The influence of manipulations to alter ambient GABA concentrations on the hypnotic and immobilizing actions produced by sevoflurane, propofol, and midazolam

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Recent studies have suggested that extrasynaptic GABA_A receptors, which contribute tonic conductance, are important targets for general anesthetics. We tested the hypothesis that manipulations designed to alter ambient GABA concentrations (tonic conductance) would affect hypnotic (as indicated by loss of righting reflex, LORR) and immobilizing (as indicated by loss of tail-pinch withdrawal reflex, LTWR) actions of sevoflurane, propofol, and midazolam. Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), which purportedly reduced ambient GABA concentrations, and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor (NO-711). The influence of these manipulations on cellular and behavioral responses to the anesthetics was studied using behavioral and electrophysiological assays. HPLC revealed that GABA levels in GAD65−/− mice were reduced in the brain (76.7% of WT) and spinal cord (68.5% of WT). GAD65−/− mice showed a significant reduction in the duration of LORR and LTWR produced by propofol and midazolam, but not sevoflurane. NO-711 (3 mg/kg, ip) enhanced the duration of LORR and LTWR by propofol and midazolam, but not sevoflurane. Patch-clamp recordings revealed that sevoflurane (0.23 mM) slightly enhanced the amplitude of tonic GABA current in the frontal cortical neurons; however, these effects were not strong enough to alter discharge properties of cortical neurons. These results demonstrate that ambient GABA concentration is an important determinant of the hypnotic and immobilizing actions of propofol and midazolam in mice, whereas manipulations of ambient GABA concentrations minimally alter cellular and behavioral responses to sevoflurane.

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1. Introduction

The GABAergic system in the central nervous system (CNS) is a key target of general anesthetics (Mihic et al., 1997; Sonner et al., 2003; Rudolph and Antkowiak, 2004; Hemmings et al., 2005; Franks, 2006). Two types of GABAergic inhibition are known: a phasic form (phasic inhibition) regulating neural excitability via the activation of postsynaptic GABA_A receptors by interneuronal release from presynaptic terminals, and a persistent tonic form (tonic inhibition) generated by continuous activation of extrasynaptic GABA_A receptors by low concentrations of ambient GABA (Bickley et al., 1996). Growing evidence suggests that tonic inhibition mediated by extrasynaptic GABA_A receptors might contribute to the actions of intravenous anesthetics such as propofol (Bai et al., 2001; Bieda and Maclver, 2004). These extrasynaptic GABA_A receptors have different pharmacological and kinetic properties compared with synaptic GABA_A receptors, as a result of the distinct subunit compositions (Glykys and Mody, 2007). Given that extrasynaptic GABA_A receptors respond to low ambient levels of GABA, manipulations of ambient GABA concentrations may affect cellular and behavioral responses to general anesthetics.

Two manipulations studied were 1) the genetic absence of glutamate decarboxylase (GAD) 65 gene (GAD65−/−), and 2) the pharmacological manipulation of GABA uptake using GABA transporter inhibitor. GAD is the only synthetic enzyme responsible for the conversion of l-glutamic acid to GABA. The brain contains two forms of GAD, which differ in molecular size, amino acid sequence,
antigenicity, cellular and subcellular locations, and interaction with the GAD cofactor pyridoxal phosphate (Erlander et al., 1991). The G7-KDa protein (GAD67) is found mainly in the cell body, whereas GAD65 is localized to the nerve terminal and is reversibly bound to the membrane of synaptic vesicles (Namchuk et al., 1997). GAD65—/— mice remain viable without apparent anatomical deficits and postsynaptic GABA_A receptor density is unchanged (Kash et al., 1997), although the survival rate of GAD65—/— mice was slightly reduced with age, largely due to spontaneous seizures (Stork et al., 2000). As a result of reduced GABAergic tone, enhanced spontaneous inhibition (Nishikawa and MacIver, 2001; Nishikawa and Harrison, 2003; Nishikawa et al., 2005), suggesting that GABA_A receptor is one of the plausible molecular targets. In addition, several targets have been also proposed for inhalational general anesthetics; glycine receptors (Mascia et al., 1996), two-pore-domain potassium channels (Sirois et al., 2000), NMDA receptors (Sonner et al., 2003), HCN channels (Chen et al., 2005), and some subtypes of sodium channels (Wu et al., 2004), whereas a specific point mutation in GABA_A receptor is critical for propofol and etomidate (Jurd et al., 2003). These data suggest that the relative contributions of GABAergic inhibition to in vivo anesthetic actions are different between sevoflurane and intravenous anesthetics. We first tested the hypothesis that genetic and pharmacological manipulations to alter ambient GABA concentrations would affect loss of righting reflex (LORR), a surrogate measure of hypnosis, and loss of tail-pinch withdrawal reflex (LTWR), a measure of immobilization, produced by sevoflurane, propofol, and midazolam. We then studied the influence of these manipulations on in vitro sevoflurane actions on membrane properties of frontal cortical layer V neurons using patch-clamp methods. The present study provides evidence that genetic and pharmacological manipulations to alter ambient GABA concentrations (tonic conductance) affect the response to propofol and midazolam, but minimally affect the actions of sevoflurane.

