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Isoflurane modulates excitability in the mouse thalamus *via* GABA-dependent and GABA-independent mechanisms

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Summary

GABAergic neurons in the reticular thalamic nucleus (RTN) synapse onto thalamocortical neurons in the ventrobasal (VB) thalamus, and this reticulo-thalamocortical pathway is considered an anatomic target for general anesthetic-induced unconsciousness. A mutant mouse was engineered to harbor two amino acid substitutions (S270H, L277A) in the GABA_A receptor (GABA_A-R) $\alpha 1$ subunit; this mutation abolished sensitivity to the volatile anesthetic isoflurane in recombinant GABA_A-Rs, and reduced *in vivo* sensitivity to isoflurane in the loss-of-righting-reflex assay. We examined the effects of the double mutation on GABA_A-R-mediated synaptic currents and isoflurane sensitivity by recording from thalamic neurons in brain slices. The double mutation accelerated the decay, and decreased the $\frac{1}{2}$ width of, evoked inhibitory postsynaptic currents (eIPSCs) in VB neurons and attenuated isoflurane-induced prolongation of the eIPSC. The hypnotic zolpidem, a selective modulator of GABA_A-Rs containing the $\alpha 1$ subunit, prolonged eIPSC duration regardless of genotype, indicating that mutant mice incorporate $\alpha 1$ -subunit containing GABA_A-Rs into synapses. In RTN neurons, which lack the $\alpha 1$ subunit, eIPSC duration was longer than in VB, regardless of genotype. Isoflurane reduced the efficacy of GABAergic transmission from RTN to VB, independent of genotype, suggesting a presynaptic action in RTN neurons. Consistent with this observation, isoflurane inhibited both tonic action potential and rebound burst firing in the presence of GABA_A-R blockade. The suppressed excitability in RTN neurons is likely mediated by isoflurane-enhanced Ba²⁺-sensitive, but 4-aminopyridine-insensitive, potassium conductances. We conclude that isoflurane enhances inhibition of thalamic neurons in VB *via* GABA_A-R-dependent, but in RTN *via* GABA_A-R-independent, mechanisms.

Keywords

volatile anesthetic; GABA_A receptor; potassium channel; reticular thalamic nucleus (RTN); VB

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1. INTRODUCTION

The thalamus regulates levels of consciousness (Llinás and Ribary, 2001; Ribary, 2005; Steriade, 2000) and has been proposed as an anatomic substrate underpinning the state of unconsciousness associated with general anesthesia (Alkire et al., 2000; Alkire and Miller, 2005; Angel, 1993; Kaisti et al., 2003; White and Alkire, 2003). One proposed mechanism underlying that effect is modulation of GABA_A receptors (GABA_A-Rs) on thalamic neurons (Detsch et al., 2002; Vahle-Hinz et al., 2001). Phasic and tonic inhibition of thalamocortical relay neurons is mediated by distinct populations of synaptic and extrasynaptic GABA_A-Rs (Belelli et al., 2005; Chandra et al., 2006; Cope et al., 2005; Jia et al., 2005) that consist of different subunit combinations (Belelli et al., 2005; Jia et al., 2005) and references therein).

The volatile anesthetic isoflurane prolongs the decay time of GABA_A-R-mediated inhibitory postsynaptic currents (IPSCs) in hippocampal neurons (Banks and Pearce, 1999; Jones and Harrison, 1993; Nishikawa and MacIver, 2001). In heterologously expressed GABA_A-Rs, serine270 (S270) in the second transmembrane (TM2) segment of the GABA_A-R α 1 (and α 2) subunit influences receptor gating and isoflurane-induced potentiation (Koltchine et al., 1999; Mihic et al., 1997; Nishikawa et al., 2002). In heterologously expressed GABA_A-Rs, when α 1S270 is replaced by histidine (S270H), the GABA concentration-response curve (CRC) is left-shifted compared to wild-type, and the potentiating effects of isoflurane are abolished (Koltchine et al., 1999; Mihic et al., 1997; Nishikawa et al., 2002). In contrast, when leucine277 (L277) is replaced by alanine (L277A), the GABA CRC is right-shifted and sensitivity to isoflurane is retained (Topf et al., 2003).

Those studies led to the prediction that in α 1-subunit-containing GABA_A-Rs harboring both the S270H and L277A mutations (α 1S270H/L277A), GABA sensitivity should be relatively unchanged while isoflurane sensitivity should be reduced. Indeed, the double mutation (α 1S270H/L277A) does produce a receptor with preserved GABA sensitivity but which is relatively insensitive to isoflurane (Borghese et al., 2006). Hippocampal CA1 neurons express a variety of GABA_A-Rs (Fritschy and Möhler, 1995; Sperk et al., 1997) including those containing α 1 subunits (Goldstein et al., 2002). In brain slices taken from the double knock-in (HA/HA) mouse (Borghese et al., 2006), we found that miniature IPSCs recorded in hippocampal neurons from HA/HA mice are less sensitive to isoflurane modulation than those recorded in wild-type (WT) littermates (Sonner et al., 2007). HA/HA mice require significantly greater inspired concentrations of isoflurane to inhibit the righting reflex (a surrogate marker for anesthetic-induced unconsciousness) than WT littermates (Sonner et al., 2007), suggesting that α 1 subunit-containing GABA_A-Rs contribute to isoflurane-induced unconsciousness.

Anesthetics modulate excitability in thalamic neurons through multiple mechanisms, and these neurons are part of a pathway intimately linked to the control of states of consciousness (Llinás and Ribary, 2001; Ribary, 2005; Steriade, 2000). We used the HA/HA mouse model to examine the contribution of GABA to isoflurane's effects in the thalamus, by virtue of the ability of the anesthetic to prolong synaptic inhibition mediated by α 1-subunit containing GABA_A-Rs. We investigated the effects of isoflurane on synaptic transmission and neuronal excitability in acutely prepared brain slices from WT and HA/HA mice.

2. Materials and methods

2.1 Mutant mouse production

Mutant mice harboring the serine270 to histidine (S270H) and leucine277 to alanine (L277A) mutations were produced using ES cell and gene targeting technologies as described previously (Borghese et al., 2006). Offspring from heterologous mating pairs were genotyped using standard Southern blotting techniques of tail biopsy tissue.

2.2 Electrophysiology

All experiments were approved by the institutional animal care and use committee at Weill Cornell Medical College and were performed in accordance with federal guidelines. Brain slice preparations and electrophysiological experiments were carried out as previously described (Ying and Goldstein, 2005; Ying et al., 2007) with slight modifications. Briefly, both horizontal and 45° angled brain slices were prepared from wild-type and homozygous knock-in mice (P60-90), and were incubated in artificial cerebral spinal fluid (ACSF) solution saturated with 95% O₂/5% CO₂ at 34 °C for 60 min and at room temperature (22-24 °C) for at least 60 min prior to use. Whole cell patch recordings were made in both voltage and current clamp modes at room temperature using an Axopatch 700B amplifier (Molecular Devices Corporation, Sunnyvale, CA).

