

Original Article

Inhibition of GABA_A receptors in the nucleus tractus solitarius induced cardiovascular depression during isoflurane inhalation anesthesia

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Abstract: Objective: Isoflurane is widely used for inhaled general anesthesia. However, the mechanisms of isoflurane-induced cardiovascular depression are not fully understood. The effect of isoflurane on GABA_A receptor expression in the NTS may underlie cardiovascular depression. Methods: The blood pressure (BP) and heart rates (HR) of conscious rats were confirmed initially using a CODA non-invasive blood pressure monitor system. Rats were anesthetized with inhaled isoflurane or an intraperitoneal injection of urethane, and BP and HR were measured. BP and HR changes evoked by microinjections of L-glutamate, GABA_A receptor agonists or antagonists into the NTS were observed. GABA_A receptor mRNA and protein expression level changes following agonist or antagonist administration were detected using real-time PCR and Western blot analyses. Results: The depressor response to L-glutamate microinjection into the NTS was significantly increased in the urethane-anesthetized group compared with isoflurane group. GABA_A receptor agonist and antagonist microinjections into the NTS did not significantly change the BP and HR in the isoflurane group, but the increasing or decreasing amplitudes of BP and HR of urethane group were significantly higher than isoflurane group. GABA_A receptor agonist and antagonist microinjections significantly altered GABA_A receptor mRNA and protein expression levels in the urethane group. Conclusion: These findings suggest that the function of GABA_A receptors in the NTS was inhibited by inhaled isoflurane, which induced cardiovascular depression.

Keywords: Isoflurane, urethane, GABA_A receptor, hypotension

Introduction

Isoflurane is a widely used inhaled general anesthetic that is well suited for rapid induction and recovery [1]. However, depressed cardiovascular function is a commonly reported side effect, and the mechanisms of isoflurane-altered cardiovascular regulation are not well studied. The current widely held opinion about this effect is that isoflurane-induced sympathetic nerve depression causes hypotension and bradycardia, and this hypotension results in hemangiectasis.

Central nervous system γ -aminobutyric acid (GABA) systems in the nucleus tractus solitarius (NTS) play a key role in cardiovascular regulation via the stimulation of GABA_A and GABA_B receptors [2]. GABA is an inhibitory neurotransmitter in the brain. GABA actions are mediated

by GABA receptors, including ionotropic GABA_A receptors and metabotropic GABA_B receptors [3]. The ionotropic GABA_A receptor includes an intrinsic Cl⁻ channel that induces fast inhibitory postsynaptic potentials.

Our previous study demonstrated that GABA_A receptor expression inhibition in the NTS up-regulated NTS function and induced hypotension and bradycardia [4]. General anesthetic agents primarily target GABA_A receptors and enhance inhibitory neurotransmission [5]. Therefore, the expression levels of GABA_A receptors during isoflurane-inhaled anesthesia may influence the hemostatic regulation of the NTS and cause further changes in blood pressure (BP) and heart rate (HR).

Homeostatic regulation is generally controlled by visceral afferents of the IX and X cranial

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nerves, which travel centrally via the solitary tract and release glutamate onto neurons in the NTS to produce excitation [6]. L-glutamate is commonly used to confirm brain microinjection location before experimental drugs are microinjected.

Most general anesthetics exert their principal neuronal functions through the promotion of inhibitory GABAergic transmission or the suppression of excitatory glutamatergic transmission, which induces a sedative state characterized by a unitary level of brain activity [7]. However, urethane anesthesia is a model of natural sleep that may be maintained for long durations in a surgical plane without any additional pharmacological administration, and it displays consistent, reproducible and predictable alteration cycles in brain activity according to electroencephalography (EEG) [8]. Therefore, urethane is frequently used in respiratory studies because of its reduced effect on cardiorespiratory parameters and long-lasting anesthetic action [9]. The central nervous system anesthetic action of urethane is different from those of other agents. Urethane potentiates the resting potassium conductance that induces hyperpolarization in central neurons [10]. One study indicated that isoflurane enhanced GABA-mediated inhibitory currents and suppressed glutamate-mediated excitatory currents on NTS neurons via distinct pre- and postsynaptic mechanisms, which may be associated with depressed cardiorespiratory function [6].