2. Materials and methods

2.1. Mice

All animal procedures and protocols used in this study were approved by the Animal Care Committee of Gunma University Graduate School of Medicine (protocol # 05–71) and performed according to NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The generation of glutamate decarboxylase 65 (GAD65) knockout mice used in the present study was described by Yanagawa et al. (1999) and Yamamoto et al. (2004). In brief, we designed a targeting vector to disrupt most of the open reading frame by inserting an in-frame stop codon in the exon 3. The linearized targeting vector was introduced by electroporation into E14.1 embryonic stem (ES) cell derived from strain 129/Ola mice, and we obtained ES cell clones carrying the GAD65 targeted mutation through homologous recombination. The correctly targeted ES cells were injected into C57BL/6J mouse (CLEA Japan, Inc, Tokyo, Japan) blastocysts to make chimeras. The chimeric male mice were mated with female C57BL/6J, and germ-line transmission was achieved. The resultant GAD65 heterozygous (+/—) mice were backcrossed for more than ten generations onto the C57BL/6J background. Wild-type (+/+) and knockout (—/—) littermates were produced from heterozygous mating pairs. GAD65—/— mice were viable and fertile and gross behaviors appeared to be normal without apparent anatomical deficits. Adult (12–16 weeks old) male WT and GAD65—/— mice weighing 23–27 g were used for experiments. Mice were group-housed in a pathogen-free transgenic facility, and water and food were available ad libitum. None of the animals were used for more than two experiments and at least 1 week was allowed for the mice to recover.

2.2. Measurement of neurotransmitter contents

For analysis of neurotransmitter tissue content, WT mice and GAD65—/— mice at 12 weeks of age were sacrificed by decapitation under deep sevoflurane anesthesia. Tissue samples of the whole brain and the spinal cord were removed quickly and tissue weight was measured. The tissue was added to 3–5 ml of saline (saline volume was approximately ten folds of tissue weight), and then homogenized in phosphate-buffered saline (PBS) containing 0.2% protease inhibitor using a polytron homogenizer (24,000 rpm, 15 s, 2–3 times). Following removal of cell debris by centrifugation at 3000 rpm (20 min, 4 °C), the supernatant (500 μl), which was added to sulfosalicylic acid (750 ml), was centrifuged again at 3000 rpm (20 min, 4 °C). The supernatant after pH adjustment was analyzed using high-performance liquid chromatography (HPLC) and fluorescence detection. HPLC was performed by the company (SRL, Tokyo, Japan). Neurotransmitter content (nmol/g) was calculated as follows: measured tissue neurotransmitter concentration (nmol/ml) = saline volume added (ml)/tissue weight (g).

2.3. Behavioral assays for intravenous drugs

Loss of righting reflex (LORR) was used as a surrogate measure for hypnosis. Each animal was received an intraperitoneal (ip) injection of propofol (Marushii Pharmaceuticals Co, Ltd., Osaka, Japan) or midazolam (Astellas Pharma Inc, Tokyo, Japan) with a volume of 10 μl/g of body weight, and then placed on their backs in a chamber (20 × 28 × 15 cm). The ability to right themselves was evaluated as described (Kubo et al., 2009a). Because we have previously reported that GAD65—/— mice showed altered responses to propofol (100 mg/kg, ip) (Kubo et al., 2009a), propofol (125 mg/kg, ip) was tested in the present study. Midazolam (50 mg/kg, ip) was used as described previously (Quinlan et al., 1998). Mice were judged to have lost this reflex when unable to right itself within 10 s. The time from ip injection of the drug to LORR was considered as the latency, and the time between the LORR and the time mice regained the ability to right themselves within 2 s was considered the duration of LORR. Loss of tail-pinch withdrawal response (LTWR) was used as a surrogate measure for immobilization (Quaschishin et al., 1980). A surgical spring clip (6 mm in size, Applied Medical, CA, USA) was placed at the base of an animal’s tail for 5 s.

Vehicle solutions for behavioral studies were as follows: propofol, lipofundin MCT(10%) (B. Braun Melsungen AG, Melsungen, Germany); midazolam, saline. An intraperitoneal injection of lipofundin MCT(10%) (10 μl/g) alone had no hypnotic/analgic effect on mouse behavior (n = 5). NO-711 hydrochloride (Sigma-Aldrich Chemical Japan, Tokyo, Japan), a potent and selective GAT-1 inhibitor that cross the blood–brain barrier (Borden et al., 1994), was diluted in sterile saline and injected 20 min prior to experiments (a volume of 10 μl/g of body weight). Other drugs were purchased from Sigma–Aldrich Chemicals (Tokyo, Japan).

2.4. Behavioral studies of sensitivity to sevoflurane

Mice were placed into a sealed Plexiglas chamber (32 × 32 × 22 cm), warmed by heating pads from below. After 20-min equilibration period with a chosen concentration of sevoflurane (0.5–5.0% atm, Marushi, Osaka, Japan) delivered via an anesthetic-specific vaporizer (Sevotec S, Ohmeda, UK) with fresh air flow at a rate of 3.0 l/min, a blinded observer scored the mice for LORR and LTWR in a quantal fashion. Sevoflurane concentration was continuously controlled by the infrared gas analyzer (BP-508, Nippon Colin Co. Ltd., Tokyo, Japan). In LORR assays, mice were judged to have lost righting reflex when unable to right itself within 10 s. In LTWR assays, movement to tail-pinch was tested by the placement of the surgical clip at the base of an animal’s tail for 5 s. If any movement to tail-pinch was detected, the concentration of sevoflurane was increased for another 20-min equilibration period, and the response was tested again. The concentration at which the mouse lost its righting reflex was used as a surrogate measure for hypnosis. Other drugs were purchased from Sigma–Aldrich Chemicals (Tokyo, Japan).
containing the frontal cortex was quickly dissected out and glued to a DTK-1000 vibratome tray (Dosaiki EM, Tokyo, Japan) using oxygenated cold modified Ringer solution. Slices (300 μm) were cut from the brain and then kept in the pre-chamber (Brain Slice Chamber System; Harvard Apparatus, Holliston, MA) filled with artificial cerebrospinal fluid (ACSF) consisting of (in mM): 125 NaCl, 2.5 KCl, 2.5 CaCl2, 2 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 11 glucose, bubbled with 95% O2 and 5% CO2 at room temperature (22–24 °C). Slices were allowed 1 h for recovery in the pre-chamber, which was designed to keep 8–12 slices viable for several hours.