2.3 Extracellular electrical stimulation

Electrical stimulation to evoke synaptic responses in thalamic neurons has been described previously (Ying and Goldstein, 2005; Ying et al., 2007). Briefly, to evoke IPSCs in VB neurons, a concentric bipolar tungsten electrode was placed in the somatosensory sector of the RTN, and to evoke IPSCs in RTN neurons, the stimulation electrode was placed in the RTN; stimulus parameters were adjusted before drug application. Our previous experiments indicated that the amplitude and time constant of evoked IPSCs (eIPSCs) are related to stimulus current intensity and duration as well as the distance between recording and stimulation sites. To facilitate comparisons between data obtained from wild-type and knock-in mice, care was taken to maintain a constant distance. The stimulating electrode was positioned using a fine adjustment micro-drive, after the whole-cell configuration was obtained. eIPSCs occurred with a latency of 2.8–3.0 ms in VB and 2.2–2.3 ms in RTN; responses were considered monosynaptic if the latency jitter was < 0.4 ms and the eIPSC rise times were consistent between trials (three trials). Gabazine (10 μM) was used to block responses mediated by GABA_A-Rs in the presence of a GABA_B receptor antagonist (2-OH-saclofen; 100 μM). In four VB neurons, RTN stimulation simultaneously evoked both IPSCs and excitatory postsynaptic currents (EPSCs), in which case ionotropic glutamate receptor antagonists (6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 20 μM) and D-2-amino-5-phosphopentanoic acid (D-AP5, 40 μM) were used to block EPSCs.

2.4 Solutions and chemicals

The artificial cerebrospinal fluid (aCSF) used for current-clamp and voltage-clamp recordings contained (in mM): 117 NaCl, 25 NaHCO₃, 3.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 2.5 CaCl₂ and 11 glucose; osmolarity was adjusted to 300 mOsm with sucrose. Intracellular solution for current-clamp recordings contained (in mM): 130 K⁺-gluconate, 5 NaCl, 2 MgCl₂, 10 HEPES, 0.5 EGTA, 2 ATP-K⁺, 0.3 GTP-Na⁺, pH adjusted to 7.25 with KOH. In another set of voltage-clamp recordings that studied potassium channel currents in RTN neurons, the same K⁺-gluconate-based solution was used; bath solution contained 2, 5 or 7 mM KCl, and the calculated Nernst equilibrium potentials for the K⁺ current are −105, −82 and −74 mV, respectively (in the absence of isoflurane). Voltage-clamp recordings of inhibitory postsynaptic currents (IPSCs) were made using a Cs⁺-based internal solution (Goldstein et al., 2002). Liquid junction potentials for all experiments were calculated, based on pipette and bath solutions, and were corrected off-line (Ying and Goldstein, 2005).

Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane; Abbott Laboratories, North Chicago, IL) was added to aCSF previously equilibrated with 95% O₂/5% CO₂ to obtain the desired concentration; isoflurane-containing solutions were kept in air-tight glass syringes and applied to brain slices *via* perfusion through polytetrafluoroethylene tubing to minimize anesthetic loss (Elsen et al., 2006).

Gabazine, CNQX, and D-AP5 were from Tocris Cookson (Ellisville, MO) and tetrodotoxin (TTX) was from Alomone Labs (Jerusalem, Israel); all other salts were from Sigma (St. Louis, MO).

2.5 Data Analysis

Membrane voltages and currents were analyzed using both Clampfit 9.2 and MiniAnalysis 6 (Synaptosoft, Decatur, GA). The IPSC decay phase was fit using a two exponential function, and a weighted decay time constant (τ_w) was calculated using the formula: $\tau_w = [A_1 \times T_1 + A_2 \times T_2]/(A_1 + A_2)$, where A_1 and A_2 are the current amplitudes, and T_1 and T_2 are the time constants, of the first and second exponential components, respectively (Ying and Goldstein, 2005). SigmaPlot 8.0 (SPSS, Chicago, IL) was used to generate figures and graphs. Averaged data are expressed as mean $\pm$ SEM; statistical significance was assessed using SigmaStat 3.01. Student's *t*-test or one-way ANOVA with pairwise comparisons was performed when appropriate, and $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Evoked IPSCs in VB and RTN neurons from knockin and wild-type mice

GABAergic neurons of the RTN form synapses on glutamatergic thalamocortical relay neurons in the VB, and are the sole source of GABAergic inhibition in the rodent VB, which is devoid of local interneurons (Arcelli et al., 1997). The $\alpha 1$ subunit is the most abundant of the GABA_A-R α subunits present in VB neurons (Fritschy and Möhler, 1995; Pirker et al., 2000), and IPSCs recorded in VB are very sensitive to low concentrations of the $\alpha 1$ -subunit-selective modulator, zolpidem (Jia et al., 2005; Sohal et al., 2006). The reticulo-thalamocortical circuit in the double knock-in mutation (HA/HA) mouse presents, therefore, an excellent model to study anesthetic effects on the function of synaptic GABA_A-Rs containing the $\alpha 1$ subunit.

We first investigated how evoked IPSCs (eIPSCs) in VB neurons were affected by the mutation. Monosynaptic GABA_A-R-mediated currents were evoked in VB neurons by extracellular stimulation of RTN in the absence of drug (control) to assess differences in synaptic currents between wild-type and HA/HA mice (Fig. 1A1-3). As shown, the decay time and $\frac{1}{2}$ width of the eIPSC recorded in a VB neuron from a HA/HA mouse were reduced as compared to those recorded in a wild-type mouse, while the amplitude was unaffected. eIPSCs were completely blocked by gabazine (Fig. 1A1), indicating that the recorded synaptic current was mediated by GABA_A-Rs. Pooled data indicate that the double mutation significantly decreased eIPSC decay time and $\frac{1}{2}$ width as compared to wild-type parameters ($P < 0.05$, Fig. 1A3 and Table 1). eIPSC rise-time was similar in wild-type and knock-in mice (data not shown). The mutation also decreased decay time and $\frac{1}{2}$ width of miniature IPSCs in VB neurons (Fig. 1B, Table 1).

Although the $\alpha 1$ subunit is densely expressed in the VB, it is absent in the RTN (Fritschy and Möhler, 1995; Pirker et al., 2000), where the $\alpha 3$ subunit predominates (Browne et al., 2001; Fritschy and Möhler, 1995; Pirker et al., 2000; Studer et al., 2006). As the $\alpha 1$ subunit does not apparently contribute to GABAergic inhibition in the RTN (Huguenard and Prince, 1994; Huntsman and Huguenard, 2000; Huntsman et al., 1999), we investigated whether the mutation had any effect on IPSCs recorded from RTN neurons. Local stimulation of RTN evoked relatively slow IPSCs in RTN neurons from both genotypes; the decay time and $\frac{1}{2}$ width of eIPSCs were substantially greater than that observed in VB neurons (Fig. 1C; Table 1), but there was no difference in the decay time or $\frac{1}{2}$ width in RTN neurons from HA/HA mice as compared to WT littermates. These data are consistent with previous reports demonstrating that IPSCs in RTN neurons decay more slowly than those in VB neurons (Huntsman and Huguenard, 2000; Schofield and Huguenard, 2007; Zhang et al., 1997).

3.2 The effect of isoflurane on eIPSCs in VB neurons from HA/HA and wild-type mice

To determine the effect of isoflurane in VB neurons, isoflurane was applied to brain slices from wild-type and knockin mice by superfusion (see Methods). In wild-type VB neurons, isoflurane (0.16 and 0.32 mM) markedly prolonged the time-course of the eIPSC as compared to control (pre-drug; Fig. 2A); in contrast, the isoflurane-induced prolongation was relatively small in HA/HA mice (Fig. 2B). Analysis of the pooled data shows that although both 0.16 mM and 0.32 mM isoflurane prolonged eIPSCs significantly in VB neurons from wild-type mice ($P < 0.05$, Fig. 2D - E), in the HA/HA mice, only the higher concentration had a significant effect. Isoflurane decreased the current amplitude slightly in both genotypes, but this change was not significant (Fig. 2A-C, F, G). Comparison between the two genotypes indicates that the double mutation significantly decreased the potentiating effects of isoflurane on eIPSCs ($P < 0.05$, Fig. 2F, G).