The present study investigated the effects of urethane and isoflurane anesthetics on rat circulation and characterized GABA_A receptor expression in the NTS. The results suggest that the circulatory depression effects of isoflurane anesthetic were not altered by a GABA_A receptor agonist or glutamate microinjection into the NTS, unlike urethane anesthesia, and GABA_A receptor expression in the NTS was not altered. Taken together, this study demonstrates that hypotension and bradycardia during isoflurane anesthesia were induced by the inhibition of the circulatory regulating function of GABA_A receptors in the NTS.

Methods

Animals and materials

Adult male Wistar rats were obtained from Jilin University (Jilin, China). Rats weighing 290±10 g were used in this study to ensure an accurate

NTS location according to The Rat Brain in Stereotaxic Coordinates [11]. All rats were housed individually and under controlled conditions with a 12:12-h light-dark cycle. Rat chow and water were available ad libitum. The First Medical Hospital of Jilin University Institutional Animal Care and Committee approved all protocols.

Recording of chronic BP and HR

Chronic BPs and HR were recorded at 9:00 AM for 7 consecutive days to obtain the baseline BP of Wistar rat without anesthetics. The CODA non-invasive blood pressure monitoring system was used to record rat chronic BP measurements (Kent Scientific, USA).

Anesthetics

Rats in the isoflurane inhalation group were induced by 3% isoflurane and maintained with 1.4% isoflurane as 1.3 MAC, which was mixed with oxygen and delivered through a nose cone. Rats in the urethane anesthetic group received an intraperitoneal injection of 0.8 g/kg 20% urethane diluted in a 0.9% salt solution.

Recording of acute BP and HR

Acute BP and HR recordings were performed using PE-10 catheters fused to PE-50 catheters. The catheter was perfused with heparinized saline (100 IU/ml), placed in the right femoral artery after anesthesia, and connected to a BP transducer and a bridge amplifier (ADInstruments, Colorado Springs, CO, USA) as previously reported [4]. The BP and HR data were collected and analyzed using Powerlab software (ADInstruments, Colorado Springs, CO, USA).

NTS microinjection

L-glutamate, GABA_A receptor agonists and antagonists were microinjected bilaterally into the NTS using a previously described procedure [12]. Briefly, anesthetized rats were placed in a stereotaxic frame as described in The Rat Brain in Stereotaxic Coordinates [11]. NTS coordinates, which were 0.5 mm rostral to the caudal tip of the area postrema, 0.5 mm lateral to the midline, and 0.5 mm below the dorsal surface of the brain stem, were determined, and a multiple-barrel glass injection pipette (tip size 20-40 μm) was positioned in the NTS. L-glutamate (200 pmol in 50 nl), a GABA_A receptor agonist (muscimol, 100 pmol in 50 nl, Sigma-Aldrich) or a GABA_A receptor antagonist (bicu-

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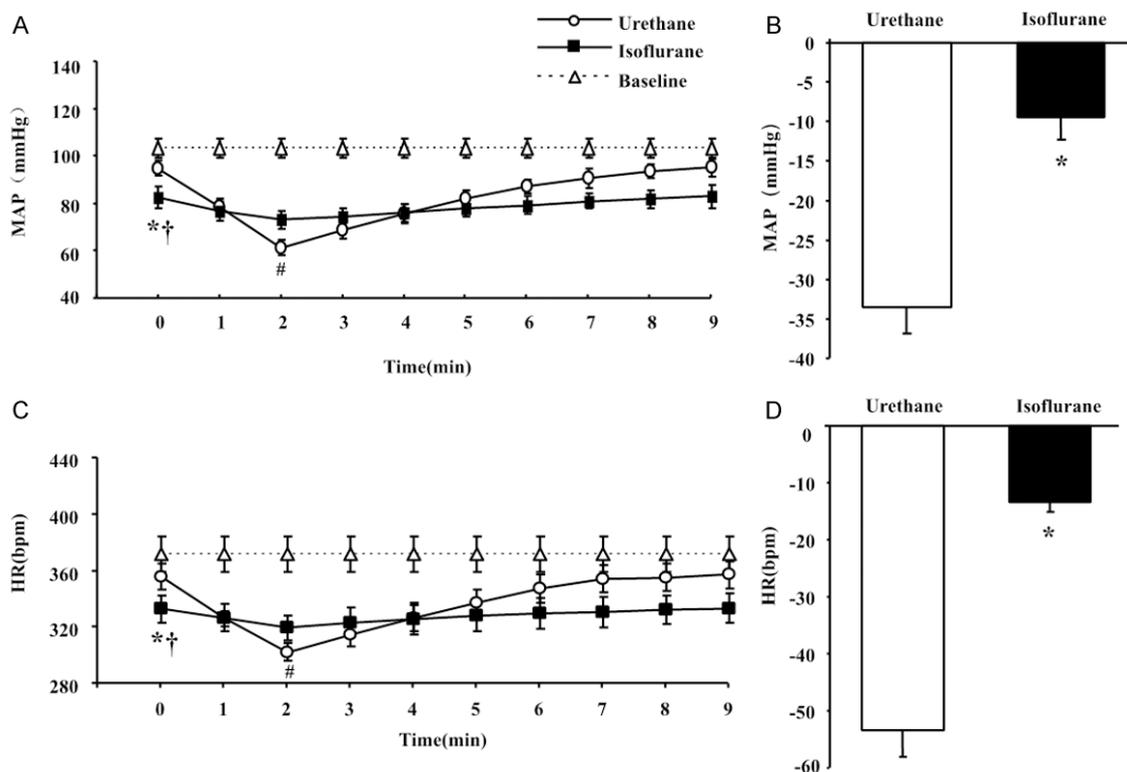


