

Ketamine Inhibits Inspiratory-evoked γ -Aminobutyric Acid and Glycine Neurotransmission to Cardiac Vagal Neurons in the Nucleus Ambiguus

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Background: Ketamine can be used for perioperative pain management as well as a dissociative anesthetic agent in emergency situations. However, ketamine can induce both cardiovascular and respiratory depression, especially in pediatric patients. Although ketamine has usually been regarded as sympathoexcitatory, recent work has demonstrated that ketamine has important actions on parasympathetic cardiac vagal efferent activity. The current study tests the hypothesis that ketamine, at clinical relevant concentrations, alters central cardiorespiratory interactions in the brainstem and, in particular, the inspiration-evoked increase in γ -aminobutyric acid-mediated and glycinergic neurotransmission to parasympathetic cardiac efferent neurons.

Methods: Cardiac vagal neurons were identified by the presence of a retrograde fluorescent tracer. Respiratory evoked γ -aminobutyric acid-mediated and glycinergic synaptic currents were recorded in cardiac vagal neurons using whole cell patch clamp techniques while spontaneous rhythmic respiratory activity was recorded simultaneously.

Results: Ketamine, at concentrations from 0.1 to 10 μ M, evoked a concentration-dependent inhibition of inspiratory burst frequency. Inspiration-evoked γ -aminobutyric acid-mediated neurotransmission to cardiac vagal neurons was inhibited at ketamine concentrations of 0.5 and 1 μ M. The increase in glycinergic activity to cardiac vagal neurons during inspiration was also inhibited at ketamine concentrations of 0.5 and 1 μ M.

Conclusions: At clinically relevant concentrations (0.5 and 1 μ M), ketamine alters central respiratory activity and diminishes both inspiration-evoked γ -aminobutyric acid-mediated and glycinergic neurotransmission to parasympathetic cardiac efferent neurons. This reduction in inhibitory neurotransmission to cardiac vagal neurons is likely responsible for the compromised respiratory sinus arrhythmia that occurs with ketamine anesthesia.

HEART rate is primarily determined by the activity of brainstem preganglionic cardioinhibitory vagal neurons (CVNs) in the nucleus ambiguus.¹⁻⁴ CVNs are intrinsically silent and thus synaptic inputs dictate their activity.⁵ For example, with each respiratory cycle, heart rate increases during inspiration, termed *respiratory sinus*

arrhythmia, and this cardiorespiratory interaction is primarily mediated by inspiration evoked increases in γ -aminobutyric acid-mediated (GABAergic) and glycinergic inhibitory neurotransmission to CVNs.⁶ Respiratory sinus arrhythmia helps to match pulmonary blood flow to lung inflation and maintain the appropriate diffusion gradient for oxygen in the lungs in spontaneously breathing individuals.⁷ However, cardiovascular reflexes and respiratory sinus arrhythmia can be attenuated or abolished with anesthetics, including ketamine.⁸⁻¹⁰

Ketamine [2-(*O*-chlorophenyl)-2-(methylamino)-cyclohexanone hydrochloride; Ketaject (Phoenix Pharmaceuticals, St. Joseph, MO)] can be used for dissociative anesthetic agent in emergency situations, as well as in perioperative pain management.¹¹ Interestingly, the effects of ketamine may interact with the opioid system. Ketamine causes a dose-dependent depression of respiration, and this depression is greater in wild-type compared with μ -opioid receptor knockout animals, suggesting that ketamine may act, at least partly, *via* interaction with μ -opioid receptors.¹² Ketamine also alters the cardiovascular system, typically eliciting an "excitation" consisting of increases in blood pressure and heart rate.¹³ This cardiovascular effect is usually regarded as sympathoexcitatory in origin.¹⁴ However, in a study examining the effects of anesthetics on the cardiac sympathetic and parasympathetic control of heart rate, the β_1 blocker propranolol did not prevent the ketamine-induced increase in heart rate,¹⁵ whereas atropine, a muscarinic antagonist that blocks parasympathetic cardiac efferent activity, inhibited the ketamine-evoked tachycardia.¹⁶ Both *in vivo* and *in vitro* studies demonstrate that parasympathetic cardiac vagal efferent activity is inhibited by ketamine.^{17,18}