The effect of isoflurane on mIPSCs was also examined. Consistent with the above results, there was a concentration-dependent increase in decay time in mIPSCs recorded in VB neurons from WT mice (Table 2). Interestingly, spontaneous IPSCs were infrequently observed in VB neurons from HA/HA mice. Of a total of 40 VB neurons recorded from HA/HA mice, only 12 (30%) showed spontaneous IPSCs (sIPSCs), and low frequency (0.2 ± 0.03 Hz) mIPSCs could only be recorded in 8 of these neurons. We could not measure an effect of isoflurane on mIPSCs in VB neurons from HA/HA mice because even at a low concentration (0.16 mM), mIPSCs disappeared. There was no significant difference in the frequency of sIPSCs and mIPSCs in hippocampal pyramidal neurons from HA/HA and wild-type mice (data not shown).

3.3 Potentiation of the eIPSC by zolpidem is independent of genotype

To examine whether the faster decay time observed in VB neurons from HA/HA mice resulted from changes in functional incorporation of the $\alpha 1$ subunit into synaptic GABA_A-Rs, we tested the effect of the imidazopyridine, zolpidem, which is a highly selective positive allosteric modulator of GABA_A-Rs containing the $\alpha 1$ -subunit (Goldstein et al., 2002; Pritchett and Seeburg, 1990; Vicini et al., 2001; Wafford et al., 1993). Zolpidem, at a low concentration (200 nM), prolonged eIPSCs in both wild-type and HA/HA VB neurons, while the amplitude was unaffected (Fig. 3A, B). The analysis of pooled data indicates that zolpidem significantly increased the decay time constant and $\frac{1}{2}$ width of eIPSCs in VB neurons from both genotypes ($P < 0.05$, compared to pre-drug values; Fig. 3C). In contrast to the observations with isoflurane (Fig. 2F, G), there was no significant difference in the effects of zolpidem on eIPSCs between wild-type and HA/HA mice (Fig. 3C).

3.4 Isoflurane decreases the reliability of synaptic transmission at RTN-VB synapses in wild-type and HA/HA mice

We investigated the effects of isoflurane on the reliability of inhibitory synaptic transmission between RTN and VB neurons; IPSCs were evoked in VB and the stimulation parameters adjusted so as to obtain a successful transmission rate of ~80% in both genotypes (Fig. 4). Application of 0.16 mM and 0.32 mM isoflurane decreased the number of eIPSCs in both wild-type (Fig. 4A) and HA/HA (Fig. 4B) mice; the transmission rate returned to baseline after washout (15 min; traces not shown). Pooled data indicate that isoflurane, at both concentrations, significantly decreased the success rate in both genotypes ($P < 0.05$), and this effect was not significantly different between genotypes (Fig. 4C). Since isoflurane did not significantly affect IPSC amplitude, the inhibition of spike-driven synaptic transmission at RTN-VB synapses observed here suggests a presynaptic site of action for isoflurane in the RTN (*Cf.* Ulrich and Huguenard, 1995).

3.5 Effect of isoflurane on intrinsic excitability of RTN neurons from wild-type and HA/HA mice

The isoflurane-induced decrease in synaptic reliability could result from a decrease in RTN excitability due to GABA_A-R-independent mechanisms. To test this possibility, we investigated whether isoflurane affected the intrinsic excitability of RTN neurons in the presence of the GABA_A-R antagonist gabazine (10 μ M). Spike firing rate and input resistance (R_{in}) in RTN neurons were very similar in the two genotypes under control conditions (Fig. 5B and E). Superfusion of isoflurane reversibly inhibited action potential firing and reduced input resistance (Fig. 5A, D). Pooled data indicate that isoflurane significantly decreased spike firing and input resistance to a similar degree in both genotypes ($P < 0.05$, Fig. 5B, C and E). These data clearly indicate that clinically relevant concentrations of isoflurane are capable of inhibiting the intrinsic excitability of RTN neurons *via* non-GABAergic mechanisms.

3.6 Isoflurane inhibits rebound burst firing in RTN neurons from wild-type mice

As we have previously shown, RTN neurons typically generate multiple rebound bursts when the membrane potential is promptly returned to baseline following application of a hyperpolarizing current step (Ying and Goldstein, 2005). To investigate how isoflurane affected this firing pattern independently of GABA_A receptor activity, rebound burst firing response was initiated by a single hyperpolarizing current pulse in the presence of gabazine (10 μ M); a representative trace is shown in Fig. 6A. The application of isoflurane (0.32 mM) hyperpolarized the membrane potential by 5.3 ± 1.2 mV ($n = 4$), dramatically reduced the ability to fire multiple rebound bursts (the number of bursts decreased from 6.5 ± 1.3 bursts/sweep to 1.5 ± 0.6 bursts/sweep), and delayed the onset of the initial rebound burst by 87.3 ± 6 ms. Intrinsic rebound burst firing was evoked in another RTN neuron using a “tuning” protocol (-150 pA, 2 Hz), which allowed testing of rhythmic rebound bursting in a time-controlled manner. Isoflurane (0.32 mM) again hyperpolarized the membrane potential and reversibly inhibited fast action potentials over-riding the low-threshold calcium spikes (Fig. 6C).

3.7 Isoflurane-mediated inhibition of firing is likely mediated via enhancement of Ba²⁺-sensitive potassium currents in RTN neurons from wild-type mice

Volatile anesthetics have long been known to hyperpolarize mammalian (Nicol and Madison, 1982) and invertebrate (Franks and Lieb, 1988) neurons due to an increase in a potassium conductance, and this is also true in thalamocortical relay neurons (Ries and Puil, 1999a). The channels underlying this anesthetic-induced hyperpolarization belong to the family of 2-pore domain K⁺ channels (K_{2P}), which include both TASK (Gray et al., 2000; Patel et al., 1999; Sirois et al., 2000) and TREK (Heurteaux et al., 2004; Patel et al., 1999) channels. Since isoflurane hyperpolarized the membrane potential in the presence of gabazine (Fig. 6C), and 2P-domain K⁺ channels are present in RTN neurons (Talley et al., 2001), voltage clamp recordings were performed in order to examine the effect of isoflurane on potassium currents in RTN neurons.

Membrane currents under control conditions were elicited by a hyperpolarizing voltage ramp in a bath solution containing CNQX (20 μ M), AP5 (40 μ M), Ni²⁺ (100 μ M), gabazine (10 μ M) and tetrodotoxin (1 μ M); averaged current traces are shown in Fig. 7A ($n = 4$), and the isolated K⁺ currents ($[K^+]_o = 5$ mM) were linear over membrane voltages between -110 to -60 mV. The addition of isoflurane (0.32 mM) alone consistently increased the current, and the isoflurane-induced current response could be abolished by the co-application of two potassium channel blockers (4-aminopyridine; 4-AP, 1 mM and BaCl₂ 1 mM). In addition, isoflurane increased the holding (baseline) current by 96 ± 15 % when membrane potential was held at -60 mV before the potassium channel blockers were applied (traces not shown).