Figure 1. Effects of L-glutamate microinjection into the NTS on BP and HR in urethane- or isoflurane-anesthetized rats. (A, C) Time course data showing the MAP (A) and HR (C) changes evoked by L-glutamate (200 pmol in 50 nl) microinjected into the NTS of urethane- or isoflurane-anesthetized rats. Data are means \pm SE (n=6 in each group). *P<0.05 vs. BP or HR baseline (conscious rats) at time 0; †P<0.05 vs. urethane group rats at time 0; #P<0.05 compared to the respective basal condition. (B, D) Bar graphs showing MAP (B) and HR (D) changes evoked by the microinjection of L-glutamate into the NTS in urethane- or isoflurane-anesthetized rats. Data represent the mean \pm SE (n=6 in each group). *P<0.05 vs. urethane group rats. BP, blood pressure; MAP, mean arterial pressure.

culline, 10 pmol in 50 nl, Sigma-Aldrich) was dissolved in saline and microinjected bilaterally into the NTS. The injection position was confirmed via microinjection of methylene blue dye (50 nl) after the protocol, and data were excluded if the injection position was inaccurate.

Western blot analysis of GABA_A receptor protein in the NTS

Based on the results of the NTS microinjection, another two groups of rats were selected as described above. GABA_A receptor agonists or antagonists were microinjected into the NTS, and the BP and HR exhibited maximum changes at approximately the third minute. The rats were euthanized after completion of the microinjection, at approximately three minutes. Some control rats were euthanized after anesthesia without agonist or antagonist microinjection to the NTS. Brains were collected and immediately placed on ice, blocked in the coro-

nal plane, frozen on dry ice, and sectioned at 100- μ m thickness in a cryostat as described previously [12]. The NTS tissue was punched using Harris Micro-Punch tools. The size of the punches varied from 0.5 to 1.0 mm, depending on the location of the brain section, which was determined using The Rat Brain in Stereotaxic Coordinates [11]. GABA_A receptor protein expression analysis was performed as described previously [12]. Briefly, membranes were probed with a primary antibody (GABA_A receptor rabbit polyclonal antibody, Santa Cruz, 1:500) and secondary antibody (goat anti-rabbit IgG horseradish peroxidase, Bio-Rad, 1:3000). Bands were visualized and analyzed using Image J software.

Real-time RT-PCR

Real-time PCR was used to detect changes in GABA_A receptor expression in the NTS after GABA_A receptor agonist or antagonist microin-