Although it is well known that ketamine directly blocks *N*-methyl-D-aspartate (NMDA) receptors, ketamine may also affect other neurotransmitters and receptors, including inhibitory neurotransmission. The current study tests the hypothesis that ketamine, at clinical relevant concentrations, inhibits central cardiorespiratory interactions in the brainstem and, in particular, the inspiration-evoked increases in GABAergic and glycinergic neurotransmission to CVNs.

Materials and Methods

All animal procedures were performed with the approval of the Animal Care and Use Committee of The

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George Washington University, Washington, D.C., in accordance with the recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.¹⁹

Fluorescent Labeling of CVNs and Medullary Slice Preparation

Neonatal Sprague-Dawley rats (P3-P7; Hilltop, Scottdale, PA) were anesthetized with ketamine-xylazine (87 and 13 mg/kg, respectively) injected subcutaneously and cooled to approximately 4°C to slow the heart rate. A right thoracotomy was performed, and the retrograde fluorescent tracer X-rhodamine-5 (and -6)-isothiocyanate (Molecular Probes, Eugene, OR; 1% solution, 10–25 μ l) was injected into the fat pads at the base of the heart. Because this fluorescent tracer can only label neurons retrogradely and cannot travel across synapses, processes from afferent neurons and cell bodies of sympathetic neurons in the brainstem are not labeled. In control experiments, sections of the cardiac branch of the vagus nerve abolished fluorescent labeling in the brainstem. After 24–48 h recovery, animals were anesthetized with halothane and killed by cervical dislocation. The brain was removed and immediately transferred into ice-cold (4°C) physiologic saline solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5 mM glucose, and 10 mM HEPES and oxygenated with 100% O₂ at a pH of 7.4. The medulla was removed with care to preserve the hypoglossal cranial nerve rootlet. The brainstem was fixed on an agar block and secured in a vibratome (Leica, Nussloch, Germany) with the rostral end up. Thin slices were sectioned serially in a rostrocaudal progression until the inferior olives and the nucleus ambiguus could be visualized on the rostral surface of the tissue. A single thick (800- μ m) section that included CVNs, the hypoglossal nerve rootlet, the pre-Bötzinger complex, and the rostral portion of the hypoglossal nucleus was cut and submerged in a recording chamber that allowed perfusion (10 ml/min) of artificial cerebrospinal fluid at room temperature containing 125 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 26 mM NaHCO₃, 5 mM glucose, and 5 mM HEPES equilibrated with carbogen (95% O₂ and 5% CO₂ at a pH of 7.4).

Recording Respiratory Network Activity

The thick medullary slice preparation contains the pre-Bötzinger complex, local circuits for motor output generation, and respiratory hypoglossal motorneurons.^{3,20} Spontaneous respiratory-related activity was recorded by monitoring motor neuron population activity from hypoglossal nerve rootlets using a suction electrode. Signals recorded from hypoglossal rootlet activity was amplified 50,000 times, band-pass filtered (low-pass 10 Hz, high-pass 300 Hz; CWE Inc., Ardmore, PA) and electronically integrated (τ = 50 ms; CWE Inc.).

Patch Clamp Techniques

Cardioinhibitory vagal neurons in the nucleus ambiguus were identified by the presence of the fluorescent tracer.⁴ Patch pipettes (2.5–3.5 M Ω) were visually guided to the surface of individual CVNs using differential interference optics and infrared illumination (Zeiss, Oberkochen, Germany). Patch pipettes contained 150 mM KCl, 4 mM MgCl₂, 2 mM EGTA, 2 mM Na-ATP, and 10 mM HEPES (pH = 7.4). This pipette solution resulted in inward Cl⁻ currents on activation of γ -aminobutyric acid and glycine receptors (calculated reversal potential of Cl⁻ = +4 mV) at a holding potential of -80 mV. Voltage clamp recordings were made with an Axopatch 200B and pClamp 8 software (Axon Instruments, Union City, CA). Only one experiment was conducted per preparation. Respiratory evoked GABAergic and glycinergic synaptic currents were recorded in cardiac vagal neurons using whole cell patch clamp techniques while spontaneous rhythmic respiratory activity was recorded simultaneously.