We next examined in another group of RTN neurons ($n = 4$) whether the isoflurane current is sensitive to a single potassium channel blocker. Currents were evoked as described above (except here $[K^+]_o = 2 \text{ mM}$); again, application of isoflurane alone increased a potassium current (reversal potential for the isoflurane current is described below). Interestingly, the isoflurane-induced current was markedly blocked by addition of Ba^{2+} (1 mM) alone (Fig. 7B) while the addition of 4-AP (1 mM) alone had no effect on the isoflurane-induced current in the same neurons.

Finally, to test whether the isoflurane-induced current was sensitive to changes in extracellular K^+ concentrations ($[K^+]_o$), the isoflurane-induced current reversal potential was examined at different $[K^+]_o$ in the absence of K^+ channel blockade (but in the presence of the same other blockers described for Fig. 7A). The reversal potential for the current modulated by isoflurane (0.32 mM) was (in mV): -82 ± 2.4 , -73 ± 1.8 , and -68 ± 2.2 ($n = 4$), when $[K^+]_o$ was 2, 5, and 7 mM, respectively, strongly suggesting that K^+ channels are involved in isoflurane-induced hyperpolarization in RTN neurons. The isoflurane-sensitive current had a reversal potential more positive than the calculated K^+ equilibrium potential (see Methods) at each $[K^+]_o$; although detailed mechanisms underlying this phenomenon are unclear (see Ries and Puil, 1999a for discussion), our data are consistent with previously reported reversal potentials for the isoflurane current in thalamic neurons (Ries and Puil, 1999a).

Discussion

Clinically relevant concentrations of the volatile anesthetic isoflurane prolong the time course of inhibitory postsynaptic currents mediated by $GABA_A$ -Rs in thalamic VB neurons of wild-type mice; this effect is significantly attenuated in litter-mates harboring a double amino acid substitution (S270H and L277A) in the $\alpha 1$ subunit of the $GABA_A$ receptor. The same low concentrations of isoflurane also decreased the reliability of synaptic transmission at RTN-VB synapses, due to a decrease in the intrinsic excitability of RTN neurons independent of $GABA_A$ -R activation.

In order to further resolve the contribution of $GABA_A$ -R mechanisms to “general anesthesia”, a mouse line was engineered harboring both the S270H and L277A mutations (Borghese et al., 2006). In VB neurons from HA/HA mice, the decay phase of evoked and miniature IPSCs was significantly faster than in neurons of their wild-type littermates (Fig. 1; Table 1), and these findings are consistent with reported data from hippocampal neurons (Sonner et al., 2007). The accelerated decay of eIPSCs in HA/HA mice presumably reflects a faster rate of receptor deactivation (Borghese et al., 2006) following the removal of GABA from the synaptic cleft.

We examined the effect of clinically relevant concentrations of isoflurane on eIPSCs and found that the anesthetic was significantly less efficacious in prolonging IPSC decay as compared to the effects observed in wild-type animals (Fig. 2). Our data are in close agreement with previous results indicating that $GABA_A$ -Rs containing both the S270H and L277A substitutions are less sensitive to isoflurane-induced potentiation than are wild-type receptors when expressed in *Xenopus* oocytes (Borghese et al., 2006) or in hippocampal neurons *in vivo* (Sonner et al., 2007). The effect of the mutation reported here may contribute to the significant increase in the inspired concentration of isoflurane required to produce the loss-of-righting reflex seen *in vivo* in the HA/HA mouse (Sonner et al., 2007).

Consistent with earlier reports (Browne et al., 2001; Huntsman and Huguenard, 2000; Jia et al., 2005; Zhang et al., 1997), these data clearly indicate that $\alpha 1$ subunit-containing $GABA_A$ receptor-mediated synaptic inhibition is highly sensitive to modulation by isoflurane in VB neurons from wild-type mice. While the low (0.16) isoflurane concentration used here clearly prolonged the synaptic current in wild-type mice (Fig. 2), it is likely that it also has an effect

at extrasynaptic GABA_A-Rs as heterologously expressed GABA_A-Rs consisting of $\alpha 4$, $\beta 2$, and δ subunits (i.e., “extrasynaptic” receptors) are more sensitive to isoflurane than receptors consisting of $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits (i.e., “synaptic” receptors) (Jia et al., 2008). The relative involvement of each population of receptors in contributing to the amnestic and hypnotic properties associated with sub-MAC (the minimum alveolar concentration required to inhibit purposeful movement in response to a noxious stimulus) concentrations of isoflurane (Eger, 2001) requires further investigation.

One possible explanation for the HA/HA associated decrease in isoflurane-modulation of GABA_A-R-mediated synaptic currents in the present study is impaired total synthesis of the $\alpha 1$ subunit, as seen in cortical (but not cerebellar) neurons (Borghese et al., 2006). A second possibility is that expression of the double mutation might impair the trafficking of $\alpha 1$ subunit-containing GABA_A-Rs to the synapse. The decay time of both evoked and miniature IPSCs is faster in HA/HA mice than in wild-type littermates (Fig. 1, Table 1), suggesting that expression of synaptically localized $\alpha 1$ subunits is preserved in HA/HA mice as incorporation of the $\alpha 1$ subunit confers rapid activation/deactivation kinetics on GABA_A-Rs (Gingrich et al., 1995; Lavoie et al., 1997; Tia et al., 1996; Vicini et al., 2001). We tested this hypothesis using the $\alpha 1$ -subunit selective imidazopyridine, zolpidem (Goldstein et al., 2002; Pritchett and Seeburg, 1990; Vicini et al., 2001; Wafford et al., 1993). Zolpidem binds to the same histidine residue at position 101 as do benzodiazepines (Rudolph et al., 1999), and mutant mice harboring the $\alpha 1$ (H101) mutation are insensitive to the hypnotic effects of zolpidem (Crestani et al., 2000). Given that zolpidem has a binding site distinct from that for isoflurane, zolpidem’s efficacy in prolonging the eIPSC in VB neurons should not be altered by the double mutation; as expected, low-concentration zolpidem (200 nM) potentiated IPSCs equally in both wild-type and HA/HA mice (Fig. 3). The present data suggest that the decrease in eIPSC modulation by isoflurane is not due to changes in the numbers of $\alpha 1$ -subunits incorporated into synaptic GABA_A-Rs in mutant mice.

Using a sub-maximal stimulation paradigm, we also tested the reliability of synaptic transmission at RTN-VB synapses as an indirect measure of excitability (i.e., the propensity to generate fast action potentials) in RTN neurons. Isoflurane reversibly decreased the number of eIPSCs in both wild-type and HA/HA mice to the same extent (Fig. 4), suggesting a decrease in the underlying excitability of RTN neurons. This effect was not due to isoflurane-induced potentiation of tonic inhibition as the GABA_A receptor subunits that generate tonic currents in VB neurons are not present in RTN neurons (Jia et al., 2005; Jia et al., 2008; Pirker et al., 2000).