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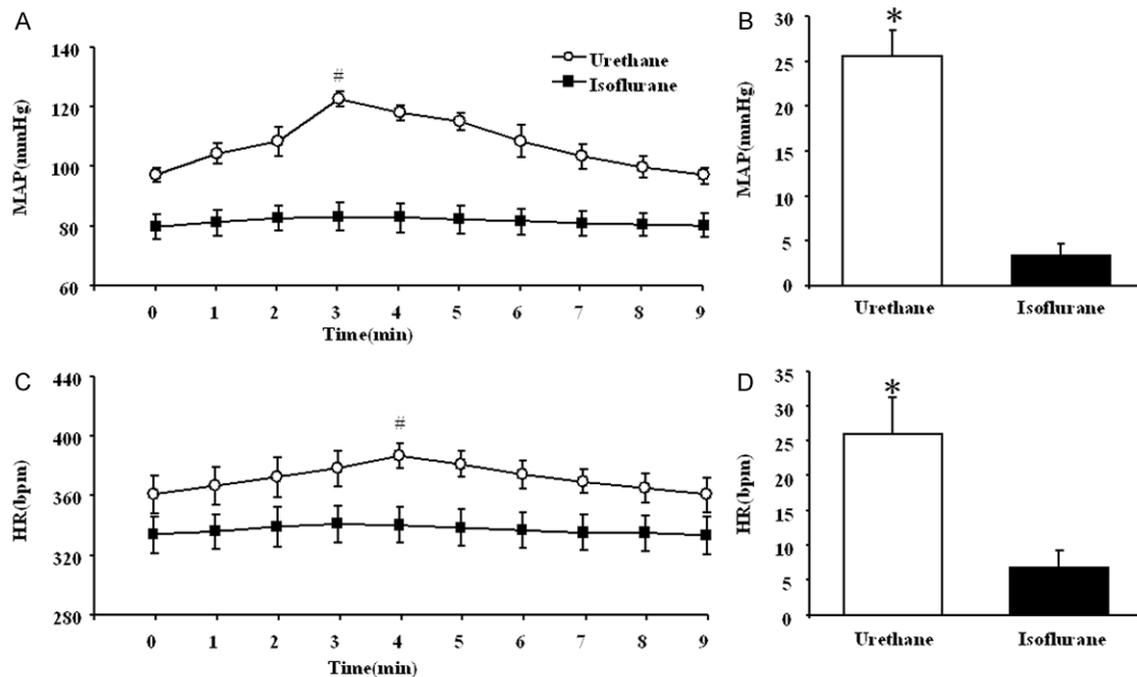


Figure 2. Effects of a GABA_A receptor agonist on BP and HR in urethane- or isoflurane-anesthetized rats. (A, C) Time course data showing the MAP (A) and HR (C) changes evoked by muscimol (100 pmol in 50 nl) microinjected into the NTS of urethane- or isoflurane-anesthetized rats. Data represent the mean \pm SE (n=6 in each group). #P<0.05 compared to the respective basal condition; (B, D) Bar graphs showing MAP (B) and HR (D) changes evoked by the microinjection of muscimol (100 pmol in 50 nl) into the NTS in urethane- or isoflurane-anesthetized rats. Data represent the mean \pm SE (n=6 in each group). *P<0.05 vs. urethane group rats. BP, blood pressure; MAP, mean arterial pressure; NTS, nucleus tractus solitarius.

jection performed over three minutes. The microinjection and NTS tissue collection protocols were followed as described above. Isolation of total RNA from NTS tissue, reverse transcription, and the specific probes for the GABA_A receptor α 1 and GABA_A receptor γ 2 were described in a previous study [13]. Samples in each experiment were analyzed in triplicate.

Statistical analysis

All data are expressed as the mean \pm SE. Comparisons between two groups were performed using 1- or 2-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at P<0.05, and individual *p* values are noted in all figures.

Results

BP and HR of conscious rats

The first experiment confirmed the BP and HR of rats without anesthetic and set the baseline for the following experiments to demonstrate

the changes in the BP and HR after two different anesthetics. BP of the urethane group is 102.9 ± 6.1 mmHg (n=6) and isoflurane group is 103.3 ± 8.2 mmHg (n=6), and the HR of the urethane group is 371.3 ± 12.5 bpm (n=6) and isoflurane group is 372.8 ± 12.0 bpm (n=6). There were no significant differences in BP and HR between the two groups. So, the BP and HR of the conscious rats were 103.2 ± 7.2 mmHg and 371.6 ± 12.2 bpm (n=12), respectively.

Effect of L-glutamate microinjection into the NTS on BP and HR in urethane- and isoflurane-anesthetized rats

The circulation of Wistar rats was significantly depressed by inhaled isoflurane compared to urethane anesthesia. BP and HR decreased significantly to 82.2 ± 4.4 mmHg and 332.7 ± 9.6 bpm in isoflurane group, respectively, compared to baseline prior to anesthesia (n=6, P<0.05). BP and HR were also significantly lower in the isoflurane group than the urethane group (94.5 ± 3.1 mmHg and 355.5 ± 9.1 bpm, respectively, n=6, P<0.05) after anesthesia.