Focal Drug Application

Focal drug application was performed using a PV830 Pneumatic PicoPump pressure delivery system (WPI, Sarasota, FL). Drugs were ejected from a patch pipette positioned within 30 μ m from the recorded CVN. The maximum range of drug application has been previously determined to 100–120 μ m downstream from the drug pipette and considerably less behind the drug pipette.²¹ GABAergic neurotransmission was isolated by focal application of D-2-amino-5-phosphonovalerate (AP-5; 50 μ M), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μ M), and strychnine (1 μ M) to block NMDA, non-NMDA glutamatergic, and glycinergic receptors, respectively. Glycinergic inhibitory postsynaptic current (IPSC) activity was isolated by focal application of NMDA, non-NMDA glutamatergic, and GABAergic antagonists AP-5 (50 μ M), CNQX (50 μ M), and gabazine (25 μ M), respectively. All drugs were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Ketamine Administration

Rhythmic inspiratory-related activity and inspiration-evoked GABAergic and glycinergic synaptic inputs in CVNs were recorded simultaneously for 20 min in artificial cerebral spinal fluid equilibrated with 95% O₂ and 5% CO₂. Slices were then exposed to ketamine by addition to the perfusate for 10 min. The exposure to ketamine was then terminated, and the slice was perfused with the control artificial cerebral spinal fluid for 60 min.

The concentration range of ketamine used in this study was within the clinically relevant range. A bolus injection of ketamine of 2 mg/kg in humans during induction results in a peak plasma ketamine concentration of between 2–10 μ M (0.4–2 μ g/ml)²² and 20 μ M (5 μ g/ml).²³ Because the unbound fraction of ketamine in plasma is

0.465,²⁴ the unbound ketamine concentrations during induction is within the range from 0.93 to 9.3 μM . Therefore, ketamine concentrations of 0.1, 0.5, 1, 5, and 10 μM were used in this study to encompass the clinically relevant range. Each slice was exposed to only one dose of ketamine. At the end of each experiment, GABAergic or glycinergic activity was reversibly blocked using gabazine (25 μM) or glycine (1 μM), respectively. Ketamine was obtained from Phoenix Pharmaceuticals (St. Joseph, MO).

Statistical Analysis

Synaptic events were detected using MiniAnalysis version 5.6.12 (Synaptosoft, Decatur, GA). IPSC frequency was analyzed in 10-s periods and was cross-correlated with respiratory activity, from 5 s before to 5 s after the onset of hypoglossal inspiratory bursts. All data are presented as average \pm SEM. Statistical comparisons were made using analysis of variance with repeated measures and Newman-Keuls posttests or paired or unpaired Student *t* tests, as appropriate. $P < 0.05$ indicated significant differences. The software programs used for statistics were Graphpad Prism 4.01 (Graphpad Software, San Diego, CA), Microcal Origin 6.0 (OriginLabs Corp., Northampton, MA), and Microsoft Excel (Microsoft Corp., Redmond, WA).

