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Isoflurane is a Potent Modulator of Extrasynaptic GABA_A Receptors in the Thalamus

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Abstract

Volatile anesthetics are used clinically to produce analgesia, amnesia, unconsciousness, blunted autonomic responsiveness, and immobility. Previous work has shown that the volatile anesthetic isoflurane, at concentrations that produce unconsciousness (250-500 μM), enhances fast synaptic inhibition in the brain mediated by GABA_A receptors (GABA_A-Rs). In addition, isoflurane causes sedation at concentrations lower than those required to produce unconsciousness or analgesia. In this study, we found that isoflurane, at low concentrations (25 – 85 μM) associated with its sedative actions, elicits a sustained current associated with a conductance increase in thalamocortical neurons in the mouse ventrobasal (VB) nucleus. These isoflurane-evoked currents reversed polarity near to the Cl⁻ equilibrium potential and were totally blocked by the GABA_A-R antagonist gabazine. Isoflurane (25 – 250 μM) produced no sustained current in VB neurons from GABA_A-R α_4 subunit knockout (*Gabra4*^{-/-}) mice, although 250 μM isoflurane enhanced synaptic inhibition in VB neurons from both wild-type and *Gabra4*^{-/-} mice. These data indicate an obligatory requirement for α_4 subunit expression in the generation of the isoflurane-activated current. In addition, isoflurane directly activated $\alpha_4\beta_2\delta$ GABA_A-Rs expressed in HEK293 cells, and was more potent at $\alpha_4\beta_2\delta$ than at $\alpha_1\beta_2\gamma_2$ receptors (the presumptive extrasynaptic and synaptic GABA_A-R subtypes in VB neurons). We conclude that the extrasynaptic GABA_A-Rs of thalamocortical neurons are sensitive to low concentrations of isoflurane. In view of the crucial role of the thalamus in sensory processing, sleep and cognition, the modulation of these extrasynaptic GABA_A-Rs by isoflurane may contribute to the sedation and hypnosis associated with low doses of this anesthetic agent.

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Introduction

General anesthesia is characterized by a variety of behavioral end-points, including amnesia, analgesia, sedation, blunted autonomic responsiveness, unconsciousness and immobility (Campagna et al., 2003). In all cases, the dose of general anesthetic required to produce amnesia, sedation and hypnosis is much lower than that required to produce immobility. For example, the commonly used inhaled anesthetic, isoflurane, prevents voluntary response to spoken commands in human subjects (Dwyer et al., 1992) at concentrations that are only 30-40% of the levels required for immobility in response to a painful stimulus.

In the waking state, the thalamus is involved in the processing and relay of sensory information to the cortex, and is also important in the maintenance of high frequency synchronous oscillatory activity that is associated with attention (Steriade et al., 1993; von Krosigk et al., 1993; Steriade, 2000; Ribary, 2005). During slow-wave sleep, however, relay neurons in the thalamus are hyperpolarized and generate 1-3Hz (delta wave) activity that entrains neurons in the cortex (Steriade, 2000; Steriade, 2003).

The thalamo-corticothalamic loop has therefore long been identified as a key target for anesthetic action to induce sedation and hypnosis (Angel, 1993; Alkire et al., 2000; White and Alkire, 2003; Alkire and Miller, 2005). *In vivo*, the firing rate of thalamocortical relay cells is strongly suppressed by isoflurane (Detsch et al., 1999), and these *in vivo* inhibitory effects of isoflurane can be reversed by local application of the GABA_A receptor (GABA_A-R) antagonist bicuculline (Vahle-Hinz et al., 2001), indicating that GABA_A-Rs contribute significantly to the effects of the anesthetic on the firing of thalamic neurons, although other mechanisms involving K⁺ conductances have also been proposed to contribute to anesthetic effects in the thalamus (Ries and Puil, 1999).

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In addition to the “classical” GABA_A-Rs that mediate synaptic inhibition, an additional population of GABA_A-Rs have recently been demonstrated to occur at extra-synaptic sites in the CNS, and a population of such receptors exists on thalamocortical relay neurons (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005; Bright et al., 2007). These extrasynaptic GABA_A-Rs, (consisting mainly of α_4 , β_2 , and δ subunits) are persistently activated by low concentrations of GABA and have distinct pharmacological properties that differentiate them from the synaptic GABA_A-Rs in relay neurons of the thalamus (which consist mainly of α_1 , β_2 , and γ_2 subunits) (Jia et al., 2005). GABA-mediated tonic inhibition in thalamic neurons requires expression of the GABA_A-R α_4 subunit, as tonic currents are absent in thalamic relay neurons from GABA_A-R α_4 subunit knockout (*Gabra4*^{-/-}) mice (Chandra et al., 2006). The inhibitory function of these extrasynaptic GABA_A-Rs has been shown to be enhanced by the novel hypnotic gaboxadol and by the intravenous anesthetic etomidate (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005).

In the present study, we investigated the actions of isoflurane on thalamocortical neurons in the mouse ventrobasal (VB) thalamus. We found that isoflurane, at clinically relevant concentrations (25 - 250 μ M) (Franks and Lieb, 1994), evoked sustained currents in VB neurons. These sustained currents were blocked by gabazine, a selective antagonist of GABA_A-Rs. In addition, we examined the actions of isoflurane on GABA_A-Rs expressed in HEK 293 cells, using subunit compositions chosen to resemble synaptic and extrasynaptic GABA_A-Rs found in the thalamus. We found that isoflurane directly activated the “extrasynaptic” $\alpha_4\beta_2\delta$ receptors and showed greater potency at $\alpha_4\beta_2\delta$ receptors than at the “synaptic” $\alpha_1\beta_2\gamma_2\delta$ receptors. The isoflurane-activated current was absent in VB neurons from *Gabra4*^{-/-} mice, which do not express extrasynaptic GABA_A-Rs (Chandra et al., 2006). Our data suggest that isoflurane is a potent modulator of extrasynaptic GABA_A-Rs in VB neurons.

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Methods

Electrophysiological recordings in brain slices

Experiments were performed in accordance with institutional and federal guidelines. Mice between 22 and 50 days old (C57BL/6, Gabra4^{+/+} and Gabra4^{-/-}) were anesthetized with halothane and sacrificed. The brains were quickly removed and placed in ice-cold slicing solution, which contained (in mM): 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 220 sucrose, 11 glucose, 10 MgSO₄ and 0.5 CaCl₂, before cutting horizontal slices (300 μm thick) on a microslicer (VT 1000S, Leica, Wetzlar, Germany).