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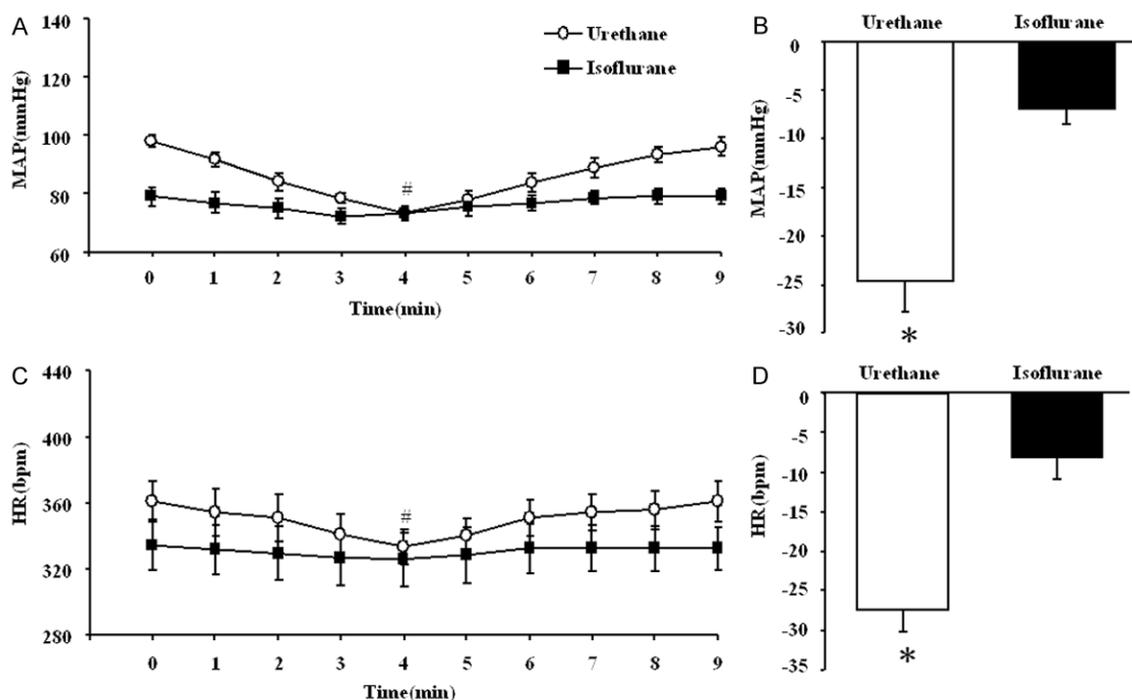


Figure 3. Effect of a GABA_A receptor antagonist on BP and HR in urethane- or isoflurane-anesthetized rats. (A, C) Time course data showing the MAP (A) and HR (C) changes evoked by bicuculline (10 pmol, in 50 nl) microinjected into the NTS of urethane- or isoflurane-anesthetized rats. Data represent the mean \pm SE (n=6 in each group). #P<0.05 compared to the respective basal condition; (B, D) Bar graphs showing MAP (B) and HR (D) changes evoked by the microinjection of bicuculline (10 pmol in 50 nl) into the NTS in urethane- or isoflurane-anesthetized rats. Data represent the mean \pm SE (n=6 in each group). *P<0.05 vs. urethane group rats. BP, blood pressure; MAP, mean arterial pressure; NTS, nucleus tractus solitarius.

L-glutamate is an excitatory neurotransmitter that up-regulates the function of some nuclei after microinjection, and it is commonly used to confirm the position of nuclei before experimental drug microinjection. **Figure 1A** and **1C** show the effects of L-glutamate on BP and HR in the two groups. NTS microinjection of L-glutamate (200 pmol, 50 nl) significantly decreased BP from 94.5 ± 3.1 to 61 ± 2.1 mmHg and HR from 355.5 ± 9.1 to 302.2 ± 6.2 bpm in the urethane anesthesia group (n=6, P<0.05), but these depressor responses were not significantly evoked in the isoflurane group (BP and HR decreased from 82.2 ± 4.4 to 72.7 ± 4.4 mmHg and 332.7 ± 9.6 to 319.2 ± 9.0 bpm, respectively). The depressor response to L-glutamate microinjection into the NTS was increased significantly in the urethane anesthetic group, and the changing of BP and HR were significantly higher in the urethane group compared to the isoflurane group (**Figure 2B** and **2D**). These data demonstrated that inhaled isoflurane decreased L-glutamate excitation in the NTS and further indicated that

inhalational isoflurane depressed the effect of the NTS on cardiovascular regulation in the central nervous system.