Results

Ketamine Inhibits Respiratory Activity

Ketamine evoked a dose-dependent inhibition of inspiratory burst frequency recorded from the hypoglossal rootlet. The frequency of inspiratory bursts decreased from an average control frequency of 4.4 ± 0.2 bursts/min to 1.6 ± 0.5 ($P < 0.001$), 0.9 ± 0.1 ($P < 0.001$), and 0.57 ± 0.02 ($P < 0.001$) bursts/min after 10 min application of ketamine at concentrations of 0.1 μM ($n = 17$), 0.5 μM ($n = 15$), and 1.0 μM ($n = 15$), respectively (fig. 1). With additional increases of ketamine concentration (5 μM , $n = 9$), the respiratory bursts were nearly completely inhibited, whereas the higher concentration of ketamine (10 μM , $n = 8$) completely blocked respiratory bursting activity (fig. 1). The respiratory responses to ketamine were reversible. After termination of ketamine application, the frequency of respiratory bursts recovered in 20–30 min with lower doses of ketamine (0.1 and 0.5 μM), whereas higher concentrations of ketamine (1.0 μM) exposure extended the recovery time from 30 min to 1 h (fig. 1). In the absence of ketamine, respiratory frequency was well maintained for the duration of these experimental procedures (control data, $n = 4$).

Ketamine Suppresses Inspiratory-evoked GABAergic Inhibitory Synaptic Inputs to CVNs

Previous work has identified the neurochemical link between the neurons essential for respiration and neu-

rons that control heart rate. The frequency of both GABAergic and glycinergic IPSCs in CVNs increases during inspiration, and the respiratory-related GABAergic but not glycinergic inhibition of CVNs is dependent on the activation of nicotinic receptors.⁶ To determine the effects of ketamine on inspiration-evoked GABAergic inputs to CVNs, GABAergic neurotransmission was isolated by focal application of glycinergic and glutamatergic antagonists. During inspiratory bursts, the average frequency of GABAergic synaptic inputs to CVNs significantly increased from a basal level of 4.6 ± 0.1 Hz to 6.7 ± 0.4 Hz during inspiratory activity ($P < 0.001$, $n = 29$ cells; fig. 2).

Ketamine did not significantly alter the inspiration elicited increase in GABAergic IPSC frequency at a concentration of 0.1 μM (control: before burst 4.5 ± 0.5 Hz, during burst 7.7 ± 0.6 Hz; 0.1 μM ketamine: before burst 3.0 ± 0.4 Hz, during burst 5.6 ± 0.4 ; $n = 10$ cells, $P < 0.05$). However, ketamine significantly suppressed the increase in GABAergic frequency during inspiration at doses of 0.5 μM (control: before burst 3.2 ± 0.3 Hz, during burst 5.6 ± 0.3 Hz; $P < 0.01$; 0.5 μM ketamine: before burst 3.9 ± 0.3 Hz, during burst 4.8 ± 0.4 ; $n = 7$, $P > 0.05$; fig. 2) and 1 μM (control: before burst 4.5 ± 0.4 Hz, during burst 8.0 ± 0.6 Hz; 1.0 μM ketamine: before burst 2.9 ± 0.3 Hz, during burst 4.2 ± 0.4 Hz; $n = 6$, $P > 0.05$; fig. 2). The inhibitory action of ketamine on GABAergic neurotransmission to CVNs during inspiration was reversible, and all IPSCs under these recording conditions were blocked by the application of the γ -aminobutyric acid type A antagonist gabazine (25 μM).

Effect of Ketamine on Inspiration-evoked Glycinergic Inhibitory Synaptic Inputs to CVNs

Glycinergic neurotransmission was isolated by focal application of GABAergic and glutamatergic antagonists. The frequency of glycinergic synaptic inputs to CVNs significantly increased during inspiratory bursts (fig. 3). The glycinergic IPSC frequency increased from a control level of 5.7 ± 0.4 Hz to 14.1 ± 0.8 Hz during inspiration ($P < 0.001$, $n = 22$ cells; fig. 3).