As shown in Figure 4, submaximal stimulation of the RTN evoked IPSCs in VB neurons with a success rate of ~80%, and isoflurane decreased the successful mono-synaptic transmission rate to ~53% at RTN-VB synapses. Pre-determined criteria were applied to identify monosynaptic IPSCs (see Methods), but given that the stimulating electrode was placed in the RTN, stimulation-evoked IPSCs conceivably could have resulted from activation of mono-synaptic (i.e., direct depolarization of RTN neurons leading to their spike firing and subsequent release of GABA) or poly-synaptic pathways (i.e., activation of excitatory afferents onto RTN neurons leading to their spike discharge and subsequent release of GABA), or both. Isoflurane modulation of a di-synaptic pathway appears unlikely, however, for a number of reasons: 1) the tip size of the stimulation electrode was very small (25 μ m), 2) stimulation intensity was low (30-100 μ A, 50-100 μ s), 3) the latency and rise times of eIPSCs were consistent before and after isoflurane application, and 4) very few evoked IPSCs were in fact di-synaptic (in four instances when IPSCs and EPSCs appeared simultaneously, CNQX and AP5 were used to block EPSCs, and glutamate receptor blockade had no effect on isoflurane-modulation of the isolated eIPSC). In addition, thalamocortical relay neurons in brain slices are typically quiescent and do not fire action potentials at resting membrane potential (Hughes et al.,

2002). Finally, a reduction in IPSC output from RTN during isoflurane application might, in theory, increase the excitability of postsynaptic VB neurons; however, direct recordings of VB spike firing failed to detect any such isoflurane-induced activation (Ries and Puil, 1999b). Thus, the decrease in the success rate by isoflurane likely reflects direct inhibition of intrinsic excitability of RTN neurons presynaptically connected with VB neurons, and evidence obtained from additional experiments (see below) supports this interpretation.

We directly tested the effects of isoflurane on RTN excitability using current clamp recordings performed in the presence of the GABA_A-R antagonist gabazine. Under these conditions, isoflurane significantly decreased action potential firing, and this was accompanied by a significant decrease in input resistance (Fig. 5). As noted above, one possible mechanism that might account for the anesthetic-induced increase in conductance is an increased potassium conductance (Franks and Lieb, 1988; Nicoll and Madison, 1982; Ries and Puil, 1999a). The reported increase in potassium conductance likely reflects activation of two-pore-domain potassium channels, particularly TREK and TASK channels, as they have been shown to be highly sensitive to volatile anesthetics both *in vitro* (recombinant channels), in brain slice preparations (Patel and Honoré, 2003; Putzke et al., 2007; Sirois et al., 2000), and *in vivo* (Heurteaux et al., 2004; Linden et al., 2006; Linden et al., 2007), and RTN neurons express moderate to high levels of several 2P-domain K⁺ channel mRNAs, including TASK-1, TREK-1, TREK-2, and TWIK-1 (Talley et al., 2001).

To study the effects of isoflurane on these related K⁺ channels, we isolated K⁺ currents by co-applying gabazine (to block GABA_A receptors), CNQX and AP5 (to block ionotropic glutamate receptors), tetrodotoxin (to block voltage-gated Na⁺ channels), and Ni²⁺ (to block voltage-gated Ca²⁺ channels). Under these conditions, we found that isoflurane markedly increased K⁺ currents in RTN neurons that were sensitive to Ba²⁺ (1 mM), but not 4-AP (1 mM) (Fig. 7), and these results are similar to earlier observations in VB neurons (Ries and Puil, 1999a). This pattern of sensitivity suggests that TASK-1 channels are likely to be involved (Duprat et al., 1997; Kim et al., 1998), and is consistent with the known expression pattern for those channels. Previous work has demonstrated that isoflurane does not affect responses mediated by GABA_B receptors or a number of ion channels (including those for Na⁺, Ca²⁺, and K⁺ channels sensitive to 4-AP, ATP, and TEA) (Ries and Puil, 1999a). Our data lend strong support to the conclusion that isoflurane increases the potassium “leak” conductance, and such a non-GABAergic action contributes to the hyperpolarization, decreased input resistance, and inhibition of tonic and rhythmic burst firing observed in RTN neurons, all of which lead to decreased action-potential-driven inhibitory output from RTN to VB.

In summary, we have shown that isoflurane can facilitate inhibition in both RTN and VB neurons by enhancing intrinsic and synaptic conductances; such inhibition may disrupt the high frequency (γ) oscillatory activity *in vivo* that is a property of the reciprocal connections between the thalamus and cortex (“thalamocortical loops”) and is associated with consciousness. Indeed, isoflurane has been shown to modulate fast oscillations in the hippocampus (Dickinson et al., 2003) and neocortex (Antkowiak and Hentschke, 1997). The inspired isoflurane concentration required to inhibit the righting reflex is increased in HA/HA mice (Sonner et al., 2007), but the righting reflex can still be inhibited at higher concentrations, indicating that the action of isoflurane on GABA_A-Rs containing the $\alpha 1$ subunit contribute to, but are not an exclusive requirement for, this effect in the mouse. Further work will further address the relative contributions of GABA_A-Rs and intrinsic conductance mechanisms to the rapid loss of consciousness induced by this inhaled anesthetic agent.

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References

- Alkire MT, Haier RJ, Fallon JH. Toward a unified theory of narcosis: brain imaging evidence for a thalamocortical switch as the neurophysiologic basis of anesthetic-induced unconsciousness. *Conscious Cogn* 2000;9:370–386. [PubMed: 10993665]
- Alkire MT, Miller J. General anesthesia and the neural correlates of consciousness. *Prog Brain Res* 2005;150:229–244. [PubMed: 16186027]
- Angel A. Central neuronal pathways and the process of anaesthesia. *Br J Anaesth* 1993;71:148–163. [PubMed: 8102065]
- Antkowiak B, Hentschke H. Cellular mechanisms of gamma rhythms in rat neocortical brain slices probed by the volatile anaesthetic isoflurane. *Neurosci Lett* 1997;231:87–90. [PubMed: 9291147]
- Arcelli P, Frassoni C, Regondi MC, De Biasi S, Spreafico R. GABAergic neurons in mammalian thalamus: a marker of thalamic complexity? *Brain Res Bull* 1997;42:27–37. [PubMed: 8978932]
- Banks MI, Pearce RA. Dual actions of volatile anesthetics on GABA_A IPSCs: dissociation of blocking and prolonging effects. *Anesthesiology* 1999;90:120–134. [PubMed: 9915321]
- Belelli D, Peden DR, Rosahl TW, Wafford KA, Lambert JJ. Extrasynaptic GABA_A receptors of thalamocortical neurons: a molecular target for hypnotics. *J Neurosci* 2005;25:11513–11520. [PubMed: 16354909]
- Borghese CM, Werner DF, Topf N, Baron NV, Henderson LA, Boehm SL 2nd, Blednov YA, Saad A, Dai S, Pearce RA, Harris RA, Homanics GE, Harrison NL. An isoflurane- and alcohol-insensitive mutant GABA_A receptor α_1 subunit with near-normal apparent affinity for GABA: characterization in heterologous systems and production of knockin mice. *J Pharmacol Exp Ther* 2006;319:208–218. [PubMed: 16807363]
- Browne SH, Kang J, Akk G, Chiang LW, Schulman H, Huguenard JR, Prince DA. Kinetic and pharmacological properties of GABA_A receptors in single thalamic neurons and GABA_A subunit expression. *J Neurophysiol* 2001;86:2312–2322. [PubMed: 11698521]
- Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE. GABA_A receptor α_4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci U S A* 2006;103:15230–15235. [PubMed: 17005728]
- Cope DW, Hughes SW, Crunelli V. GABA_A receptor-mediated tonic inhibition in thalamic neurons. *J Neurosci* 2005;25:11553–11563. [PubMed: 16354913]
- Crestani F, Martin JR, Möhler H, Rudolph U. Mechanism of action of the hypnotic zolpidem *in vivo*. *Br J Pharmacol* 2000;131:1251–1254. [PubMed: 11090095]
- Detsch O, Kochs E, Siemers M, Bromm B, Vahle-Hinz C. Differential effects of isoflurane on excitatory and inhibitory synaptic inputs to thalamic neurones *in vivo*. *Br J Anaesth* 2002;89:294–300. [PubMed: 12378670]
- Dickinson R, Awaiz S, Whittington MA, Lieb WR, Franks NP. The effects of general anaesthetics on carbachol-evoked gamma oscillations in the rat hippocampus *in vitro*. *Neuropharmacology* 2003;44:864–872. [PubMed: 12726818]
- Duprat F, Lesage F, Fink M, Reyes R, Heurteaux C, Lazdunski M. TASK, a human background K⁺ channel to sense external pH variations near physiological pH. *EMBO J* 1997;16:5464–5471. [PubMed: 9312005]
- Eger EI 2nd. Age, minimum alveolar anesthetic concentration, and minimum alveolar anesthetic concentration-awake. *Anesth Analg* 2001;93:947–953. [PubMed: 11574362]
- Elsen FP, Liljelund P, Werner DF, Olsen RW, Homanics GE, Harrison NL. GABA_A-R α_1 subunit knockin mutation leads to abnormal EEG and anesthetic-induced seizure-like activity in mice. *Brain Res* 2006;1078:60–70. [PubMed: 16490182]