Slices were perfused with carbogenated artificial cerebrospinal fluid (aCSF), which contained (in mM): 124 NaCl, 2.5 KCl, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose. Whole-cell patch clamp recordings from visually identified thalamic neurons were performed using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) at room temperature (20-22 °C) as previously described (Jia et al., 2005). The intracellular solution for voltage-clamp recordings contained (in mM): 140 CsCl, 4 NaCl, 1 MgCl₂, 10 HEPES, 0.05 EGTA, 2 ATP-Mg, and 0.4 GTP-Mg; pH was adjusted to 7.2 with CsOH. Intracellular solution for current-clamp recordings contained (in mM): 130 K⁺-gluconate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2 ATP-K⁺, and 0.3 GTP-Na⁺, pH adjusted to 7.25 with KOH. Spontaneous inhibitory postsynaptic currents (IPSCs) were recorded at -60 mV and isolated by bath application of 2-5 mM kynurenic acid. Access resistance was monitored throughout the recording period; cells were included for analysis only if the series resistance was less than 20 MΩ and the change in series resistance was less than 20% over the course of the experiment. Data were analyzed as described previously (Jia et al., 2005); very briefly, off-line analysis was performed using MiniAnalysis 5.5 (Synaptosoft, Decatur, GA), SigmaPlot 6.0 (SPSS, Chicago,

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IL) and Excel 2000 (Microsoft, Redmond, WA). The holding current shift was measured as the difference in the holding current before and during drug application. Inhibitory postsynaptic currents (IPSCs) were detected and analyzed using MiniAnalysis as described (Jia et al., 2005). Unless otherwise indicated, averaged data are expressed as mean \pm SEM. Statistical significance was assessed using Student's t test or one-way ANOVA with a Dunnett test, and $p < 0.05$ was considered statistically significant.

Recordings from HEK 293 cells expressing recombinant GABA_A-Rs

The cDNAs encoding the human α_1 , mouse α_4 , rat β_2 , human γ_{2s} , and human δ subunits were sub-cloned into the pcDNA3.1 expression vector and transiently expressed in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD), as described in Jia et al. (2005). Ligand-gated currents were recorded at room temperature (voltage clamped at -60 mV) using an Axopatch 200 amplifier (Molecular Devices). The extracellular solution contained (in mM): 145 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 6 D-glucose, 10 HEPES, and pH adjusted to 7.4 with NaOH. The intracellular solution used to fill patch pipettes contained (in mM): 145 N-methyl-D-glucamine hydrochloride, 0.1 CaCl₂, 5 ATP-K, 1.1 EGTA, 2 MgCl₂, 5 HEPES, and pH adjusted to 7.2 with KOH. GABA and/or isoflurane were applied rapidly (~ 50 ms exchange time) to the cell *via* a multi-channel motor-driven solution exchange device (Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA).

Concentration-response amplitude data were analyzed as previously described (Jia et al., 2005); concentration-response data for each individual cell was fitted (using a sum of least squares method) to a Hill equation of the form: $I = I_{max} \times [agonist]^{nH} / ([agonist]^{nH} + EC_{50}^{nH})$; where I is the peak current, I_{max} is the maximum whole-cell current amplitude, $[agonist]$ is the

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agonist concentration, EC_{50} is the agonist concentration eliciting a half-maximal current response, and n_H is the Hill coefficient. Peak current in response to an EC_{20} concentration of GABA alone ($I_{EC_{20}}$) was defined as the control response and isoflurane was then pre-applied for 20s prior to co-application with GABA(EC_{20}) to ensure that isoflurane had reached equilibrium with the receptors. Isoflurane-induced potentiation was calculated as the percentage increase in peak current relative to control. Currents elicited directly during pre-application of isoflurane were measured and normalized to $I_{EC_{20}}$ in order to quantify the direct activation of GABA_A receptors by isoflurane in the absence of GABA. Statistical significance was assessed using a one-way ANOVA with a Dunnett's multiple comparison post-test. Data are presented as mean \pm SEM.

Drugs and preparation of volatile anesthetic solutions

GABA (4-aminobutanoic acid), gabazine (4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid hydrobromide) and kynurenic acid (4-oxo-1H-quinoline-2-carboxylic acid) were purchased from Sigma (St. Louis, MO). Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) was obtained from Abbott Laboratories (North Chicago, IL). A stock solution of 10 mM GABA was prepared daily. Isoflurane solutions were prepared by injection of liquid anesthetic with a gas-tight syringe (Hamilton, Reno, NV) into intravenous solution bags containing 100 ml extracellular solution and were used within two hours (Krasowski and Harrison, 2000). Isoflurane solutions were applied to the brain slice preparations *via* perfusion through polytetrafluoroethylene tubing; the drug-containing solution was applied to the HEK cells locally using a rapid solution changer. We have previously shown that losses of anesthetic to the air and the tubing using this approach are less than 5% (Krasowski and Harrison, 2000).

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Generation and use of α_4 subunit knockout mice

α_4 subunit gene knockout mice were generated as previously described (Chandra et al., 2006). All knockout ($Gabra4^{-/-}$) and wild-type ($Gabra4^{+/+}$) littermates used were age-matched and were on the same genetic background (129X1/S1 x C57BL/6J hybrid; F2-F6 generations). Experimenters were blind to genotype in all studies.

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Results

Low concentrations of isoflurane enhance tonic inhibition in VB neurons

Voltage-clamp recordings were made in VB relay neurons held at -60 mV using a CsCl-based intracellular solution. A low concentration of isoflurane (25 μ M) elicited a sustained inward current (Figure 1A); the mean amplitude of this current was 10 ± 2 pA ($n = 14$). At higher concentrations, isoflurane elicited larger currents (85 μ M: 23 ± 3 pA, $n = 14$; 250 μ M: 51 ± 4 pA, $n = 17$; Figures 1B and C).

We next examined whether the currents induced by isoflurane were mediated by GABA_A-Rs. As shown in Figure 2A, 20 μ M gabazine, a specific GABA_A-R antagonist, not only completely blocked inhibitory synaptic currents (IPSCs), but also blocked the inward current elicited by isoflurane (250 μ M), indicating that these isoflurane-activated tonic currents are mediated by GABA_A-Rs. The additional outward current induced by the addition of gabazine (Figure 2A) indicates the presence of a persistent background current in this neuron, consistent with the reports of tonic inhibition in thalamocortical relay neurons (Belelli *et al.*, 2005; Cope *et al.*, 2005; Jia *et al.*, 2005; Chandra *et al.*, 2006; Bright *et al.*, 2007). Isoflurane is able to directly activate GABA_A-Rs (Yang *et al.*, 1992; see also Figure 5), as well as potentiate the action of GABA at these receptors (Jones and Harrison, 1993, see also Figure 5), and so for the sake of simplicity, we will refer to the sustained current recorded from neurons in brain slices as the “tonic current” or “isoflurane-activated current”.

We then performed experiments using a voltage ramp protocol to compare the reversal potential of isoflurane-activated currents (E_{Iso}) to the Cl⁻ equilibrium potential (E_{Cl}). Currents induced by a slow ramp voltage command (+40 to -40 mV, 10 seconds) were recorded before and after the perfusion of 250 μ M isoflurane, and E_{Iso} was calculated from the subtracted traces

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as shown in Figure 2B; E_{Iso} was 5 ± 1 mV ($n = 6$), which is very close to the predicted E_{Cl} (2.1 mV), calculated using the Nernst equation. These results support our interpretation that isoflurane-induced currents arise via the enhancement of a chloride conductance mediated by GABA_A -Rs.