Effects of GABA_A receptor agonists and antagonists on BP and HR in urethane- and isoflurane-anesthetized rats

We further investigated the effects of GABA_A receptor agonists and antagonists on BP and HR to examine the function of this receptor in the NTS in the urethane and isoflurane anesthesia groups. BP and HR were recorded before and after NTS microinjection of muscimol (GABA_A receptor agonist) or bicuculline (GABA_A receptor antagonist) as described in the Methods section. NTS microinjection of muscimol (100 pmol, in 50 nl) significantly increased BP from 97 ± 2.6 to 122.5 ± 2.4 mmHg and HR from 360.8 ± 13.7 to 386.6 ± 8.0 bpm in the urethane group (n=6, P<0.05), but muscimol microinjection did not significantly alter the depressor responses in the isoflurane group (**Figure 2A** and **2C**). The increases in BP

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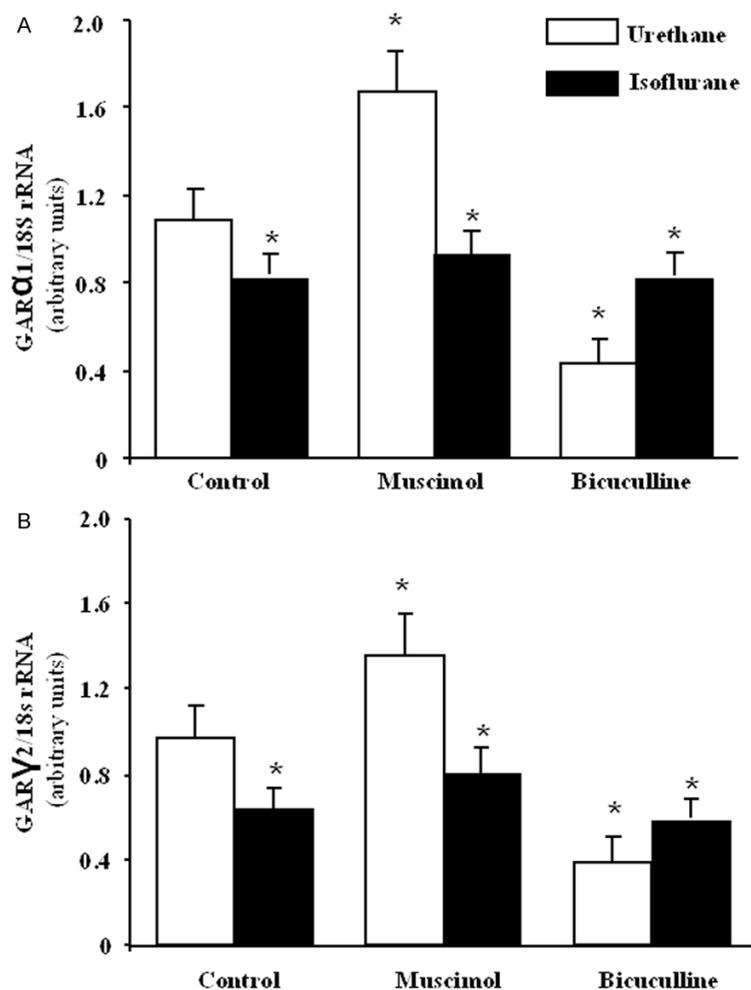


Figure 4. GABA_A receptor expression in the NTS of isoflurane- and urethane-anesthetized rats. (A) GABA_A receptor α1 mRNA and (B) GABA_A receptor γ2 changes evoked by GABA_A receptor agonist or antagonist in the NTS. GABA_A receptor mRNA levels were detected using real-time RT-PCR as described in the Methods section. Data were normalized to 18S rRNA and represent the mean ± SE of the fold increase over control (n=6 in each group). *P<0.05 vs. the control group of urethane-anesthetized rats. GABA_A, γ-aminobutyric acid type A; NTS, nucleus tractus solitarius.

and HR following muscimol microinjection in the urethane group were significantly higher than in the isoflurane group (n=6, P<0.05) (Figure 2B and 2D).

Microinjection of the GABA_A receptor antagonist bicuculline (10 pmol in 50 nl) into the NTS significantly decreased BP from 98±2 to 73.3±2.6 mmHg and HR from 357.8±12.6 to 333.5±10.9 bpm in the urethane group (n=6, P<0.05), but bicuculline microinjection did not significantly decrease BP and HR in the isoflurane group (Figure 3A and 3C). The decreases

in BP and HR induced by bicuculline microinjection in the urethane group were significantly higher than in the isoflurane group (n=6, P<0.05) (Figure 3B and 3D).

