Physiol 1993; 469:341–63

41. Schild JH, Clark JW, Hay M, Mendelowitz D, Andresen MC, Kunze DL: A- and C-type nodose sensory neurons: Model interpretations of dynamic discharge characteristics. J Neurophysiol 1994; 71:2338–58

42. Schild JH, Clark JW, Canavier CC, Kunze DL, Andresen MC: Afferent synaptic drive of rat medial nucleus tractus solitarius neurons: Dynamic simulation of graded vesicular mobilization, release, and non-NMDA receptor kinetics. J Neurophysiol 1995; 74:1529–48

43. Schild JH, Kunze DL: Experimental and modeling study of Na^+ current heterogeneity in rat nodose neurons and its impact on neuronal discharge. J Neurophysiol 1997; 78:3198–209