Isoflurane prolongs the decay time of IPSCs

We also examined the effects of isoflurane on synaptic GABA_A -Rs in the thalamus. In thalamic VB neurons, synaptic GABA_A -Rs consist primarily of α_1 , β_2 , and γ_2 subunits (Jia et al., 2005). Spontaneous IPSCs were readily observed in VB neurons and were completely blocked by gabazine (Figure 2A). Isoflurane (25 - 250 μM) had no effect on either the amplitude or frequency of these IPSCs (Figure 3). Low concentrations of isoflurane (25 and 85 μM), isoflurane had little to no effect on the decay time of IPSCs (% change: 25 μM , $4 \pm 4\%$, $n = 10$; 85 μM , $28 \pm 6\%$, $n = 10$), but a higher concentration of 250 μM isoflurane significantly increased IPSC decay time ($133 \pm 12\%$, $n = 10$). Only the higher concentrations of isoflurane (85-250 μM), therefore, prolong IPSCs mediated by synaptic GABA_A -Rs in VB relay neurons, and these findings are completely consistent with observations made in hippocampal neurons (Jones and Harrison, 1993; Banks and Pearce, 1999; Nishikawa and MacIver, 2001; Caraiscos et al., 2004; Verbny et al., 2005).

Isoflurane decreases the excitability of VB neurons

From a resting membrane potential of about -75 mV, most VB neurons in our recordings displayed “burst” firing in response to depolarizing current injection (Llinás and Jahnsen, 1982). In order to facilitate measurements of firing rate, we therefore depolarized the membrane

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potential to about -60 mV by constant current injection. At this membrane potential, VB neurons were generally silent but displayed sustained action potential (AP) firing in response to injection of depolarizing current. The amplitude of the current step (500 ms duration) was adjusted to induce ~ 10 APs (Figure 4A), corresponding to a firing frequency of ~ 20 Hz, and we then compared the numbers of APs evoked by depolarizing current steps before and after isoflurane application. Isoflurane ($25 \mu\text{M}$) significantly reduced the number of evoked APs, from 9.5 ± 1.0 to 7.5 ± 1.0 ($p < 0.01$, $n = 6$). In addition to the effect on spike firing, isoflurane ($25 \mu\text{M}$) also decreased the membrane input resistance (R_m) to $90 \pm 2\%$ of control ($p < 0.01$, $n = 6$).

Isoflurane directly activates GABA_A $\alpha_4\beta_2\delta$ receptors expressed in HEK293 cells

The GABA_A-Rs activated at inhibitory synapses onto VB neurons are thought to be of the $\alpha_1\beta_2\gamma_2$ subtype (Zhang et al., 1997; Huntsman and Huguenard, 2000) whereas the extrasynaptic receptors are thought to be $\alpha_4\beta_2\delta$ (Belelli *et al.*, 2005; Jia *et al.*, 2005; Chandra *et al.*, 2006). In order to compare the isoflurane sensitivity of these proposed “synaptic” and “extrasynaptic” GABA_A-R subtypes, we tested the effect of isoflurane on $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_4\beta_2\delta$ GABA_A-Rs expressed in HEK 293 cells. Concentration-response curves for GABA (not shown) revealed that $\alpha_4\beta_2\delta$ receptors (EC_{50} $2.1 \pm 0.1 \mu\text{M}$, $n = 43$) are more sensitive to GABA than $\alpha_1\beta_2\gamma_{2s}$ receptors (EC_{50} : $22.7 \pm 2.1 \mu\text{M}$, $n = 20$; $p < 0.001$), but the maximum GABA current was smaller in $\alpha_4\beta_2\delta$ receptors than in $\alpha_1\beta_2\gamma_{2s}$ receptors (223 ± 16 pA and $1,741 \pm 193$ pA, respectively), all consistent with earlier observations (Jia et al., 2005).

We then examined the modulation of these recombinant GABA_A-R subtypes over a range of concentrations of isoflurane (25 - 800 μM). Isoflurane was applied for 20 seconds to a HEK cell held under voltage clamp prior to co-application with GABA (EC_{20}) for another 20 seconds.

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Typical recordings from both $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$ receptors are shown in Figure 5A and 5B (two different HEK cells for each subtype). At all concentrations tested, isoflurane enhanced the amplitude of the GABA-evoked response to a greater degree in $\alpha_4\beta_2\delta$ receptors than in $\alpha_1\beta_2\gamma_2$ receptors (Figure 5C).

In the presence of isoflurane, the peak current observed in response to GABA is actually the sum of two components, I_{Direct} (*i.e.*, the current induced by isoflurane alone, due to direct receptor activation) and $I_{\text{GABAEC20+ISO}}$ (*i.e.*, the isoflurane-potentiated GABA-evoked current). In a more detailed analysis, we compared these two currents in $\alpha_1\beta_2\gamma_2$ and $\alpha_4\beta_2\delta$ receptors. As shown in Figure 6A, isoflurane, over the entire range of concentrations tested (25 to 800 μM), directly activated $\alpha_4\beta_2\delta$ receptors in the absence of GABA, whereas in $\alpha_1\beta_2\gamma_2$ s receptors, only higher concentrations of isoflurane ($\geq 200 \mu\text{M}$) were able to induce direct currents. In addition, isoflurane (at all concentrations tested) activated proportionally larger direct currents ($I_{\text{Direct}}/I_{\text{GABAEC20}}$) in $\alpha_4\beta_2\delta$ receptors than in $\alpha_1\beta_2\gamma_2$ s receptors. At the same time, isoflurane was more potent as a modulator (in terms of potentiation of the response to GABA) at $\alpha_4\beta_2\delta$ receptors than at $\alpha_1\beta_2\gamma_2$ s receptors (Figure 6B). Note that these data illustrate the potentiating effect of isoflurane, calculated by subtracting I_{Direct} (Figure 6A) from the total current (Figure 5C).

Because some of the isoflurane currents were small in amplitude ($\sim 10 \text{ pA}$), we decided to test the possibility that the currents were influenced by the change of solutions and the associated movement artifacts. We therefore used the same protocol, but omitted the isoflurane. Under these test conditions, when switching from one stream of saline to another, there was no significant enhancement of the GABA response ($\alpha_1\beta_2\gamma_2$ s: $0 \pm 10\%$, $n = 5$; $\alpha_4\beta_2\delta$: $-5 \pm 5\%$, $n = 7$) or direct current response to the solution exchange ($\alpha_1\beta_2\gamma_2$ s: $2 \pm 1\%$, $n = 5$; $\alpha_4\beta_2\delta$: $-4 \pm 2\%$, $n = 7$).

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Isoflurane-evoked currents are absent in VB neurons from Gabra4^{-/-} mice

In a final set of experiments, we used a knock-out mouse strain to determine whether extrasynaptic GABA_A-Rs are required for the generation of isoflurane-induced currents in the thalamus. We have previously demonstrated that extrasynaptic GABA_A-Rs are absent in thalamic relay neurons from Gabra4^{-/-} mice (Chandra et al., 2006). No significant isoflurane-evoked currents were detected in VB neurons from Gabra4^{-/-} mice (25 μM: 0 ± 1 pA, n = 10; 85 μM: 0 ± 1 pA, n = 12; 250 μM: 1 ± 1 pA, n = 11). In contrast, wild-type neurons showed measurable isoflurane-evoked currents (25 μM: 8 ± 2 pA, n = 8; 85 μM: 17 ± 3 pA, n = 8; 250 μM: 44 ± 4 pA, n = 9), which were comparable to isoflurane-evoked currents recorded from standard C57BL/6 mice (Figure 7). This difference between the genotypes was highly significant ($p < 0.001$ at all three concentrations of isoflurane). In contrast, we found that the modulation of IPSCs in VB neurons by isoflurane was similar in wild-type and α₄knockout mice. As shown in Figure 7C, isoflurane had a comparable effect on the decay time of IPSCs recorded in VB neurons from wild-type and Gabra4^{-/-} mice. IPSC decay times (in ms) in the absence or presence of isoflurane (at the specified concentration) were (WT vs. KO): control: 15 ± 1 vs. 14 ± 1; 25 μM, 16 ± 2 vs. 15 ± 1; 85 μM, 19 ± 1 vs. 19 ± 2; 250 μM, 33 ± 3 vs. 31 ± 3. These results are consistent with the hypothesis that extrasynaptic (and not synaptic) GABA_A-Rs mediate the isoflurane-evoked tonic current in thalamic neurons.