Slices were transferred to a recording chamber (1.0 ml in volume, Brain Slice Chamber System, Harvard Apparatus, Inc., Holliston, MA) perfused with an oxygenated ACSF at a rate of approximately 3 ml/min. Patch electrodes were made from borosilicate thin-walled capillaries (GC-1.5, Narishige Co., Ltd., Tokyo, Japan). Recording electrodes (5–7 MΩ) were filled with Cs2SO4–based ACSF (110 mM, TEA 5 mM, CaCl2 0.5 mM, MgCl2 2 mM, EGTA 5 mM, HEPES 5 mM and MgATP 5 mM; pH 7.2) to investigate spontaneous IPSCs at a holding potential of 0 mV. The liquid junction potential in these conditions was approximately 9 mV, and all data presented were corrected using this value. K-glutamate solutions (K-glucocollate 100 mM, EGTa 5 mM, HEPES 40 mM, MgCl2 5 mM, ATP 2 mM, GTP 0.3 mM, pH 7.25) were used for current clamp recordings, so the impermeable ion (glutamate) would not contribute to anesthetic-induced changes in resting membrane potential or current–voltage relations. Whole cell patch-clamp recordings were made from visualized layer V pyramidal neurons in the frontal cortex using an upright AxioLab2 FS plus microscope (Zeiss, Jena, Germany). The magnification of the image was collected with an intensified CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) with contrast enhancement. The image of the neurons was displayed on a video monitor, and glass patch pipettes were visually advanced using a micromanipulator (MWW3, Narishige Co., Ltd., Tokyo, Japan) through the slice to the surface of the neurons. Each patch was held in a microelectrode (Axon Instruments Inc., Union City, CA) was used for whole cell recordings. Whole cell currents were filtered at 2–5 kHz and digitized at 10 kHz (Digidata 1322A, Axon Instruments Inc.) and stored on a Pentium-based personal computer for later analysis. The GABAergic nature of the synaptic currents was verified by applying the GABA, receptor antagonist, picrotoxin (PIC). Series resistances were between 10 and 25 MΩ, and were then compensated approximately 80%. Recordings were performed at room temperature (22–24 °C).

In some experiments using WT mice, NO-711 (3 mg/kg) was injected intraperitoneally 20 min before decapitation. It was confirmed, 10 min later, that NO-711 prolonged the latency to jumping or licking responses in the hot-plate test (53 °C) (Kurihara et al., 2009b), and then brain slices were made as described above. The effects of NO-711 in the slice remained effective for several hours, since the amplitude of tonic inhibition was larger than that of WT mice without NO-711 injection (n = 5). Thus, in vitro experiments with NO-711 were performed within 4 h after making slice preparations. In separate experiments, NO-711 was directly added to the ACSF solution that was bathing the slice in the recording chamber. However, in this case, it was difficult to determine the concentration that should be used to match the dose injected in behavioral studies. Judging from the influence on tonic inhibition, we estimated that 1–2 μM NO-711 were relevant concentrations.

2.6. Data analysis

Data acquisition and analysis were performed with pCLAMP software version 8.1 (Axon Instruments Inc., Union City, CA) and Igor Pro version 5.0 (WaveMetrics, Lake Oswego, OR). Synaptic currents were defined as current deflections with a fast rising phase and a relatively slower decay phase. The rise time was defined as time interval between 10% and 90% of the peak amplitude and synaptic currents having the rise time <2 ms were included for analysis. The amplitude of synaptic current was measured from the initial inflection point (not from the baseline) to the peak, to avoid the effects of summation on amplitude distribution. Threshold-level crossings were set at approximately three folds of baseline noise, which was measured during the period of no detectable events. As a result, synaptic currents larger than 6 pA in the amplitude were counted for analysis. This definition eliminated the infrequently observed single channel events or synaptic currents with slow rise time, but successfully detected most IPSCs. The decay phase was fitted with a single exponential curve and a time from peak to 1/e was defined as the decay time.

2.7. Application of sevoflurane to slices and concentration measurement

Artificial cerebrospinal fluid solution at room temperature was bubbled with a carrier gas (95% O2, 5% CO2) passing through a calibrated commercial vaporizer (Sevotec S, Ohmeda, BOC Health Care, West Yorkshire, United Kingdom) at the designated concentration, and was applied to the recording chamber using a gravity-feed and vacuum system. High-quality polytetrafluoroethylene was used for tubing and valves to minimize loss of volatile anesthetic and drug binding. Sevoflurane (Maruishi Pharmaceutical, Osaka, Japan) concentrations used for this study were 2.0% (clinically relevant concentration for mice) and 5.0% (high dose for mice). To determine the actual aqueous concentrations of sevoflurane in the submerged recording chamber for each concentration used, aliquots of the solution were taken from the recording chamber and filled into airtight glass containers for gas chromatographic measurements as described previously (Ishizeki et al., 2008). In brief, aliquots of the solution were directly taken from the recording chamber for gas chromatographic measurements. The peak of sevoflurane was observed approximately 3 min after injection, and the area under the curve was measured. The aqueous sevoflurane concentration was calculated by comparing to that of sevoflurane standard Apparatus (1.0 mM), in which 20 μl of sevoflurane was dissolved in ethanol (100 ml). The final aqueous concentration of 2.8% sevoflurane was 0.23 ± 0.01 mM (n = 5), and 5.0% was 0.41 ± 0.01 mM (n = 5).