- Franks NP, Lieb WR. Volatile general anaesthetics activate a novel neuronal K⁺ current. *Nature* 1988;333:662–664. [PubMed: 2453807]
- Fritschy JM, Möhler H. GABA_A-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J Comp Neurol* 1995;359:154–194. [PubMed: 8557845]
- Gingrich KJ, Roberts WA, Kass RS. Dependence of the GABA_A receptor gating kinetics on the α -subunit isoform: implications for structure-function relations and synaptic transmission. *J Physiol* 1995;489 (Pt 2):529–543. [PubMed: 8847645]
- Goldstein PA, Elsen FP, Ying SW, Ferguson C, Homanics GE, Harrison NL. Prolongation of hippocampal miniature inhibitory postsynaptic currents in mice lacking the GABA_A receptor α_1 subunit. *J Neurophysiol* 2002;88:3208–3217. [PubMed: 12466441]
- Gray AT, Zhao BB, Kindler CH, Winegar BD, Mazurek MJ, Xu J, Chavez RA, Forsayeth JR, Yost CS. Volatile anesthetics activate the human tandem pore domain baseline K⁺ channel KCNK5. *Anesthesiology* 2000;92:1722–1730. [PubMed: 10839924]
- Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, Lang-Lazdunski L, Widmann C, Zanzouri M, Romey G, Lazdunski M. TREK-1, a K⁺ channel involved in neuroprotection and general anesthesia. *EMBO J* 2004;23:2684–2695. [PubMed: 15175651]
- Hughes SW, Cope DW, Blethyn KL, Crunelli V. Cellular mechanisms of the slow (1 Hz) oscillation in thalamocortical neurons *in vitro*. *Neuron* 2002;33:947–958. [PubMed: 11906700]
- Huguenard JR, Prince DA. Clonazepam suppresses GABA_B-mediated inhibition in thalamic relay neurons through effects in nucleus reticularis. *J Neurophysiol* 1994;71:2576–2581. [PubMed: 7931539]
- Huntsman MM, Huguenard JR. Nucleus-specific differences in GABA_A-receptor-mediated inhibition are enhanced during thalamic development. *J Neurophysiol* 2000;83:350–358. [PubMed: 10634878]
- Huntsman MM, Porcello DM, Homanics GE, DeLorey TM, Huguenard JR. Reciprocal inhibitory connections and network synchrony in the mammalian thalamus. *Science* 1999;283:541–543. [PubMed: 9915702]
- Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA. An extrasynaptic GABA_A receptor mediates tonic inhibition in thalamic VB neurons. *J Neurophysiol* 2005;94:4491–4501. [PubMed: 16162835]
- Jia F, Yue M, Chandra D, Homanics GE, Goldstein PA, Harrison NL. Isoflurane is a potent modulator of extrasynaptic GABA_A receptors in the thalamus. *J Pharmacol Exp Ther* 2008;324:1127–1135. [PubMed: 18094320]
- Jones MV, Harrison NL. Effects of volatile anesthetics on the kinetics of inhibitory postsynaptic currents in cultured rat hippocampal neurons. *J Neurophysiol* 1993;70:1339–1349. [PubMed: 7506753]
- Kaisti KK, Langsjo JW, Aalto S, Oikonen V, Sipila H, Teras M, Hinkka S, Metsahonkala L, Scheinin H. Effects of sevoflurane, propofol, and adjunct nitrous oxide on regional cerebral blood flow, oxygen consumption, and blood volume in humans. *Anesthesiology* 2003;99:603–613. [PubMed: 12960544]
- Kim D, Fujita A, Horio Y, Kurachi Y. Cloning and functional expression of a novel cardiac two-pore background K⁺ channel (cTBAK-1). *Circ Res* 1998;82:513–518. [PubMed: 9506712]
- Koltchine VV, Finn SE, Jenkins A, Nikolaeva N, Lin A, Harrison NL. Agonist gating and isoflurane potentiation in the human γ -aminobutyric acid type A receptor determined by the volume of a second transmembrane domain residue. *Mol Pharmacol* 1999;56:1087–1093. [PubMed: 10531417]
- Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB, Twyman RE. Activation and deactivation rates of recombinant GABA_A receptor channels are dependent on α -subunit isoform. *Biophys J* 1997;73:2518–2526. [PubMed: 9370445]
- Linden AM, Aller MI, Leppä E, Vekovischeva O, Aitta-Aho T, Veale EL, Mathie A, Rosenberg P, Wisden W, Korpi ER. The *in vivo* contributions of TASK-1-containing channels to the actions of inhalation anesthetics, the α_2 adrenergic sedative dexmedetomidine, and cannabinoid agonists. *J Pharmacol Exp Ther* 2006;317:615–626. [PubMed: 16397088]
- Linden AM, Sandu C, Aller MI, Vekovischeva OY, Rosenberg PH, Wisden W, Korpi ER. TASK-3 knockout mice exhibit exaggerated nocturnal activity, impairments in cognitive functions, and reduced sensitivity to inhalation anesthetics. *J Pharmacol Exp Ther* 2007;323:924–934. [PubMed: 17875609]