Ketamine at a concentration of 0.1 μM did not block the increase in inspiration-evoked glycinergic postsynaptic currents. In the presence of ketamine, glycinergic IPSC frequency changed from 4.6 ± 0.3 Hz before burst to 7.9 ± 0.5 Hz during burst ($P < 0.001$). However, at higher concentrations (0.5 and 1 μM), ketamine inhibited the increase of glycinergic IPSC frequency during inspiratory bursting (fig. 3). The average frequency was not significantly increased from 6.9 ± 0.3 before burst to 5.7 ± 0.3 Hz during the inspiratory activity ($P > 0.05$; fig. 3) at a concentration of 0.5 μM or from 8.2 ± 0.5 to 9.0 ± 0.6 Hz during inspiration ($P > 0.05$) at a dose of 1.0 μM . The inhibitory action of ketamine on glycinergic neurotransmission to CVNs during inspiration was re-

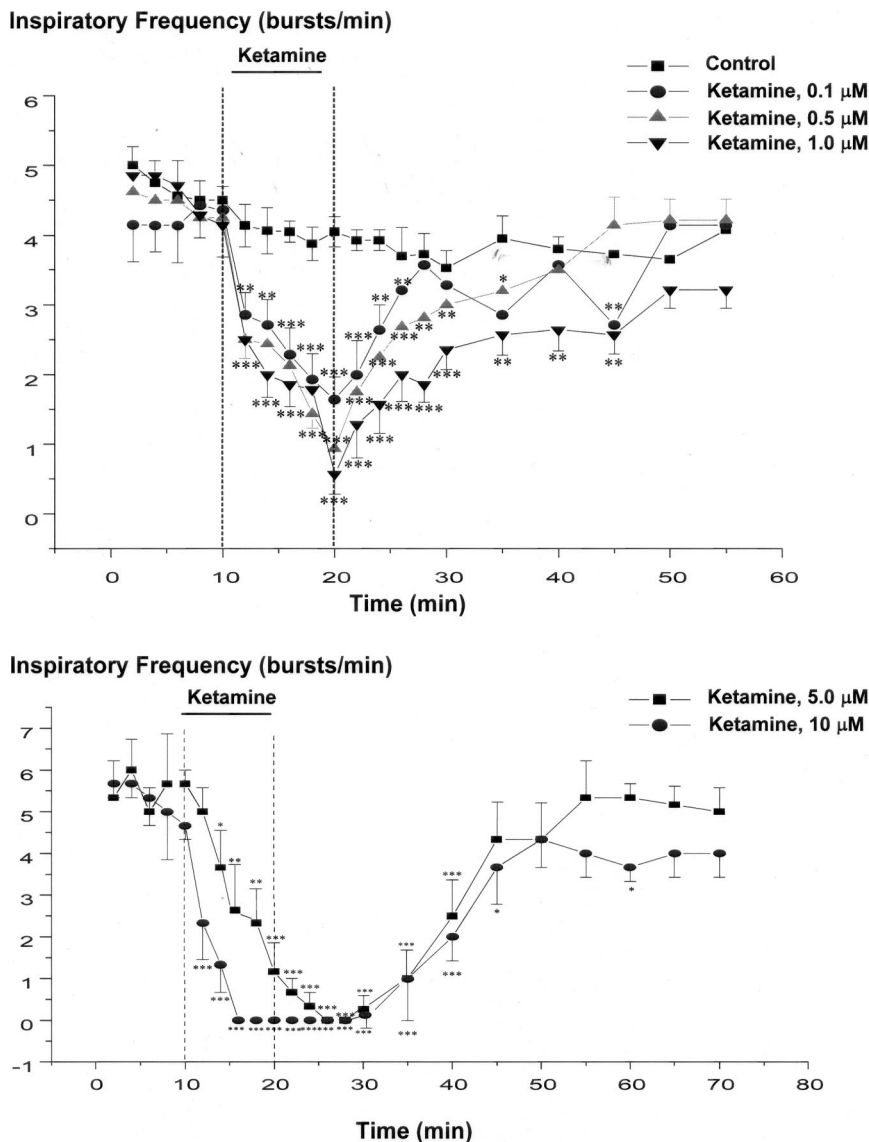


Fig. 1. Ketamine, at concentrations of 0.1–1 μM , significantly inhibited inspiratory frequency (top). At higher concentrations, ketamine either severely reduced (1 μM) or abolished (5 μM) inspiratory activity (bottom). The inhibition of respiratory activity by ketamine was gradually reversed after termination of ketamine exposure. In the absence of ketamine, respiratory frequency was well maintained for the duration of these experimental procedures (control data).

versible, and at the end of this series of experiments, all IPSCs were blocked by the application of the glycine antagonist strychnine (1 μM).