Although sevoflurane is administered to humans in the gas phase at body temperature (37 °C), in vitro electrophysiological experiments using sevoflurane were carried out at room temperature. In general, gas-phase potencies are reported to be temperature-dependent, increasing markedly with decreasing temperatures (Franks and Lieb, 1998). Procedures using gas-phase EC50 concentrations for room temperature experiments can thus result in overdosing with the in vitro preparation (Franks and Lieb, 1998) have reported that the upper estimate of mammalian minimum alveolar concentration values for sevoflurane expressed as free aqueous concentration in saline is 0.33 mM. Taking these data into considerations, sevoflurane 0.23 mM was used as a clinically relevant concentration and 0.41 mM as a relatively high concentration.

2.8. Statistics

Results are expressed as mean ± SD. The results were analyzed by using Student's t-test or one way analyses of variance (ANOVA). Post hoc comparisons between the individual groups were performed by means of the Tukey test. Statistical significance between curves fitted to LORR and LTWR data was performed by comparing EC50 values via t-test. The level of statistical significance was set at P < 0.05 in all tests.

3. Results

3.1. GABA levels in the brain and the spinal cord are reduced in GAD65−/− mice

We measured the GABA content in the whole brain and spinal cord in GAD65−/− mice at 12–16-weeks old. GAD65−/− mice showed a significant reduction in GABA levels in the brain (76.7% of WT, P < 0.001, n = 6 each, Fig. 1A) and the spinal cord (68.5% of WT, P < 0.001, n = 5 for WT and n = 6 for GAD65−/− mice, Fig. 1B).

Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might have been expected, the difference in glutamate and glycine levels did not reach statistical significance when calculating mean values from experiments on five or six animals, respectively.

3.2. Ambient GABA levels are altered in GAD65−/− mice and in WT mice following blockade of GAT-1 transporter

Because the major anesthetic targets are believed to be cortical neurons and thalamic neurons (Franks, 2006), GABA(A) receptor-mediated miniature IPSCs (mIPSCs) were recorded from the frontal cortex layer V pyramidal neurons at 0 mV, close to the reversal potential of EPSCs, using Cs2SO4-based internal solutions (Fig. 2A). Tetrodotoxin (TTX, 1 μM) was used to block sodium channels that give rise to action potentials. Under these conditions, glutamate-mediated EPSCs were negligible. These observations were further confirmed by applying a GABAergic antagonist, picrotoxin (PIC, 50 μM or 100 μM, Pittler and Alger, 1992). Because both doses produced a similar baseline shift, PIC (50 μM) was used in following experiments. These neurons were identified using IR-DIC microscopy by their large (>20 μm diameter) pyramidal shaped cell bodies with long apical dendrites extending toward the pial surface. The mean amplitude of mIPSCs was unchanged in GAD65−/− mice or in WT with NO-711 injection (12.9 ± 3.5 pA in WT mice, 11.9 ± 4.7 pA in GAD65−/− mice, and 13.9 ± 3.5 pA in WT mice with NO-711, n = 10 each). The rise time of mIPSCs was also similar among groups (WT mice, 0.8 ± 0.2 ms, n = 10; GAD65−/− mice, 0.9 ± 0.3 ms, n = 10; WT mice with NO-711 (ip), 0.8 ± 0.3 ms, n = 10).

Ambient GABA levels were then evaluated by measuring the amplitude of tonic conductance of layer V pyramidal neurons in the
Frontal cortex of mice. The effects of PIC on the holding current were examined in the absence of the anesthetic. Although PIC (50 μM, 5 min) produced an inward shift in all groups, the amplitude of tonic current was slightly but significantly reduced in GAD65−/− mice (13.3 ± 5.5 pA in GAD65−/− slices, n = 12; 24.4 ± 6.5 pA in WT slices, n = 11, P < 0.001, Fig. 2B). On the other hand, the tonic conductance was significantly increased in WT mice treated with a GAT-1 inhibitor NO-711 (3 mg/kg, ip) (34.5 ± 7.8 pA, n = 12, P < 0.01 vs. WT slices, Fig. 2B). These data provide indirect evidence that ambient GABA levels in the frontal cortex are altered by genetic and pharmacological manipulations used in the present study.

3.3. Behavioral responses to sevoflurane are unchanged in GAD65−/− mice

We next examined whether the anesthetic sensitivity to sevoflurane is altered by GAD65 gene knockout. The LORR assay was performed to examine the anesthetic sensitivity of mice to sevoflurane. Mice were judged to have lost this reflex when unable to right itself within 10 s. The dose–response curves for the ED50 determination for LORR are presented in Fig. 3A. The calculated ED50 values for LORR were 1.24% in WT mice (n = 14) and 1.26% in GAD65−/− mice (n = 14). Thus, GAD65 gene knockout did not affect anesthetic sensitivity to sevoflurane. LTWR was used as a surrogate measure for immobilization (Quasha et al., 1980). Sevoflurane concentration was increased and the ED50 value for LTWR was determined. The calculated ED50 values for LTWR were 2.07% in WT mice (n = 21) and 2.04% in GAD65−/− mice (n = 21). These data suggest that hypnotic and immobilizing actions of sevoflurane are unaffected by GAD65 gene knockout and resulting reduction in GAD65−/− mice are unaffected by GAD65 gene knockout in the brain and the spinal cord. Data bars show mean ± SD.