GABA_A receptor agonist and antagonist microinjections in the isoflurane group did not induce the same circulation changes as in the urethane group. Therefore, we hypothesized that the inhaled isoflurane inhibited GABA_A receptor function in the NTS and further disturbed the depressor responses of the NTS in the central nervous system.

GABA_A receptor expression in the NTS of isoflurane- and urethane-anesthetized rats

The GABA_A receptor α1 and GABA_A receptor γ2 mRNA levels were measured as described in the Methods section to confirm the effects of the two different anesthetic methods on GABA_A receptors. GABA_A receptor α1 and GABA_A receptor γ2 mRNA levels were decreased significantly by isoflurane anesthesia compared to the urethane group, increased significantly by the GABA_A receptor agonist muscimol (100 pmol in 50 nl) and decreased by the GABA_A receptor antagonist

bicuculline (10 pmol in 50 nl) in the urethane group (Figure 4). However, GABA_A receptor α1 and GABA_A receptor γ2 mRNA levels were not significantly changed by the GABA_A receptor agonist muscimol (100 pmol in 50 nl) or the antagonist bicuculline (10 pmol in 50 nl) in the isoflurane group compared to the urethane anesthetic. Therefore, GABA_A receptors in the NTS were strongly depressed by inhaled isoflurane and were not correspondingly affected by an agonist or antagonist as urethane group, which suggests that the BP-regulating effect of the NTS was interrupted by isoflurane.

Isoflurane inhibits GABA_A receptors in NTS

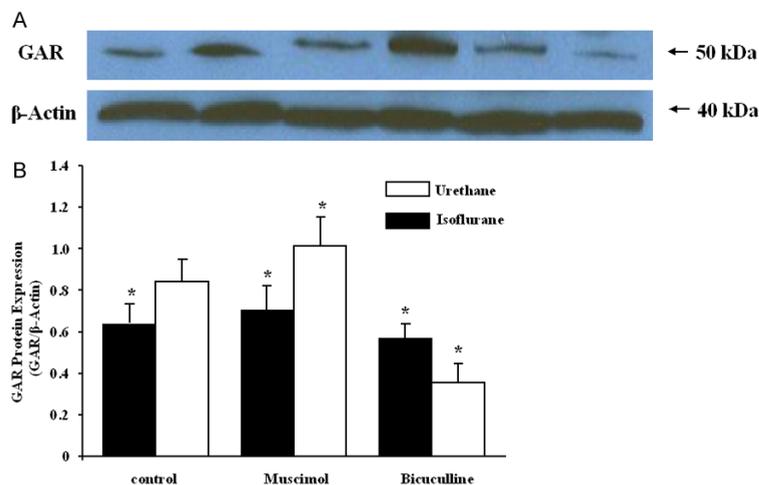


Figure 5. GABA_A receptor protein levels in the NTS of isoflurane- and urethane-anesthetized rats. A. Representative blot showing GABA_A receptor protein level changes evoked by GABA_A receptor agonist or antagonist in the NTS of isoflurane- and urethane-anesthetized rats. B. Bar graphs summarizing GABA_A receptor protein levels in the NTS. Data were normalized to β-actin and represent the mean ± SE (n=3 experiments from 6 rats in each group). GABA_A, γ-aminobutyric acid type A; NTS, nucleus tractus solitarius.

GABA_A receptor protein levels in the NTS of isoflurane- and urethane-anesthetized rats

GABA_A receptor protein levels were significantly decreased by isoflurane anesthesia compared to urethane anesthesia, and protein levels were significantly increased by the GABA_A receptor agonist muscimol (100 pmol in 50 nl) and decreased by the GABA_A receptor antagonist bicuculline (10 pmol in 50 nl) in the urethane anesthetic group. However, microinjection of the GABA_A receptor agonist muscimol (100 pmol in 50 nl) or the antagonist bicuculline (10 pmol in 50 nl) did not significantly alter GABA_A receptor protein expression levels in the NTS of rats anesthetized by inhaled isoflurane (**Figure 5**). These results demonstrated that GABA_A receptors in the NTS were inhibited by inhaled isoflurane, and the central nervous system-mediated cardiovascular actions of NTS were abolished to some degree by isoflurane.