Discussion

Three potential cellular targets of anesthetics within the brainstem include excitatory and inhibitory synaptic transmission and voltage-gated ion channels. Ketamine is a noncompetitive NMDA antagonist as well as an inhibitor of sodium, potassium, and calcium channels.^{23,25,26} This study tests the hypothesis that ketamine alters central respiratory activity and respiratory-related inhibitory synaptic neurotransmission to cardiac vagal neurons in the nucleus ambiguus. There are three findings from the study: (1) Ketamine inhibited central respiratory activity; (2) ketamine attenuated the increase of inspiratory-evoked GABAergic neurotransmission to cardiac vagal neurons; and (3) ketamine eliminated the inspiratory-induced increase in glycinergic activity to cardiac vagal neurons.

Respiratory rhythm is generated by neurons contained within the lower brainstem. A particularly important region is the pre-Bötzinger complex.²⁰ Isolating the pre-Bötzinger complex in a slice preparation preserves rhythmic activity, which can be recorded from either the pre-Bötzinger complex or the hypoglossal (XII) motor nucleus.^{27,28} NMDA receptors are critical for respiratory rhythmogenesis.^{21,29} Because ketamine is a potent noncompetitive NMDA antagonist,³⁰ this mechanism of action may be responsible for the observed ketamine inhibition and ultimately abolishment of central respiratory activity at higher concentrations. This finding is also consistent with clinical application of ketamine in which respiratory depression occurs in patients.^{31,32}

Ketamine has been associated with positive chronotropic and inotropic cardiovascular responses attributed to sympathoexcitation but may include direct peripheral actions on the heart and blood vessels. However, several lines of evidence indicate that ketamine also acts on

Fig. 2. Inspiratory-related bursting activity was recorded from the hypoglossal rootlet (XII) and electronically integrated (\int XII). Fluorescently identified cardiac vagal neurons (CVNs) were patch clamped in the whole cell configuration. During inspiratory bursts, the average frequency of γ -aminobutyric acid-mediated (GABAergic) synaptic inputs to CVNs significantly increased (*left*). Ketamine suppressed the increase in GABAergic frequency during inspiration at a dose of $0.5 \mu\text{M}$ (*middle*) and further reduced the inspiratory evoked increase in GABAergic neurotransmission during inspiration at $1 \mu\text{M}$ (*right*). The inhibitory action of ketamine on GABAergic neurotransmission to CVNs during inspiration is reversible, and all inhibitory postsynaptic currents (IPSCs) under these recording conditions were blocked by the application of the γ -aminobutyric acid type A antagonist gabazine ($25 \mu\text{M}$).