![Fig. 1. GABA contents of GAD65−/− mice are reduced in the brain (A) and the spinal cord (B). Neurotransmitter contents were analyzed by high-performance liquid chromatography (HPLC). (A) In the whole brain, GABA contents were significantly reduced in GABA65−/− mice (n = 6, P < 0.001, t-test) compared with those of WT mice (n = 6). Data bars show mean ± SD. (B) In the spinal cord, GABA contents were also significantly reduced in GAD65−/− mice (n = 6, P < 0.001, t-test). Note that glutamate and glycine contents were unaffected by GAD65 gene knockout in the brain and the spinal cord. Data bars show mean ± SD.](image)

3.4. Behavioral response to propofol and midazolam are diminished in GAD65−/− mice

We then compared anesthetic sensitivity to propofol and midazolam, a positive allosteric modulator of GABA receptors. The latency to LORR produced by propofol (125 mg/kg, ip) was signifi-
cantly prolonged in GAD65−/− mice (188.9 ± 23.6 s, n = 10, P < 0.01) than that of WT mice (150.0 ± 27.5 s, n = 10). The duration of LORR produced by propofol (125 mg/kg, ip) was significantly reduced in GAD65−/− mice (1568.0 ± 689.0 s, n = 10, P < 0.05) than that of WT mice (2263.0 ± 533.6 s, n = 10, Fig. 3B left). Similarly, the duration of LTWR produced by propofol (125 mg/kg, ip) was significantly reduced in GAD65−/− mice (n = 8, P < 0.01, Fig. 3B right). As shown in Fig. 3C, the duration of LORR produced by midazolam (50 mg/kg, ip) was significantly reduced in GAD65−/− mice (2498.4 ± 1351.0 s, n = 9, P < 0.05) than that of WT mice (3564.0 ± 854.0 s, n = 21) without affecting the latency to LORR (P = 0.60 between genotypes). In addition, the duration of LTWR produced by midazolam (50 mg/kg, ip) was significantly reduced in GAD65−/− mice (n = 8 each, P < 0.05).

3.5. Behavioral responses to sevoflurane are unchanged by GABA transporter 1 inhibition

We then tested whether the selective GAT-1 inhibitor, NO-711, would affect LORR produced by sevoflurane, propofol and midazolam. Because enhanced GABA concentration by NO-711 may produce hypnotic actions in the absence of the anesthetics, we first confirmed that NO-711 at low doses (1 or 3 mg/kg/ip) had no effect on righting reflex in both genotypes. Righting reflex was slightly impaired at 6 mg/kg (ip) and severely impaired at high doses (>10 mg/kg). These data suggest that NO-711 alone can induce hypnosis in a dose-dependent manner by enhancing tonic inhibitions. NO-711 (3 mg/kg, ip) did not affect dose–response relationship of LORR produced by sevoflurane (Fig. 4A). The calculated ED50...
values for LORR were 1.28% in WT mice \( (n=10) \) and 1.36% in GAD65\(^{-/-}\) mice \( (n=10) \). In LTWR assays, NO-711 \( (3.0 \text{ mg/kg, ip}) \) had no effect on immobilizing actions produced by sevoflurane. The calculated \( \text{ED}_{50} \) values for LTWR were 2.15% in WT mice \( (n=8) \) and 2.12% in WT mice with NO-711 \( (3 \text{ mg/kg, ip}) \) \( (n=9) \).

3.6. Behavioral responses to propofol and midazolam are enhanced by GAT-1 inhibition

NO-711 \( (3 \text{ mg/kg, ip}) \) significantly increased the duration of LORR produced by propofol \( (125 \text{ mg/kg, ip}) \) from 2263.0 ± 533.6 s \( (n=10) \) to 3471.7 ± 1076.1 s \( (n=8) \), * \( P < 0.05 \), Fig. 4B) in WT mice without affecting the latency to LORR \( (n=8) \), \( P = 0.66 \). NO-711 \( (3 \text{ mg/kg, ip}) \) also increased the duration of LTWR \( (P < 0.01) \) produced by propofol \( (125 \text{ mg/kg, ip}, \text{Fig. 4B right}) \). NO-711 \( (3.0 \text{ mg/kg, ip}) \) increased the duration of LORR produced by midazolam \( (50 \text{ mg/kg, ip}) \) from 3564.0 ± 854.0 s to 4601.0 ± 951.0 s \( (n=7) \), ** \( P < 0.01 \) and LTWR \( (**P < 0.01) \) produced by propofol \( (125 \text{ mg/kg, ip}) \).

3.7. Sevoflurane-induced enhancement of phasic and tonic inhibition

Intravenous anesthetic such as propofol and thiopental strongly enhance both phasic and tonic inhibition (Bai et al., 2001; Bieda and MacIver, 2004; Bieda et al., 2009). However, little is known about the effects of sevoflurane on phasic and tonic components of GABAergic inhibition. Although basal inhibitory synaptic transmission was normal in GAD65\(^{-/-}\) mice (Kubo et al., 2009a; Tian et al., 1999) and WT mice with NO-711, sevoflurane enhanced GABAergic inhibition in cortical pyramidal neurons. Sevoflurane \( (0.23 \text{ mM}) \) significantly increased the frequency of mIPSCs in WT