- Llinás R, Ribary U. Consciousness and the brain. The thalamocortical dialogue in health and disease. *Ann N Y Acad Sci* 2001;929:166–175. [PubMed: 11349424]
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature* 1997;389:385–389. [PubMed: 9311780]
- Nicoll RA, Madison DV. General anesthetics hyperpolarize neurons in the vertebrate central nervous system. *Science* 1982;217:1055–1057. [PubMed: 7112112]
- Nishikawa K, Jenkins A, Paraskevakis I, Harrison NL. Volatile anesthetic actions on the GABA_A receptors: contrasting effects of $\alpha 1$ (S270) and $\beta 2$ (N265) point mutations. *Neuropharmacology* 2002;42:337–345. [PubMed: 11897112]
- Nishikawa K, MacIver MB. Agent-selective effects of volatile anesthetics on GABA_A receptor-mediated synaptic inhibition in hippocampal interneurons. *Anesthesiology* 2001;94:340–347. [PubMed: 11176100]
- Patel AJ, Honoré E. 2P domain K⁺ channels: novel pharmacological targets for volatile general anesthetics. *Adv Exp Med Biol* 2003;536:9–23. [PubMed: 14635644]
- Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M. Inhalational anesthetics activate two-pore-domain background K⁺ channels. *Nat Neurosci* 1999;2:422–426. [PubMed: 10321245]
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G. GABA_A receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 2000;101:815–850. [PubMed: 11113332]
- Pritchett DB, Seeburg PH. γ -aminobutyric acid_A receptor $\alpha 5$ -subunit creates novel type II benzodiazepine receptor pharmacology. *J Neurochem* 1990;54:1802–1804. [PubMed: 2157817]
- Putzke C, Hanley PJ, Schlichthorl G, Preisig-Muller R, Rinne S, Anetseder M, Eckenhoff R, Berkowitz C, Vassiliou T, Wulf H, Eberhart L. Differential effects of volatile and intravenous anesthetics on the activity of human TASK-1. *Am J Physiol Cell Physiol* 2007;293:C1319–1326. [PubMed: 17699638]
- Ribary U. Dynamics of thalamo-cortical network oscillations and human perception. *Prog Brain Res* 2005;150:127–142. [PubMed: 16186020]
- Ries CR, Puil E. Ionic mechanism of isoflurane's actions on thalamocortical neurons. *J Neurophysiol* 1999a;81:1802–1809. [PubMed: 10200214]
- Ries CR, Puil E. Mechanism of anesthesia revealed by shunting actions of isoflurane on thalamocortical neurons. *J Neurophysiol* 1999b;81:1795–1801. [PubMed: 10200213]
- Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Möhler H. Benzodiazepine actions mediated by specific γ -aminobutyric acid_A receptor subtypes. *Nature* 1999;401:796–800. [PubMed: 10548105]
- Schofield CM, Huguenard JR. GABA affinity shapes IPSCs in thalamic nuclei. *J Neurosci* 2007;27:7954–7962. [PubMed: 17652586]
- Sirois JE, Lei Q, Talley EM, Lynch C 3rd, Bayliss DA. The TASK-1 two-pore domain K⁺ channel is a molecular substrate for neuronal effects of inhalation anesthetics. *J Neurosci* 2000;20:6347–6354. [PubMed: 10964940]
- Sohal VS, Pangratz-Fuehrer S, Rudolph U, Huguenard JR. Intrinsic and synaptic dynamics interact to generate emergent patterns of rhythmic bursting in thalamocortical neurons. *J Neurosci* 2006;26:4247–4255. [PubMed: 16624945]
- Sonner JM, Werner DF, Elsen FP, Xing Y, Liao M, Harris RA, Harrison NL, Fanselow MS, Eger EI II, Homanics GE. Effect of isoflurane and other potent inhaled anesthetics on minimum alveolar concentration, learning, and the righting reflex in mice engineered to express $\alpha 1$ γ -aminobutyric acid type A receptors unresponsive to isoflurane. *Anesthesiology* 2007;106:107–113. [PubMed: 17197852]
- Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W. GABA_A receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. *Neuroscience* 1997;80:987–1000. [PubMed: 9284055]
- Steriade M. Corticothalamic resonance, states of vigilance and mentation. *Neuroscience* 2000;101:243–276. [PubMed: 11074149]

- Studer R, von Boehmer L, Haenggi T, Schweizer C, Benke D, Rudolph U, Fritschy JM. Alteration of GABAergic synapses and gephyrin clusters in the thalamic reticular nucleus of GABA_A receptor $\alpha 3$ subunit-null mice. *Eur J Neurosci* 2006;24:1307–1315. [PubMed: 16987218]
- Talley EM, Solorzano G, Lei Q, Kim D, Bayliss DA. CNS distribution of members of the two-pore-domain (KCNK) potassium channel family. *J Neurosci* 2001;21:7491–7505. [PubMed: 11567039]
- Tia S, Wang JF, Kotchabhakdi N, Vicini S. Distinct deactivation and desensitization kinetics of recombinant GABA_A receptors. *Neuropharmacology* 1996;35:1375–1382. [PubMed: 9014154]
- Topf N, Jenkins A, Baron N, Harrison NL. Effects of isoflurane on γ -aminobutyric acid type A receptors activated by full and partial agonists. *Anesthesiology* 2003;98:306–311. [PubMed: 12552186]
- Ulrich D, Huguenard JR. Purinergic inhibition of GABA and glutamate release in the thalamus: implications for thalamic network activity. *Neuron* 1995;15:909–918. [PubMed: 7576639]
- Vahle-Hinz C, Detsch O, Siemers M, Kochs E, Bromm B. Local GABA_A receptor blockade reverses isoflurane's suppressive effects on thalamic neurons *in vivo*. *Anesth Analg* 2001;92:1578–1584. [PubMed: 11375850]
- Vicini S, Ferguson C, Prybylowski K, Kralic J, Morrow AL, Homanics GE. GABA_A receptor $\alpha 1$ subunit deletion prevents developmental changes of inhibitory synaptic currents in cerebellar neurons. *J Neurosci* 2001;21:3009–3016. [PubMed: 11312285]
- Wafford KA, Whiting PJ, Kemp JA. Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant γ -aminobutyric acid_A receptor subtypes. *Mol Pharmacol* 1993;43:240–244. [PubMed: 8381510]
- White NS, Alkire MT. Impaired thalamocortical connectivity in humans during general-anesthetic-induced unconsciousness. *Neuroimage* 2003;19:402–411. [PubMed: 12814589]
- Ying SW, Goldstein PA. Propofol-block of SK channels in reticular thalamic neurons enhances GABAergic inhibition in relay neurons. *J Neurophysiol* 2005;93:1935–1948. [PubMed: 15563549]
- Ying SW, Jia F, Abbas SY, Hofmann F, Ludwig A, Goldstein PA. Dendritic HCN2 channels constrain glutamate-driven excitability in reticular thalamic neurons. *J Neurosci* 2007;27:8719–8732. [PubMed: 17687049]
- Zhang SJ, Huguenard JR, Prince DA. GABA_A receptor-mediated Cl⁻ currents in rat thalamic reticular and relay neurons. *J Neurophysiol* 1997;78:2280–2286. [PubMed: 9356381]

Figure 1. Comparison of IPSCs in thalamic neurons from wild-type and knock-in mice
(A1) Representative current traces demonstrating evoked IPSCs (eIPSCs) in VB neurons in response to extracellular stimulation of the RTN under control conditions; each trace represents the average of 10-20 events in this and following figures; thick trace: wild-type, thin trace: HA/HA. The evoked current could be completely blocked by the GABA_A receptor antagonist gabazine (10 μ M).
(A2) Current traces in (A1) scaled to peak to facilitate comparison of the current decay time between genotypes.

(A3) Bar graph shows eIPSC parameters in VB neurons from HA/HA mice as percent of the wild-type response. *: $P < 0.05$, wild-type vs. HA/HA, t-test, $n = 16$ (wild-type) and $n = 13$ (HA/HA).

(B1) Averaged current traces for miniature IPSCs (mIPSCs) recorded in VB neurons from wild-type (thick trace) and HA/HA (thin trace) mice.

(B2) Current traces in **(B1)** scaled to peak to facilitate comparison of the current decay time between genotypes.

(B3) Bar graph shows IPSC parameters for mIPSCs recorded in VB neurons from HA/HA mice as percent of the wild-type response. *: $P < 0.05$, wild-type vs. HA/HA, t-test, $n = 8$ /each group.

(C1) IPSCs in RTN were evoked by local stimulation of RTN; representative traces were obtained from neurons in a wild-type (thick trace) and a HA/HA (thin trace) mouse.

(C2) Current traces in **(C1)** scaled to peak (as above).