Discussion

This study demonstrated that the general anesthetic isoflurane significantly inhibited GABA_A receptor expression in the NTS compared to urethane, and a GABA_A receptor agonist or glutamate microinjection did not reverse the effects of urethane anesthesia. A GABA_A receptor agonist, antagonist, or glutamate microin-

jection did not significantly alter isoflurane-induced circulatory depression, which indicates that GABA_A receptors in the NTS were over inhibited by inhaled isoflurane. Urethane anesthesia did not significantly induce circulatory depression, and GABA_A receptor agonist and antagonist microinjections significantly altered BP and HR, as described in a previous study [4].

Urethane is widely used for neurophysiological experiments because it is a non-typical general anesthetic due to its only moderate effects on excitatory and inhibitory neurotransmission compared with other commonly used anesthetics [14]. We demonstrated similar results in this study. A GABA_A receptor agonist and antagonist significantly altered GABA_A receptor expression in the NTS in urethane-anesthetized rats. Urethane anesthesia exerted a reduced effect on circulation compared with conscious rats.

The NTS is located in the dorsomedial medulla oblongata at the site of primary cardiovascular sensory afferents, such as those arising from arterial baroreceptors, cardiopulmonary receptors terminate and peripheral chemoreceptors [15]. L-glutamate is the main excitatory amino acid transmitter in the transmission of cardiovascular reflexes within the NTS, and it is the major excitatory transmitter released by visceral afferent input to the NTS [16, 17]. The current study compared the cardiovascular effects of L-glutamate microinjection in the NTS in two different anesthetic rats. L-glutamate did not enhance or attenuate bradycardia and hypotension after isoflurane anesthesia. Clearly, this observation is a controversial result, but it is similar to a previous report that demonstrated that isoflurane inhibited glutamate uptake via a noncompetitive mechanism, which was an indirect effect of isoflurane on the ion gradients that power glutamate uptake [18]. We hypothesized that isoflurane would produce an indirect excitatory effect on NTS neurons, which would affect the efficiency of reflex adjustments of BP.

Isoflurane inhibits GABA_A receptors in NTS

Available evidence in the literature indicates that baroreceptor and chemoreceptor afferents terminate in the NTS, which plays an important role in central cardiovascular regulation and the pathogenesis of hypertension [19, 20]. GABA is a well-known inhibitory amino acid that exhibits potent neurotransmitter activity within the NTS. The NTS contains a high density of ionotropic GABA_A receptors and metabotropic GABA_B receptors [2]. The ionotropic GABA_A receptor has an intrinsic Cl⁻ channel that induces fast inhibitory postsynaptic potentials [4, 12]. The GABA_B receptor is a G-protein-coupled receptor that mediates presynaptic and postsynaptic inhibition via reductions in calcium conductance or increases in potassium conductance [21]. Yamakura T et al. reported that isoflurane exhibits effects on various receptor systems, including GABA_A, N-methyl-D-aspartate, and acetylcholine receptors [22]. The current study examined GABA_A receptor expression in the NTS using two different anesthesia methods. Notably, inhaled isoflurane did not enhance GABA_A receptor expression in the NTS. In contrast, isoflurane suppressed GABA_A receptor expression in NTS tissue compared to the urethane group. Low levels of GABA_A receptor expression decreased inhibitory postsynaptic currents, which induced an increase in NTS activity. Similarly, enhanced baroreceptor afferent increases NTS activity, which exhibits central depression functions. Shuiping Dai et al. reported that isoflurane enhanced both types of phasic GABA_A receptor-mediated inhibition to similar degrees at amnesic concentrations, and the actions of GABA_A receptors increased in the hippocampus [23]. This study indicated that isoflurane exhibits different effects on GABA_A expression in various nuclei.

James et al. indicated that isoflurane acts through multiple distinct mechanisms to inhibit neurotransmission within the NTS, which would underlie the suppression of homeostatic reflexes. In their study, isoflurane dose-dependently enhanced GABA-mediated excitatory neurotransmission to NTS neurons in vitro. Our in vivo study demonstrated that isoflurane attenuated the expression of GABA_A receptors in NTS tissue. Therefore, we speculated that the enhanced GABA-mediated excitatory neurotransmission may occur through a decrease in GABA_A receptor expression to depress circulation.

In conclusion, we examined the effects of inhaled isoflurane on circulation and compared these effects with urethane anesthesia. Isoflurane significantly decreased BP and HR and attenuated GABA_A receptor expression in the NTS. The NTS lost circulatory responses to microinjections of GABA_A receptor agonists and antagonists, which was different from urethane-anesthetized rats. These effects of isoflurane within the NTS suggest that the depressed cardiovascular function was induced by the inhibition of GABA_A receptor expression in the NTS.

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Disclosure of conflict of interest

None.

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