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Discussion

Volatile anesthetics have been shown to modulate GABA_A-R function in neurons and heterologous expression systems (Jones and Harrison, 1993; Krasowski et al., 1998; Banks and Pearce, 1999; Krasowski and Harrison, 2000; Li and Pearce, 2000; Nishikawa and MacIver, 2001; Nishikawa and Harrison, 2003; Hemmings et al., 2005). In the present study, we found that isoflurane (25 - 250 μ M) induced sustained currents in thalamic relay neurons and that this was completely dependent on the presence of extrasynaptic GABA_A-Rs ($\alpha_4\beta_2\delta$ subtype). Recordings from $\alpha_4\beta_2\delta$ receptors expressed in HEK 293 cells demonstrated that isoflurane not only potentiated the GABA response, but also directly activated these receptors in the absence of GABA. The sustained current evoked by isoflurane in thalamic relay neurons therefore likely results from the sum of the direct activation of extrasynaptic GABA_A-Rs and the enhancement of the action of ambient GABA on these receptors.

General anesthesia in mammals is a complex phenomenon, involving a combination of desirable effects such as amnesia, hypnosis and immobility (Campagna et al., 2003). Although immobilization is commonly used as a measure of anesthetic potency (Hemmings et al., 2005), 3-fold lower concentrations are required for sedation and hypnosis (Dwyer et al., 1992). GABA_A-Rs may not be involved in the immobilizing action of inhaled anesthetics, which seems to occur at the level of the spinal cord, and is resistant to GABA antagonists (Sonner et al., 2003), but there is strong evidence that GABA_A-Rs are involved in the sedation and hypnosis induced by many general anesthetics (reviewed by Hemmings et al., 2005).

The thalamus relays sensory information to the appropriate modality-specific areas in the sensory cortex, and also participates in the transitions between waking and sleep states (Saper et al., 2001; Steriade, 2003). The thalamus is therefore a potential target for the sedative and

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hypnotic actions of volatile anesthetics, and it has been argued previously that the thalamus is a principal site of drug action for generating the anesthetized state (Angel, 1993). A variety of studies support the idea that volatile anesthetics modulate thalamic function. At the whole brain level, positron emission tomography (PET) imaging demonstrates that the volatile anesthetics isoflurane and halothane both produce a particularly large decrease in glucose utilization in the thalamus (White and Alkire, 2003). Consistent with a decrease in metabolic activity, the firing rate of thalamocortical relay cells *in vivo* is strongly suppressed by anesthetic concentrations of isoflurane (Detsch et al., 1999).

A K^+ conductance mechanism has been implicated in the inhibitory actions of isoflurane in the thalamus *in vitro* (Ries and Puil, 1999b; Ries and Puil, 1999a), but in other studies the effect of isoflurane to inhibit spike firing induced by a mechanical-stimulus in the thalamus *in vivo* is blocked by local application of the GABA_A-R antagonist bicuculline (Vahle-Hinz et al., 2001). In the present study, we demonstrate that tonic currents activated by isoflurane in thalamic neurons *in vitro* are mediated by GABA_A-Rs, because the isoflurane-induced currents were totally blocked by a specific GABA_A-R antagonist, gabazine. In addition, the reversal potential of isoflurane-induced currents (E_{Iso}) is close to the calculated equilibrium potential for chloride ions (E_{Cl}) in our experiments, and far from E_K .

Extrasynaptic GABA_A-Rs and tonic inhibition have been observed in the thalamus, hippocampus, dentate gyrus, cortex and cerebellum (Brickley et al., 1996; Nusser and Mody, 2002; Jia et al., 2005; Keros and Hablitz, 2005). The presence of a low concentration of GABA in the extracellular space leads to persistent activation of the extrasynaptic GABA_A-Rs, and the resulting tonic inhibition regulates the excitability of individual neurons and the activity of neural networks (Mody and Pearce, 2004; Semyanov et al., 2004; Farrant and Nusser, 2005; Jia

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et al., 2007). In the thalamus, δ -subunit containing extrasynaptic GABA_A-Rs appear to be a common target for a variety of sedative and hypnotic agents, including gaboxadol (THIP; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005) and tetrahydrodeoxycorticosterone (THDOC) (Cope et al., 2005). Several labs also report that extrasynaptic GABA_A-Rs containing the δ -subunit are sensitive to low concentrations of ethanol (Mody et al., 2007; Olsen et al., 2007; Santhakumar et al., 2007; Smith and Gong, 2007; but see: Borghese and Harris, 2007; Botta et al., 2007).

Of note, not all extrasynaptic GABA_A-Rs contain δ subunits; in hippocampal CA1 pyramidal neurons, for example, one population of extrasynaptic GABA_A-Rs contains the α_5 subunit (Caraiscos et al., 2004). Like extrasynaptic receptors containing δ subunits, α_5 -subunit containing GABA_A-Rs are sensitive to a spectrum of anesthetic agents. At the same concentration of isoflurane as used in the present study, Caraiscos and colleagues observed that 25 μ M isoflurane potentiated the tonic current in cultured hippocampal pyramidal neurons obtained from wild-type, but not α_5 -subunit knockout, mice (Caraiscos et al., 2004). In addition, the intravenous anesthetic etomidate was shown to potentiate tonic currents recorded in cultured hippocampal pyramidal neurons expressing the GABA_A-R α_5 -subunit. In this case, the amnestic, and not sedative-hypnotic, effect of the drug is dependent on α_5 -subunit expression (Cheng et al., 2006). These studies suggest that extrasynaptic GABA_A-Rs are a common molecular target for CNS depressants (Orser, 2006).

In the thalamus, extrasynaptic GABA_A-Rs that contain the δ subunit also seem to require inclusion of the GABA_A-R α_4 subunit as tonic currents are absent in relay neurons from GABA_A-R α_4 subunit knockout (*Gabra4*^{-/-}) mice (Chandra et al., 2006). Our experiments on thalamic

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relay neurons from *Gabra4*^{-/-} mice suggest that this population of extrasynaptic GABA_A-Rs exclusively mediates the sustained currents elicited by low concentrations of isoflurane, although the prolongation of synaptic currents by isoflurane ($\geq 85 \mu\text{M}$) is independent of α_4 subunit expression, since IPSCs were potentiated to a similar extent by isoflurane in wild-type and α_4 -subunit knockout mice. We conclude that the α_4 -subunit containing GABA_A-Rs provide the molecular basis for the isoflurane-induced current, and that this current is completely independent of synaptic GABA_A-Rs.