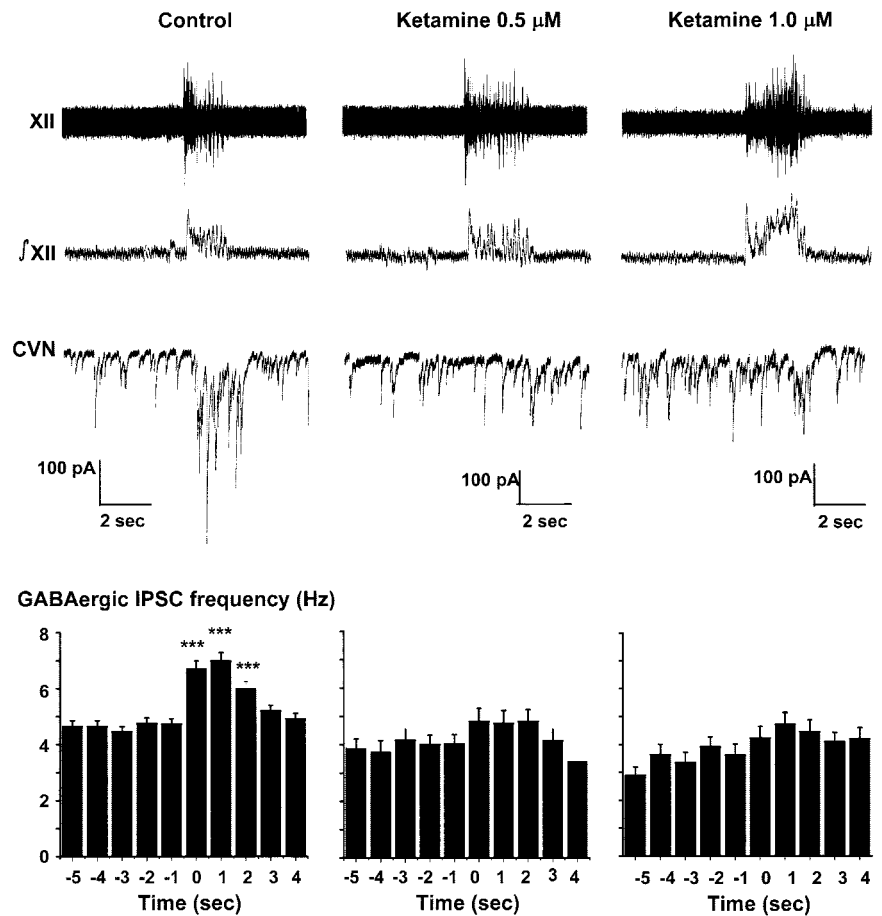
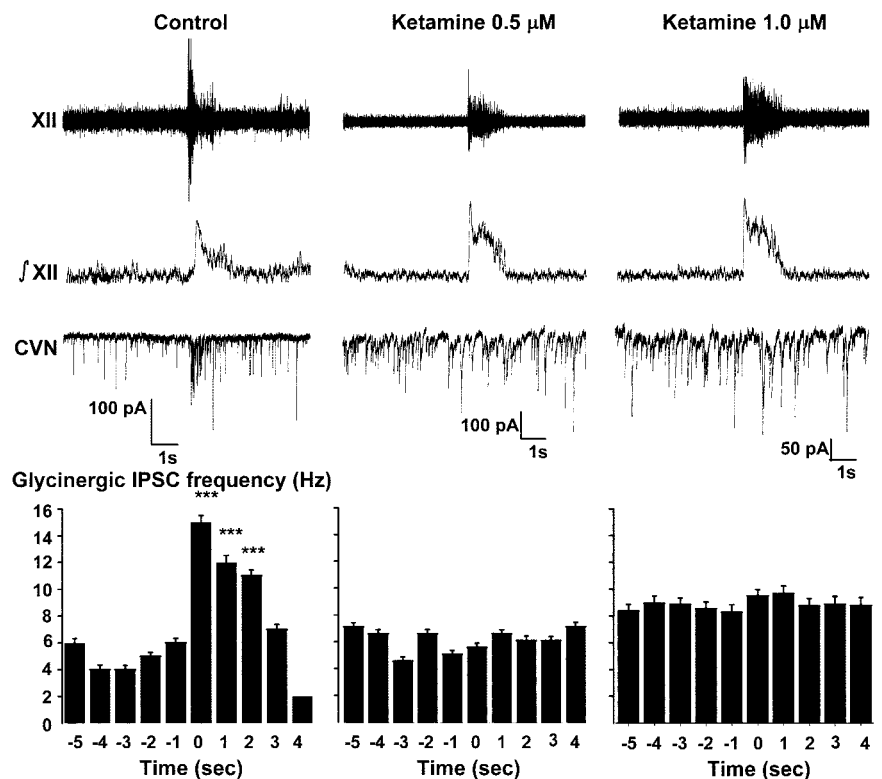