(C3) Bar graph shows IPSC parameters for eIPSCs recorded in RTN neurons from HA/HA mice as percent of the wild-type response ($n = 7$ /each group). There is no difference in current parameters between the two genotypes.

Figure 2. Effects of isoflurane on evoked IPSCs in VB neurons

(A, B) eIPSCs in VB neurons from wild-type (A) and HA/HA (B) mice. The volatile anesthetic isoflurane (Iso) was applied by superfusion at concentrations of 0.16 mM and 0.32 mM as indicated.

(C - E) Bar graphs summarizing the effects of isoflurane on the amplitude (C), decay time (D), and 1/2 width (E) of eIPSCs in VB neurons from wild-type and HA/HA mice. *: $P < 0.05$, **: $P < 0.001$, one way ANOVA, vs. control (no isoflurane); $n = 16$ and 13 for wild-type and HA/HA mice, respectively.

(F - G) Bar graphs summarizing the effects of isoflurane on eIPSCs between genotypes at concentrations of 0.16 mM (F) and 0.32 mM (G). The mutation significantly reduced the ability of isoflurane to alter the eIPSC decay time and 1/2 width. *: $P < 0.01$, **: $P < 0.001$, vs. wild-types; data are derived from C-E.

Figure 3. The mutation does not influence the slowing of eIPSC decay kinetics in response to zolpidem

(A, B) eIPSCs in VB neurons from wild-type (A) and HA/HA (B) mice. Superimposed traces demonstrate the effect of superfusion of the GABA_A receptor $\alpha 1$ subunit-selective imidazopyridine, zolpidem (200 nM), on eIPSCs. Scale in panels A and B are the same. (C) Bar graph indicates that percent increase elicited by zolpidem in the decay time and 1/2 width in VB neurons is similar between genotypes. *: $P < 0.05$, t-test, vs. control (no zolpidem), $n = 13$ (wild-type) or 10 (HA/HA). There is no difference in percent change between the two genotypes (wild-type vs. HA/HA).

Figure 4. Isoflurane decreases the reliability of GABAergic synaptic transmission in wild-type and double-knockin mice

(A, B) eIPSCs recorded in VB neurons in wild-type (A) and HA/HA (B) mice. Initial stimulation parameters were set to obtain ~80% successful synaptic transmission rate. Single eIPSCs are displayed in a concatenated fashion for clarity. Stimulation signals are indicated by digital stimulus markers. Isoflurane was superfused at the indicated concentrations; traces for washout are not shown.

(C) Bar graph indicates that isoflurane significantly decreased the successful synaptic transmission rate in both genotypes. *: $P < 0.05$, one way ANOVA, vs. control, $n = 10$ (each genotype); there is no difference between the two genotypes.

Figure 5. Isoflurane suppresses intrinsic excitability of RTN neurons from wild-type and double-knockin mice

(A) Tonic spike firing was initiated in RTN neurons from wild-type (top traces) and HA/HA (bottom traces) mice by intracellular injection of square-wave depolarizing current (500 ms duration, protocol not shown) in the presence of gabazine (10 μ M). Isoflurane reversibly decreased spike firing in a concentration-dependent fashion in RTN neurons from both wild-type and HA/HA mice. Initial membrane potential (in mV) is indicated to the left.

(B, C) Input-output curves indicate that isoflurane decreased tonic spike firing in a concentration-dependent manner in RTN neurons from wild-type (B) and HA/HA (C) mice.

*: $P < 0.05$, one-way ANOVA, vs. control, $n = 8$ (each genotype).

(D) Representative voltage traces indicate that isoflurane decreases input resistance in a concentration-dependent manner.

(E) Grouped data summarizing the effect of isoflurane on input resistance (R_{in}). Data are derived from the same neurons in **(B)** and **(C)**. *: $P < 0.05$, one-way ANOVA, vs. control.

Figure 6. Isoflurane inhibits rebound burst firing in RTN neurons

(A) Voltage response in an RTN neuron from a wild-type mouse showing characteristic multiple rebound bursts induced by a single hyperpolarizing current step (-90 pA, 500 ms) in control (black trace). The presence of 0.32 mM isoflurane (red trace) reduced the number of bursts. The thin dotted line indicates the membrane potential. Gabazine (10 μ M) was present in the extracellular solution to block GABA_A receptors in this and subsequent records. The thick black bar indicates the region of the two voltage traces that are shown in (B).

(B) The bursts indicated by the thick black line in A shown on an expanded time scale. Note the delay in the onset of the rebound burst in the presence of isoflurane.

(C) Rebound burst firing was evoked in a different RTN neuron using a tuning protocol (-150 pA current steps at 2 Hz, protocol shown beneath). Isoflurane reversibly hyperpolarized the membrane potential and inhibited over-riding Na⁺-dependent fast action potentials.

Figure 7. Isoflurane enhances K⁺ currents in RTN neurons from wild-type mice

(A) Voltage-clamp recordings showing voltage-current relationship in RTN neurons.

Membrane currents were evoked by hyperpolarizing voltage ramps (from -60 to -110 mV, 4 s in duration; hyperpolarization rate: 0.0125 mV/ms) in control and in the presence of isoflurane (Iso, 0.32 mM; red trace) alone or isoflurane co-applied with 4-aminopyridine (4-AP; 1 mM) and BaCl₂ (Ba²⁺; 1 mM). Each trace is the average of the current response from four neurons (here and in **B**; error bars omitted for clarity). In this set of experiments, [K⁺]_o = 5 mM.

(B) Currents were evoked in a different group of RTN neurons (n = 4) using the same protocol as in **A**. Recording were made as in **A** (except here [K⁺]_o = 2 mM); isoflurane (0.32 mM) was applied by itself (red trace) or in the presence of either Ba²⁺ (1 mM) or 4-AP (1 mM) as indicated.

Table 1
IPSC characteristics in thalamic neurons from wild-type and HA/HA mice

Region	IPSC	Genotype	(n)	Amplitude (pA)	Decay (ms)	½ Width (ms)
VB	eIPSC	Wild-type	(16)	315 ± 18	22.2 ± 1.1	11.6 ± 0.7
		HA/HA	(13)	292 ± 26	12.9 ± 1.4*	7.5 ± 0.4*
VB	mIPSC	Wild-type	(8)	27.0 ± 1.6	8.4 ± 0.2	7.1 ± 0.3
		HA/HA	(8)	24.3 ± 1.0	4.6 ± 0.3*	4.9 ± 0.3*
RTN	eIPSC	Wild-type	(7)	99.1 ± 9.6	98.2 ± 2.6	67.7 ± 2.9
		HA/HA	(7)	105.1 ± 10.6	92.4 ± 5.1	63.5 ± 3.1

Data are mean ± SEM. mIPSCs were recorded in the presence of TTX (0.5 μM).

* P < 0.001, vs. wild type, *t*-test.

Table 2

Effects of isoflurane on mIPSCs in VB neurons from wild-type mice

	Control	Iso 0.16 mM	Iso 0.32 mM
Amplitude (pA)	27.3 ± 4.9	32.7 ± 3.9	30.6 ± 3.2
Decay time (ms)	9.1 ± 0.5	16.8 ± 0.8*	28.07 ± 1.7*
½ width (ms)	7.6 ± 0.4	10.6 ± 0.5	14.7 ± 1.7*
Frequency (Hz)	13.2 ± 0.9	14.1 ± 1.1	12.7 ± 0.8

* P < 0.05, one-way ANOVA, vs. control, n = 8