We examined the pharmacological properties expressed GABA_A-Rs configured so as to resemble natively expressed extrasynaptic ($\alpha_4\beta_2\delta$) receptors, which are more sensitive to GABA than $\alpha_1\beta_2\gamma_{2s}$ receptors (Brown et al., 2002; Storustovu and Ebert, 2006). We found that isoflurane is a more potent modulator at $\alpha_4\beta_2\delta$ GABA_A-Rs (which are analogous to native extrasynaptic receptors) than at $\alpha_1\beta_2\gamma_2$ receptors (which are analogous to native synaptic receptors). One interesting finding is that isoflurane, at a concentration as low as 25 μM , directly activates $\alpha_4\beta_2\delta$ receptors in the absence of GABA. In contrast, only high concentrations of isoflurane ($\geq 200 \mu\text{M}$) are able to induce significant direct currents in $\alpha_1\beta_2\gamma_{2s}$ receptors, consistent with earlier observations (Krasowski et al., 1998; Krasowski and Harrison, 2000; Raines et al., 2003).

In conclusion, we show that extrasynaptic GABA_A-Rs in the thalamus are potently activated by isoflurane. Since the thalamus plays a critically important role in both sensory processing and sleep regulation, we suggest that the sedative and hypnotic actions of isoflurane at sub-anesthetic concentrations may be mediated in part through this population of receptors.

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Footnotes

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Legends for Figures

Figure 1. Isoflurane evokes tonic currents in VB neurons.

A, The left-hand panel shows a small current shift elicited by 25 μ M isoflurane in a VB neuron. The right-hand panel shows the corresponding all-points histograms for two 60 second data epochs; the black and gray histograms illustrate the membrane current in the absence and presence of isoflurane, respectively, and the dashed lines represent the best-fit curves for Gaussian distributions.

B, A much larger current in a VB neuron in response to the application of 250 μ M isoflurane (note the different vertical scale bar here from that in *A*).

C, The concentration-dependent amplitude of tonic currents evoked by isoflurane at different concentrations (25 μ M: 10 ± 2 pA, $n = 14$; 85 μ M: 23 ± 3 pA, $n = 14$; 250 μ M: 51 ± 4 pA, $n = 17$).

Figure 2. Isoflurane-evoked tonic currents are mediated by GABA_A-Rs.

A, The left-hand panel shows the current shift induced by 250 μ M isoflurane, which was totally blocked by the specific GABA_A-R antagonist, gabazine (20 μ M). The right-hand panel shows the corresponding all-points histograms.

B, The left-hand panel shows the whole-cell currents induced by a slow voltage ramp command (shown in inset) in the absence (control; black trace) or presence of 250 μ M isoflurane (gray trace). Right panel shows the subtracted ($I_{\text{isoflurane}} - I_{\text{control}}$) current trace (from data in left panel). The reversal potential (~ 5 mV) is close to the calculated equilibrium potential for Cl⁻ (+2 mV).

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Figure 3. Isoflurane prolongs the decay time of inhibitory postsynaptic currents (IPSCs) mediated by synaptic GABA_A-Rs.

A, Examples of IPSCs recorded in the absence or presence of isoflurane (25 or 250 μM).

B, Averaged IPSCs are shown superimposed. 25 μM isoflurane had no effect on IPSC kinetics, whereas 250 μM isoflurane prolonged the IPSC decay time.

C, Pooled data illustrating the effects of isoflurane on IPSC parameters. The low (25 and 85 μM) concentrations of isoflurane have no effect on any IPSC parameter (decay time: control 13.8 ± 1.4 ms; 25 μM isoflurane, 14.1 ± 1.2 ms; $n = 10$; 85 μM, 17.0 ± 1.3 ms, $n = 10$) while 250 μM isoflurane significantly increased the decay time to 31.0 ± 2.5 ms ($n = 10$, *** $p < 0.001$, one-way ANOVA).

Figure 4. Isoflurane decreases the excitability of VB neurons.

A, Representative current clamp traces demonstrate action potential (AP) firing evoked by a 0.09 nA current step (duration 500 ms) in a VB neuron. Membrane resistance (R_m) was measured by injecting hyperpolarizing current (-0.02 nA). After isoflurane (25 μM) perfusion, AP firing decreased.

B, Pooled data shows that isoflurane (25 μM) reduces the firing rate of VB neurons to $79 \pm 4\%$ of control (**: $p < 0.01$, $n = 6$); isoflurane also reduced R_m from 298 ± 50 MΩ to 272 ± 49 MΩ (**: $p < 0.01$).

Figure 5. Isoflurane is more potent at $\alpha_4\beta_2\delta$ than $\alpha_1\beta_2\gamma_2s$ GABA_A-Rs expressed in HEK 293 cells.

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A, Typical GABA-activated currents are shown before and during isoflurane application in experiments from two different HEK 293 cells expressing $\alpha_1\beta_2\gamma_{2s}$ GABA_A-Rs. The bars above the current traces indicate the period of drug application at the specified concentration.

B, Similar experiments in two different HEK 293 cells expressing $\alpha_4\beta_2\delta$ GABA_A receptors.

C, Averaged concentration-effect curves for the potentiation of GABA by isoflurane in $\alpha_1\beta_2\gamma_{2s}$ (n = 5 - 15) and $\alpha_4\beta_2\delta$ (n = 7 - 16) GABA_A receptors. Isoflurane (25 - 800 μ M) is more potent at $\alpha_4\beta_2\delta$ than $\alpha_1\beta_2\gamma_{2s}$ GABA_A receptors. Total potentiation is expressed as the sum of I_{Direct} and $I_{\text{GABAEC50+Iso}}$, where I_{Direct} is the current induced by isoflurane alone and $I_{\text{GABAEC50+Iso}}$ is the isoflurane-potentiated GABA-evoked current. The curve fits were obtained as described in Methods.

Figure 6. Isoflurane directly activates and potentiates GABA-evoked responses in, $\alpha_4\beta_2\delta$ receptors.

A, Normalized concentration-effect curves for the direct activation of current by isoflurane in $\alpha_1\beta_2\gamma_{2s}$ (n = 5 - 15) and $\alpha_4\beta_2\delta$ (n = 7 - 16) GABA_A receptors.

B, Isoflurane concentration-dependent GABA response potentiation curves for $\alpha_1\beta_2\gamma_{2s}$ and $\alpha_4\beta_2\delta$ GABA_A receptors.

Figure 7. Isoflurane-evoked tonic currents are absent in VB neurons from Gabar4^{-/-} mice.

A, Isoflurane (250 μ M) evoked a marked tonic current (~50 pA) in a VB neuron from a wild-type mouse. Isoflurane also increased the decay time of the IPSC (from the time points indicated; the trace shown is the ensemble average of more than 100 individual IPSCs).

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B, Isoflurane (250 μ M) had no effect on the tonic current in a VB neuron from an α_4 knockout mouse, but did prolong the IPSC.

C, IPSC decay time is modulated by isoflurane to the same extent in VB neurons from wild-type and Gabar4^{-/-} mice. The percentage change (from control) in IPSC decay time was: 25 μ M (WT vs. KO): 7 ± 6 %, $n = 7$ vs. 2 ± 3 %, $n = 10$; 85 μ M: 25 ± 5 %, $n = 7$ vs. 27 ± 5 %, $n = 10$; 250 μ M: 130 ± 15 %, $n = 6$ vs. 123 ± 12 % $n = 6$.