**Fig. 3.** The effects of reduced GABA contents by GAD65 gene knockout on behavioral responses to sevoflurane, propofol, and midazolam. (A) Dose–response curves for the determination of the \( \text{ED}_{50} \) value for loss of righting reflex (left) and for loss of tail-pinch withdrawal response (right) produced by sevoflurane. Data presented are the fraction of mice that did not respond, i.e., failed to right themselves on the LORR or failed to move in response to the tail clamp vs. sevoflurane concentration. The fitted curves were generated using a weighted sum of least-squares method (KaleidaGraph version 3.5, Reading, PA) to a Hill equation: \( \text{LORR} \% = \text{ED}_{50} \% / (\text{ED}_{50} \% + \text{LORR} \%); \) where LORR \( \% \) is a percentage of LORR, \( [\text{drug}] \) is the anesthetic concentration, and \( n \) is the Hill coefficient. \( \text{EC}_{50} \) did not differ between genotypes for either assay. (B) The duration of LORR and LTWR were compared between WT mice and GAD65\(^{-/-}\) mice. A significant reduction in the duration of LORR \( (**P < 0.01 \) between genotypes, \( n=10) \) and LTWR \( (**P < 0.01) \) was observed in GAD65\(^{-/-}\) mice. (C) A significant reduction in the duration of LORR and LTWR was observed in GAD65\(^{-/-}\) mice \( (**P < 0.01) \) without affecting the latency to LORR \( (n=7) \), \( P = 0.89 \). NO-711 \( (3 \text{ mg/kg, ip}) \) increased the duration of LTWR \( (n=7) \), \( P < 0.05 \) produced by midazolam \( (50 \text{ mg/kg, ip}, \text{Fig. 4C right}) \).

**Fig. 4.** The effects of increased GABA contents by NO-711 on behavioral responses to sevoflurane, propofol and midazolam. (A) Pre-injection of NO-711 \( (3 \text{ mg/kg, ip}) \) had little effect on the dose–response relationship of LORR (left) and LTWR (right) produced by sevoflurane. (B) NO-711 \( (3 \text{ mg/kg, ip}) \) significantly increased the duration of LORR \( (n=8) \), \( P < 0.05 \) and LTWR \( (**P < 0.01) \) produced by propofol \( (125 \text{ mg/kg, ip}) \). (C) The effects of NO-711 \( (3 \text{ mg/kg, ip}) \) on the duration of LORR and LTWR produced by midazolam \( (50 \text{ mg/kg, ip}) \). NO-711 increased the duration of LORR \( (n=7) \), ** \( P < 0.01 \) and LTWR \( (**P < 0.05) \). Data bars show mean ± SD.
mice (180.1% of WT control, n = 8, P < 0.001), GAD65−/− mice (168.9% of GAD65−/− mice control, n = 8, P < 0.001), and WT with NO-711 (ip) (195.0% of NO-711 control, n = 8, P < 0.001). There was no group difference in the frequency in the presence of sevoflurane (0.23 mM, ANOVA). Sevoflurane (0.23 mM) also increased the decay time of mIPSCs in WT mice (241.1% of WT control, n = 8, P < 0.001), GAD65−/− mice (232.9% of GAD65−/− mice control, n = 8, P < 0.001), and WT with NO-711 (ip) (267.0% of NO-711 control, n = 8, P < 0.001). There was no group difference in the decay time in the absence or presence of sevoflurane (ANOVA). In addition, sevoflurane (0.23 M) produced a relatively small but significant baseline shift in hhold in all groups (Fig. 5A). The amplitudes of baseline shift produced by sevoflurane were 13.1 ± 5.0 pA in WT mice (n = 8, P < 0.001 vs. control), 11.2 ± 3.9 pA in GAD65−/− mice (n = 8, P < 0.001 vs. control), and 14.9 ± 5.9 pA in WT mice with NO-711 (ip) (n = 8, P < 0.001 vs. control, Fig. 5B). These sevoflurane effects were reversible after washout of the anesthetic.

A specific GABA<sub>A</sub> receptor antagonist, SR95531 (1 μM), selectively blocks only synaptic GABA<sub>A</sub> receptors that generate IPSCs (Yamada et al., 2007), while PIC blocks both synaptic and extrasynaptic tonic conductances (Bieda and Maclver, 2004). SR95531 does not affect GABA-transaminase or GAD activities. To examine the relative contributions of synaptic components in GABAergic inhibition, we tested the effects of SR95531 (1 μM) on mIPSCs in the presence of sevoflurane (0.23 mM). SR95531 (1 μM, 5 min) abolished mIPSCs in all groups but had little effect on baseline holding current (WT slices, n = 5; GAD65−/− slices, n = 4; WT slices with NO-711, n = 5). Prolonged application (20 min) produced a small baseline shift in hhold in all group, these changes did not reach statistical significance because of their variability.

3.8. Sevoflurane-induced depression of pyramidal neuron excitability

Whole cell recordings were obtained from visually identified layer V pyramidal neurons located within the medial PFC. Resting membrane potentials were −71.3 ± 2.6 mV (n = 15) in WT slices, −70.6 ± 3.5 mV (n = 14) in GAD65−/− slices, and −71.7 ± 3.9 mV (n = 15) in WT slices with NO-771. The majorities of neurons were typically silent at rest and were held for periods for >15 min. Although bath application of sevoflurane (0.23 mM, 20 min) produced a small hyperpolarization in all groups, but these effects did not reach statistical significance because of cell to cell variability. Sevoflurane (0.41 mM, 20 min) significantly hyperpolarized the neuron (−5.3 ± 2.5 mV in WT slices, n = 8; −6.3 ± 3.6 mV in GAD65−/− slices, n = 8; −7.3 ± 4.0 mV in WT slices with NO-711, n = 8).