Fig. 3. During inspiratory bursts, the average frequency of glycinergic synaptic inputs to cardiac vagal neurons (CVNs) significantly increased during inspiratory activity (*left*). Ketamine suppressed the increase in glycinergic frequency during inspiration at a dose of $0.5 \mu\text{M}$ (*middle*) and further reduced the inspiratory-evoked increase in glycinergic neurotransmission during inspiration at $1 \mu\text{M}$ (*right*). The inhibitory action of ketamine on glycinergic neurotransmission to CVNs during inspiration was reversible, and all inhibitory postsynaptic currents (IPSCs) under these recording conditions were blocked by the application of the glycine antagonist strychnine ($1 \mu\text{M}$).



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parasympathetic control of heart rate. Although the non-competitive blockade of NMDA receptors by ketamine is a major target in the central nervous system, various reports indicate that ketamine also alters GABAergic neurotransmission. Ketamine prevented the arrhythmogenic response to the hypothalamic stimulation and its facilitation by baclofen in rats through both central GABAergic and glutamatergic mechanisms.³³ Furthermore, ketamine increased the γ -aminobutyric acid type A receptor-mediated response in rat hippocampus,³⁴ brainstem, and cortex.³⁵ In expression systems, glycine receptors have been considered quite resistant to ketamine,^{36,37} but this contrasts markedly with the inhibition of native glycinergic neurotransmission onto CVNs by low-micromolar ketamine as shown in this study.

Another possible site of action of ketamine is on nicotinic cholinergic receptors. Nicotinic receptors are involved in respiratory activity in the brainstem.³⁸ Previous studies demonstrated that activation of $\alpha_4\beta_2$ subunit containing nicotinic receptors mediated the respiratory-related GABAergic inhibition of CVNs.⁶ Ketamine decreases both the open and closed times of nicotinic acetylcholine receptors³⁹ and inhibits presynaptic and postsynaptic nicotinic excitation in CVNs.⁴⁰

The increase in heart rate during inspiration, respiratory sinus arrhythmia, is likely due to respiratory-related increases in GABAergic and glycinergic neurotransmission to cardioinhibitory parasympathetic cardiac efferent neurons during inspiration. Although feedback from pulmonary stretch receptors and direct respiratory-related changes in venous return and cardiac stretch can evoke respiratory related fluctuations in heart rate, the dominant source of respiratory sinus arrhythmia originates from the brainstem.⁷ Respiratory sinus arrhythmia persists when the lungs are stationary (caused by muscle paralysis or constant flow ventilation) and the respiratory modulation of heart rate remains synchronized with brainstem respiratory rhythms even if artificial ventilation of the lungs and chemoreceptor activation occur at different intervals.⁴¹⁻⁴⁵ In both animals and humans, respiratory sinus arrhythmia is mediated *via* cardiac vagal activity. Respiratory sinus arrhythmia persists in experimental animals upon sectioning sympathetic pathways and in quadriplegic patients with spinal cord injury and sympathetic dysfunction.^{41-44,46} Blockade of parasympathetic cardiac activity abolishes respiratory sinus arrhythmia.⁴⁷ During normal eupneic respiration, cardiac vagal neurons receive inhibitory GABAergic and glycinergic neurotransmission during inspiration but receive no excitatory synaptic inputs during any phase of the respiratory cycle.^{6,48}

Because ketamine inhibits inspiration-evoked increases in GABAergic and glycinergic neurotransmission, one would predict that ketamine inhibits this cardiorespiratory interaction. This reduction in inhibitory neurotransmission to cardiac vagal neurons is likely responsible for

the compromised respiratory sinus arrhythmia that occurs with ketamine anesthesia. Consistent with this finding, recent work has found that ketamine significantly suppresses respiratory sinus arrhythmia.⁹ In summary, this study demonstrates that ketamine causes respiratory depression and inhibits the mechanisms responsible for respiratory sinus arrhythmia, both inspiratory-evoked increases in GABAergic and glycinergic neurotransmission to parasympathetic cardiac vagal neurons.

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