D, Isoflurane evokes tonic currents in VB neurons from wild-type, but not Gabar4^{-/-}, mice.

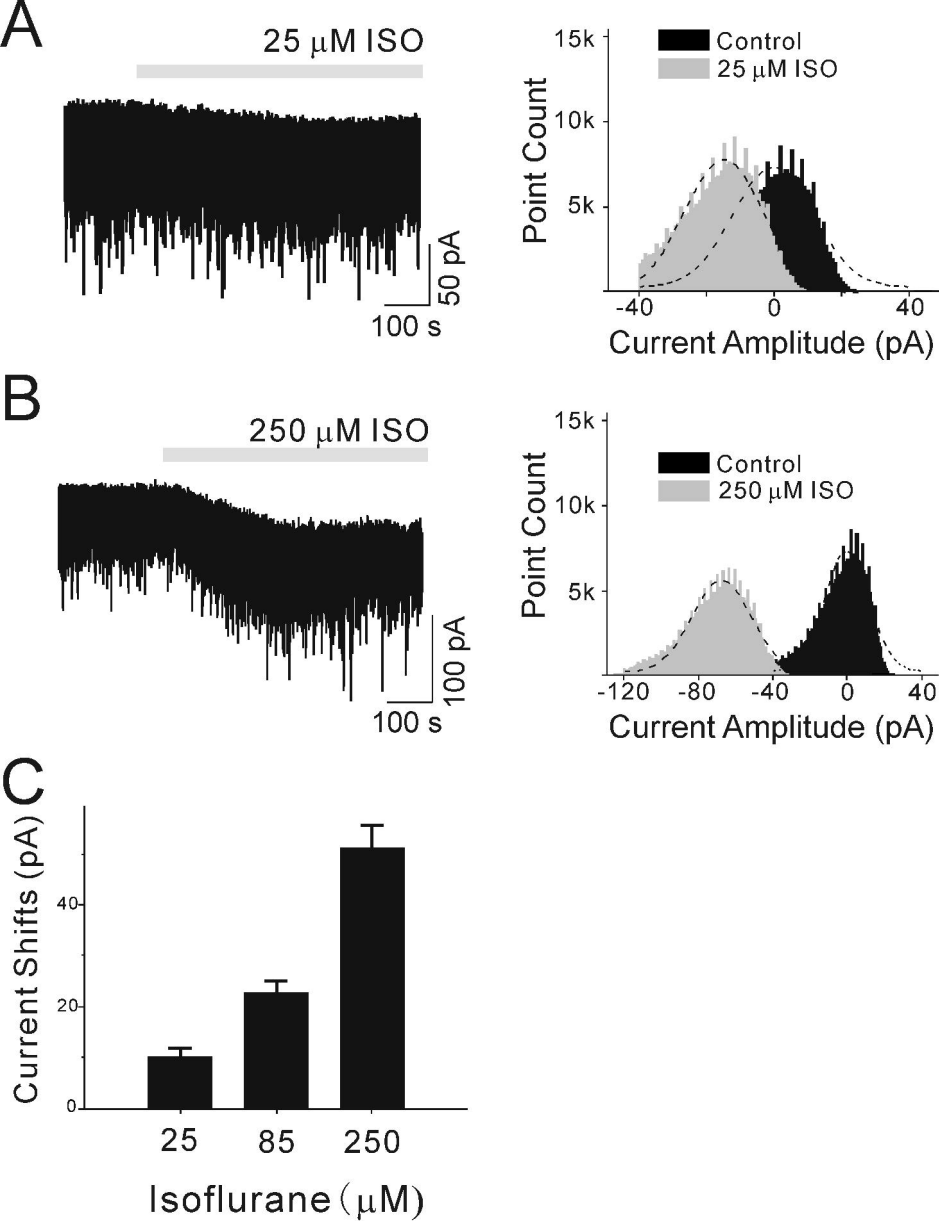
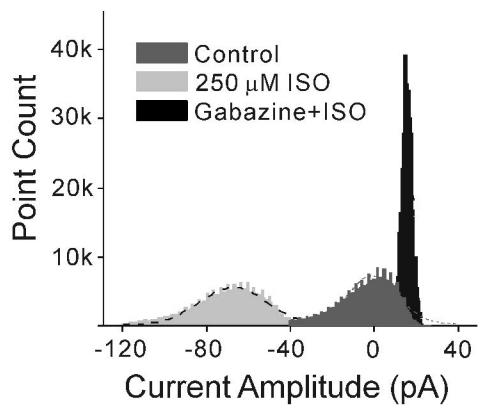
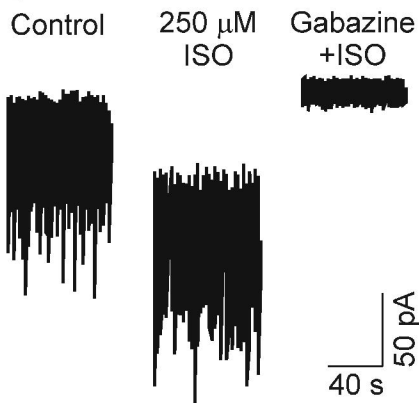
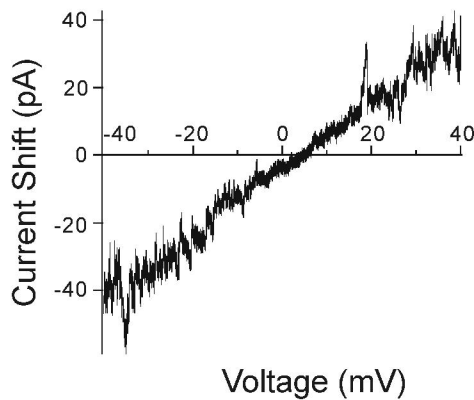
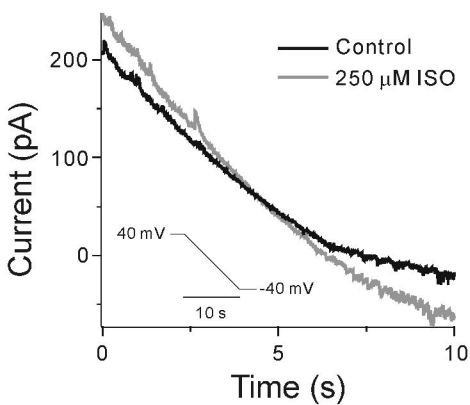
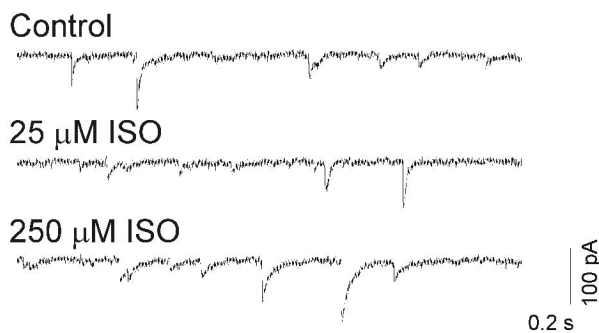
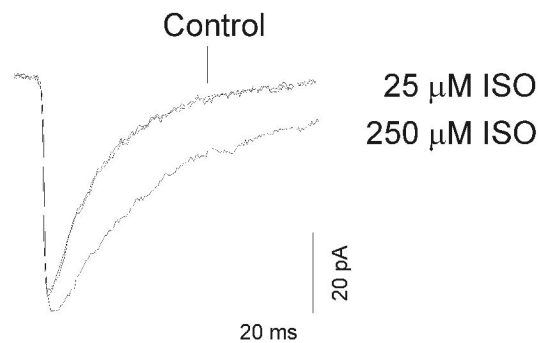
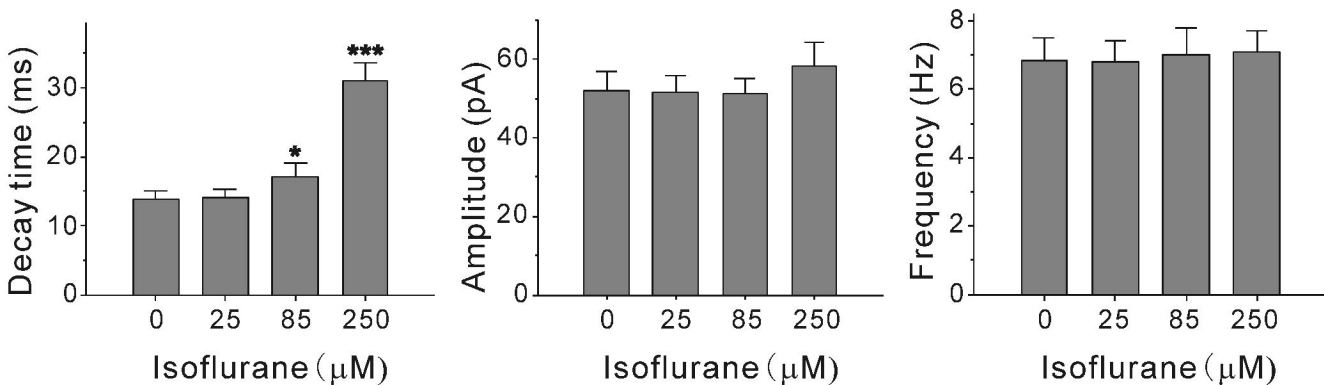