In response to supra-threshold currents, some pyramidal neurons exhibited modest spike frequency accommodation, other neurons responded to current injection with regularly regular inter-spike intervals. Sevoflurane-induced neural depression was compared by measuring the number of APs to depolarizing current injection, as a measure of effects on intrinsic excitability. We focused on pyramidal neurons with relatively regular inter-spike intervals (Fig. 6A). In WT mice, sevoflurane (0.23 mM, 20 min) did not change action potential discharge responses to depolarizing
current in pyramidal neurons. The number of APs was significantly depressed when sevoflurane (0.41 mM) was applied to the chamber (Fig. 6B). In WT mice, sevoflurane at 0.41 mM significantly depressed the number of APs from 10.5 ± 1.3 to 6.5 ± 2.4 (n = 10, P < 0.05 vs. control, ANOVA–Tukey). Similar results were observed in GAD65−/− mice and WT mice with NO−711 (ip). Although sevoflurane at 0.23 mM had no effect on neuronal excitability of pyramidal neurons in GAD65−/− mice (n = 9) and WT mice with NO−711 (ip) (n = 9), sevoflurane at 0.41 mM significantly depressed the number of APs in GAD65−/− mice (n = 9, P < 0.05 vs. control, ANOVA–Tukey) and in WT mice with NO−711 (ip) (n = 9, P < 0.01 vs. control, ANOVA–Tukey). The membrane input resistance in pyramidal neurons was very similar in all groups under control conditions. Superfusion of sevoflurane 0.41 mM, but not 0.23 mM, reversibly reduced input resistance to a similar degree in all groups (91.3 ± 3.4% of control in WT slices, n = 5; 93.3 ± 4.3% of control in GAD65−/− slices, n = 5; 89.8 ± 5.4% of control in WT slices with NO−711, n = 5).

In separate experiments, NO−711 was directly added to the ACSF solution that was bathing the slice in the recording chamber. The effects of NO−711 on sevoflurane-induced depression of pyramidal neuron excitability were basically similar to those observed with intraperitoneally injected NO−711 (3 mg/kg). In the presence of NO−711 (2 mM), sevoflurane (0.23 mM) had little effect on neuronal excitability of pyramidal neurons (n = 6), whereas sevoflurane (0.41 mM) significantly depressed the number of APs (n = 5, P < 0.01 vs. control, ANOVA–Tukey).

4. Discussion

GAD65−/− mice appeared to show normal sensitivity to sevoflurane despite a 20–30% reduction in GABAergic inhibitory tone, whereas they showed reduced sensitivity to propofol and midazolam. In contrast, enhanced GABA concentrations by NO−711 prolonged the duration of LORR and LTWR by propofol and midazolam, but not sevoflurane. Sevoflurane enhanced tonic inhibition of layer V cortical neurons; however, these effects were not strong enough to alter discharge properties of cortical neurons. These data show that genetic and pharmacological manipulations designed to alter ambient GABA concentrations differentially regulate hypnotic and immobilizing actions of general anesthetics.

4.1. Reduced ambient GABA concentrations in GAD−/− mice

GAD65−/− mice appeared to show partial reductions in GABA concentrations in the whole brain and whole spinal cord. However, HPLC data include synaptic vesicular, intracellular, and extracellular (ambient) GABA concentrations. Patch-clamp studies confirmed that the amplitude of tonic inhibition was significantly reduced in GAD65−/− mice and that this reduction was relevant to HPLC data. Given that the amplitude of tonic inhibition reflects the ambient GABA concentration (Semyanov et al., 2004), we conclude that ambient GABA concentrations are reduced in GAD65−/− mice, and that reduced ambient GABA concentrations are responsible for altered behavioral responses to propofol (Kubo et al., 2009a) and midazolam.

Although a compensatory mechanism involving the balance between inhibitory and glutamatergic excitatory neurotransmission might have been expected, glutamate and glycine levels were unchanged. As a result, the ratio of excitatory to inhibitory neurotransmitter levels actually increased in GAD65−/− mice. It has been reported that the survival rate of GAD65−/− mice was thus significantly reduced, largely due to spontaneous seizures; only 2–3% of WT mice died before postnatal 25 weeks, whereas 25% of GAD65−/− mice died during the same period (Stork et al., 2000). To minimize the possibility of a generalized increase in CNS excitability and behavioral abnormality during experiments, 12–16 week old mice were used in our study. In fact, we have previously confirmed that excitatory synaptic transmission in hippocampal slices was normal in GAD65−/− mice, and that the survival rate was almost similar between genotypes at this stage (Kubo et al., 2009a).

4.2. Reduced sensitivity to propofol and midazolam, but not sevoflurane, in GAD65−/− mice

Our data are consistent with previous reports showing that GAD65−/− mice had reduced sensitivity to pentobarbital, which is known to potentiate GABAergic inhibition (Kash et al., 1999). Although the molecular and cellular factors underlying differences in anesthetic sensitivity are currently unknown, one possibility is that sevoflurane may have multiple molecular targets contributing to in vivo hypnotic actions (Masica et al., 1996; Sirois et al., 2000; Sonner et al., 2003; Chen et al., 2005; Wu et al., 2004). If so, the present data lead us to believe that relatively small changes in ambient GABA concentrations are insufficient to alter behavioral responses to sevoflurane. To support this notion, sevoflurane has been shown to depress excitatory synapses in synaptosomes (Moe et al., 2003), the brainstem (Stucke et al., 2005), and the hippocampus (Ishizeki et al., 2008), whereas propofol has little effect on excitatory synapses in cortical neurons (Kitamura et al., 2003), the hippocampus (Kubo et al., 2009a), and the spinal cord (Takazawa et al., 2009). Together, available data suggest that imbalance in excitatory and inhibitory neurotransmitter contents in the brain significantly affect hypnotic and immobilizing actions of propofol and midazolam.