Figure 1

A**B****Figure 2**

A**B****C****Figure 3**

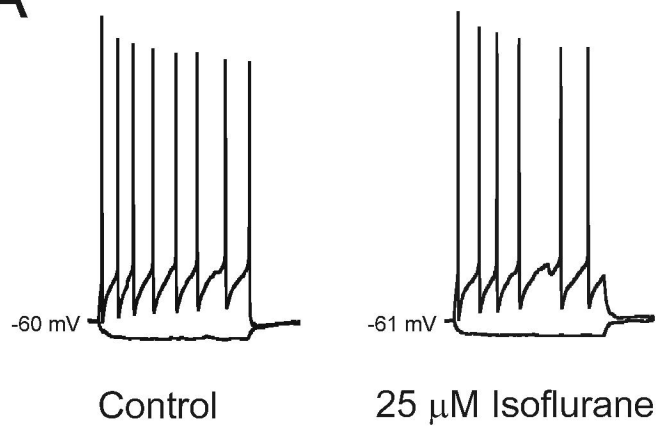
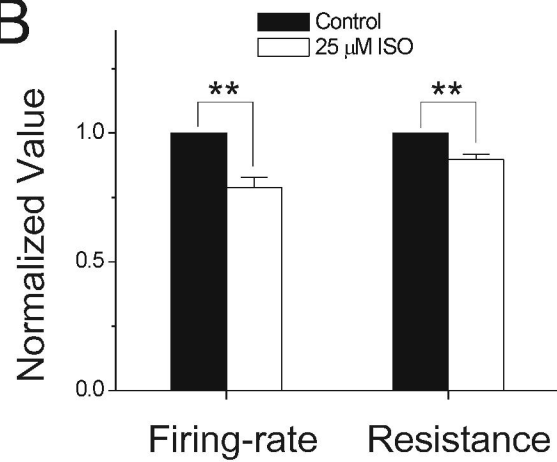
A**B**

Figure 4

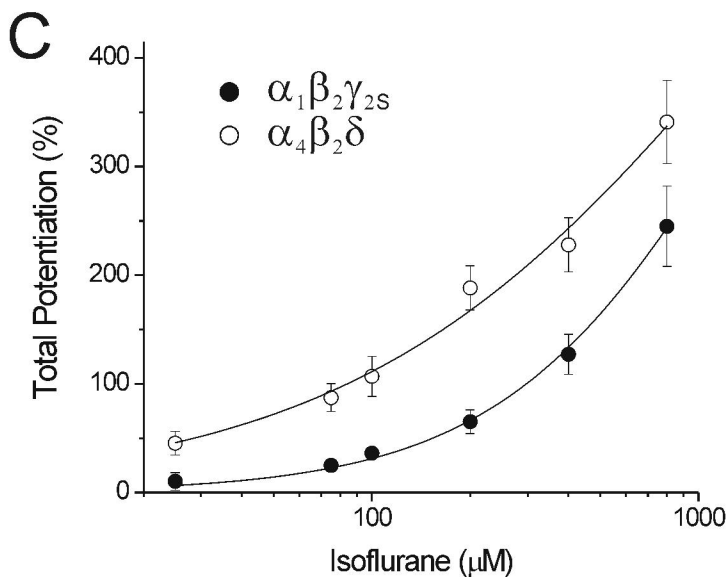
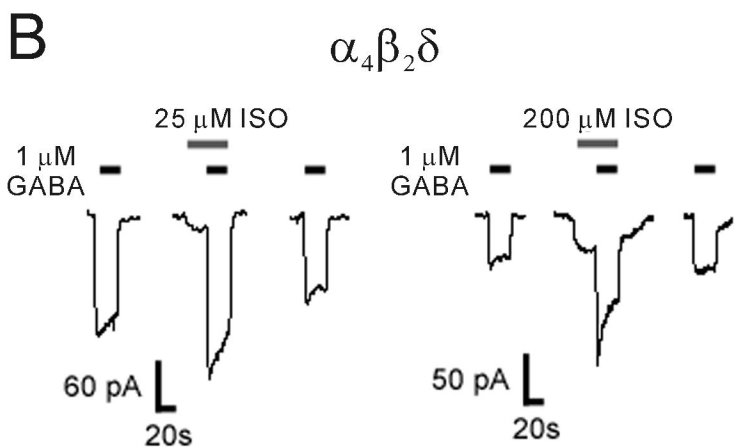
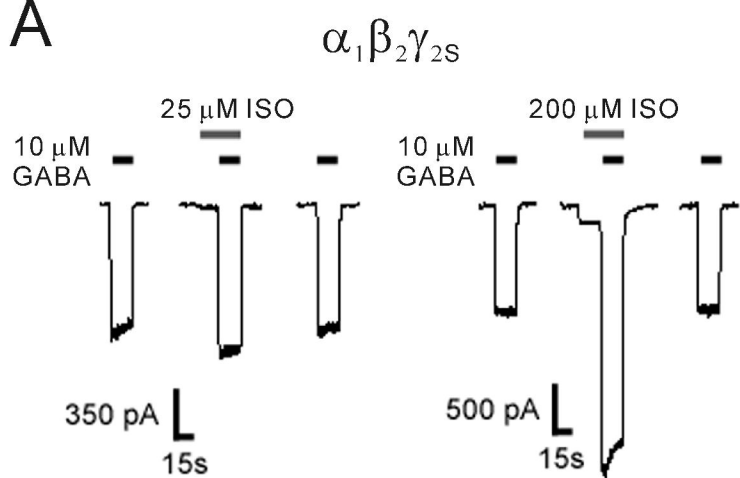
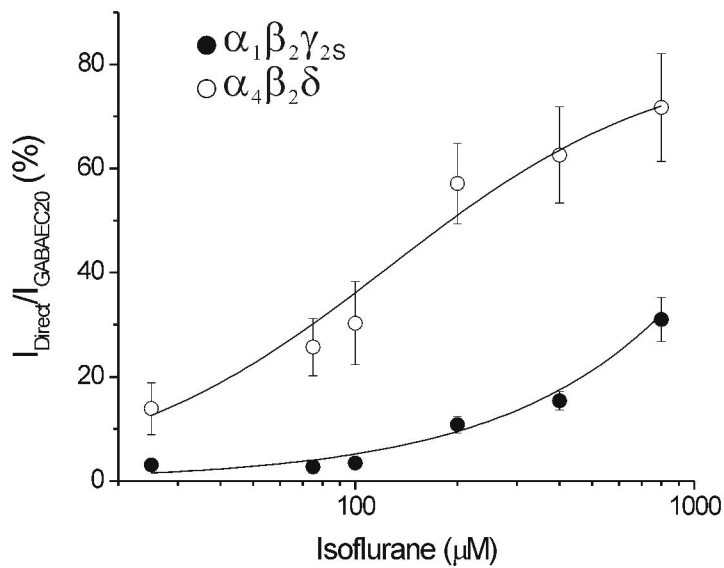
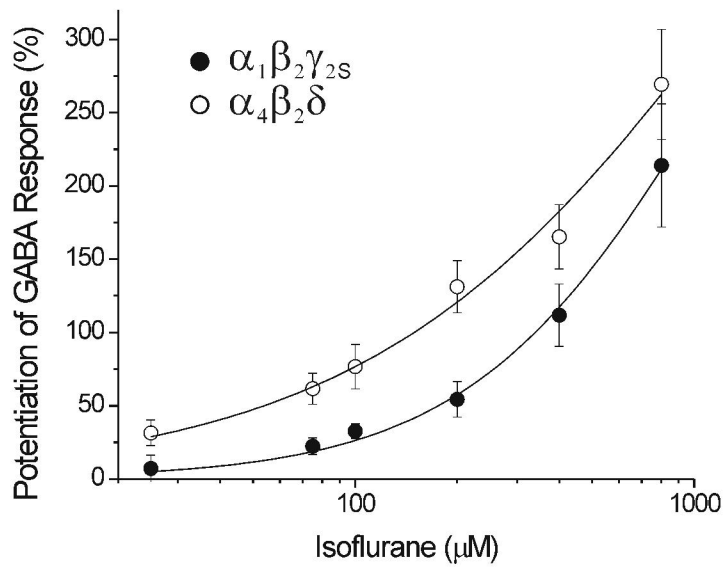


Figure 5

A**B****Figure 6**

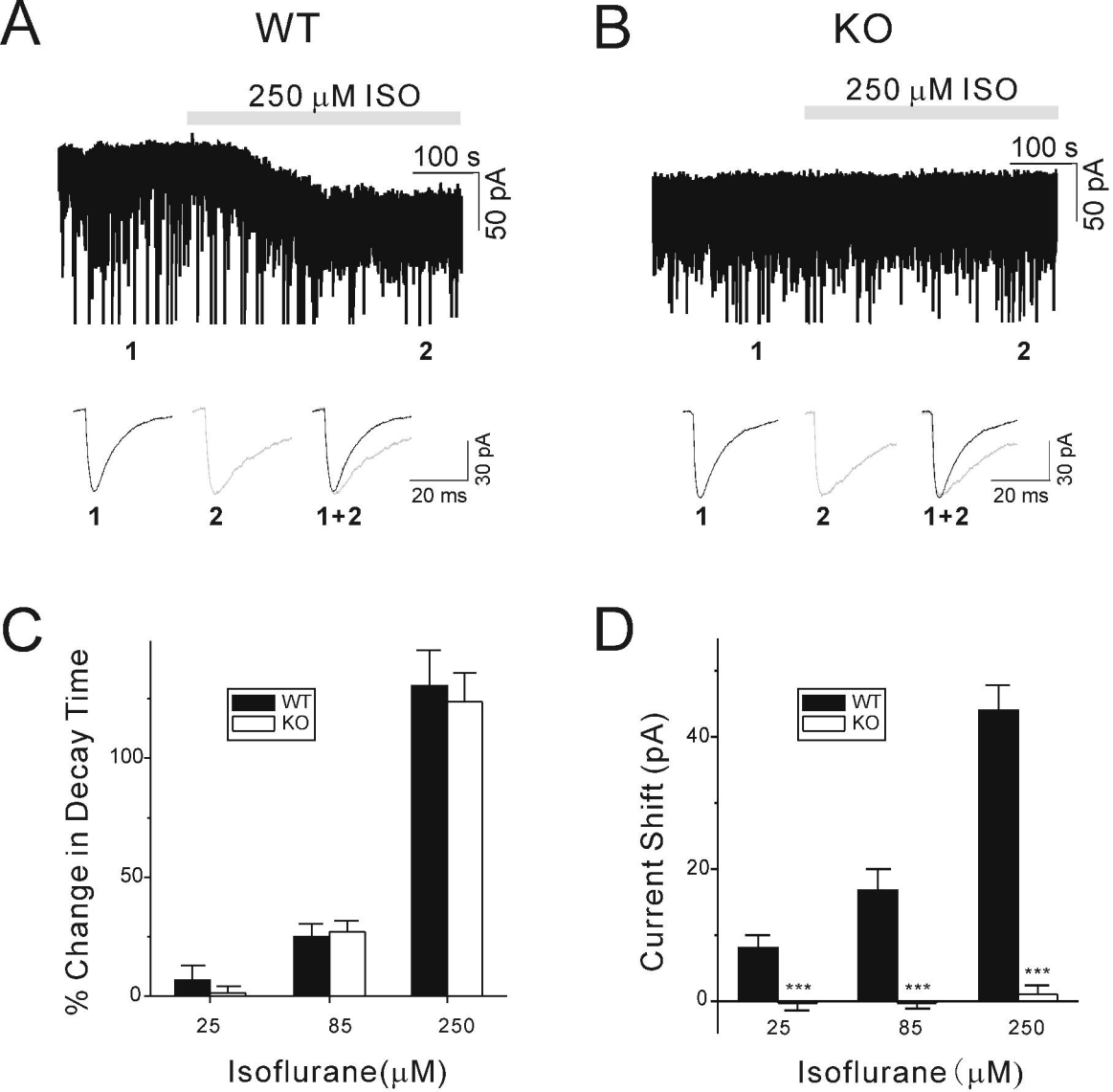


Figure 7