It must be emphasized, however, that the influence of GAD65 knockout is relatively small in terms of anesthetic sensitivity. In fact, hypnotic actions could be elicited in GAD65−/− mice with the use of higher concentrations of propofol and midazolam. The most likely interpretation of these findings is that other molecular targets may mediate behavioral responses to these agents. In this context, mutations in postsynaptic GABAA receptors have appeared to modulate in vivo propofol actions (Jurd et al., 2003). The mutation of asparagine to methionine (N265M) in the β3 subunit greatly reduces the ability of propofol and etomidate to cause LORR and eliminates their abilities to prevent response to painful stimuli, but has little effect on the actions of volatile anesthetics. In contrast, the GABAA α1 subunit (S270) is a critical determinant that influences a variety of behaviors in the mouse in view of volatile anesthetic sensitivity (Homanics et al., 2005). These data show that postsynaptic GABAA receptors play more important roles with in vivo anesthetic potencies.

4.3. Increased extracellular GABA concentrations by GABA transporter 1 inhibition

The actions of synaptically released GABA are terminated by uptake into presynaptic terminals and surrounding glial cells via Na+/K+-dependent transporters. Molecular biological studies have cloned four subtypes of mouse GABA transporter (GAT): GAT-1, GT-2, GAT-3, and GAT-4 (Borden, 1996). NO−711 is a potent and selective GABA uptake inhibitor that exhibits the highest affinity for human GAT-1. Hybridization signals for GAT-1 mRNA have been observed over many regions of the rat brain, including the retina, olfactory bulb, neocortex, ventral pallidum, hippocampus, and cerebellum (Durkin et al., 1995), indicating that NO−711 induces an increase in endogenous GABA concentrations at many brain regions. However, the degree of increased GABA concentrations produced by NO−711 is difficult to determine. We estimated changes in GABA concentrations using two methods. First, we
confirmed physiologically that the amplitude of tonic conductance in cortical pyramidal neurons of brain slices from WT mice with NO-711 (3 mg/kg, ip) was greater than that of WT mice without apparent changes in IPSC amplitude, indicating that the tonic current is not a summation of IPSCs, but is instead attributable to continuous GABA(A) receptor activation by endogenous GABA. Second, we confirmed behaviorally that NO-711 (1.0–10 mg/kg, ip) dose-dependently increased the latency in the hot-plate test in WT mice and GAD65−/− mice (Kubo et al., 2009b). Judging from these data, NO-711 (1.0 mg/kg, ip) to GAD65−/− mice reinstated GABA levels similar to WT mice, because the latency was similar between groups. High-dose NO-711 (6.0–10 mg/kg) produced slight hypnotic actions in the absence of anesthetics (Kubo et al., 2009b). Together, we believe that NO-711 (3.0 mg/kg, ip) enhances ambient GABA contents without producing hypnotic properties.

One critical issue is the selectivity of the manipulations that were used to alter tonic but not phasic inhibition. Although the manipulations may well alter ambient GABA concentrations and thereby alter tonic inhibition, they may also alter phasic inhibition. Concerning this issue, we have previously confirmed that GAD65 gene knockout reduced tonic inhibition without affecting phasic inhibition (IPSCs) (Kubo et al., 2009a). Tian et al. (1999) have also reported that the frequency of sIPSCs was intact in retinal ganglion cells in GAD65−/− mice. Although we do not know the source of compensation for the synthesis of GABA being released, deleting GAD65 gene may have little effect on the GABA content of spontaneously released synaptic vesicles.

4.4. Molecular targets of general anesthetics: synaptic and extrasynaptic GABA receptors

Growing evidence suggests that tonic GABA(A) receptors may be more important mediators of anesthetic-induced neural depression than phasic GABA(A) receptors (Orser, 2006). This concept is largely based on findings that tonic GABA(A) receptors are very sensitive to low concentrations of anesthetics. The continuous, non-desensitizing nature of tonic GABA(A) receptors could be expected to contribute to a larger charge transfer or shunting inhibition. The present study suggests that this notion may be true for some, but not all, types of general anesthetics. In contrast to the strong depressant effects produced by propofol (Bieda and MacIver, 2004) and thio-pental (Bieda et al., 2009), actions of sevoflurane (0.23 mM) were not strong enough to alter discharge properties of layer V cortical neurons. In support of our data, isoflurane appears to enhance phasic GABA(A) inhibition, but does not enhance tonic GABA(A) receptors to depress synaptically evoked discharge of hippocampal CA1 neurons (Bieda et al., 2009). In addition, halothane at clinical concentrations (0.35 mM) has only minimal depressant effects on the postsynaptic membrane excitability of CA1 pyramidal cells (Sonner et al., 2003). AP discharge in response to depolarizing current injection is not altered by halothane, nor is the threshold, rise time or amplitude of spikes altered. Furthermore, Ogawa et al. (2011) have examined the effects of sevoflurane (0.3 mM) on extrasynaptic GABA receptors in mechanically dissociated hippocampal CA1 neurons. GABA (1 mM)-induced currents were enhanced by sevoflurane (138% of control), suggesting that extrasynaptic GABA receptors may contribute to the enhancement of the inhibitory responses to some degree. All over the result suggest that the role of ambient GABA concentrations (tonic conductance) may be different in volatile vs. intravenous anesthetics.

In this context, we are interested in the influence of ambient GABA contents on amnestic actions of anesthetics, because it has been shown that GABA(A) receptor z5 subunit null mutant mice had very little etomidate-induced amnesia, long-term potentiation, and tonic current augmentation (Cheng et al., 2006).

5. Conclusions

The present study provides in vivo evidence that genetic and pharmacological manipulations to alter ambient GABA concentrations have significant effects on the hypnotic and immobilizing actions of propofol and midazolam, whereas these manipulations minimally alter cellular and behavioral responses to sevoflurane.

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