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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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ABSTRACT

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.

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 intermittent GABA 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 (Brickley 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 MacIver, 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,

Abbreviations: ACSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; mIPSC, miniature inhibitory postsynaptic current; GAD, glutamate decarboxylase; GAT, GABA transporter; LORR, loss of righting reflex; LTWR, loss of tail-pinch withdrawal response; NO-711, 1-[2-[[(Diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride ($C_{21}H_{22}N_2O_3 \cdot HCl$).

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antigenicity, cellular and subcellular locations, and interaction with the GAD cofactor pyridoxal phosphate (Erlander et al., 1991). The 67-KDa isoform (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, GAD65−/− mice appear to show increased anxiety levels (Kash et al., 1999; Kubo et al., 2009a), different sensitivity to pentobarbital (Kash et al., 1999), and hyperalgesia to thermal, but not chemical, stimulation (Kubo et al., 2009b). On the other hand, inhibition of GABA uptake and/or metabolism is a strategy for enhancing ambient GABA concentrations. GABA is cleared from the synaptic cleft by specific, high-affinity, sodium- and chloride-dependent transporters, which are thought to be located on presynaptic terminals and surrounding glial cells, i.e., four distinct GABA transporters, GAT-1, GAT-2, GAT-3 and BGT-1 (Borden, 1996). NO-711, a potent and selective GAT-1 inhibitor, was used because GAT-1 is responsible for the majority of neuronal GABA transport.

We have reported that sevoflurane enhances GABAergic 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 through 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 C67BL/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 mice 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 whole 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 μl), 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 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 (Maruishi 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 (Quasha 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/LCT 10% (B. Braun Melsungen AG, Melsungen, Germany); midazolam, saline. An intraperitoneal injection of lipofundin MCT/LCT 10% (10 μl/g) alone had no hypnotic/analgesic effect on mice behavior ($n = 5$). NO-711 hydrochloride (Sigma-Aldrich 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, Maruishi, Osaka, Japan) delivered via an anesthetic-specific vaporizer (Sevotec 5, 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 tail-pinch reflex was noted. Sevoflurane concentration also was confirmed by gas chromatograph analysis (GC-4000, GL Sciences Inc., Tokyo, Japan) of samples drawn from the chamber.

2.5. Electrophysiology

The methods of brain slice electrophysiology were described previously (Nishikawa et al., 2005; Ishizeki et al., 2008). Briefly, mice were decapitated under deep isoflurane anesthesia, and the brain was then removed and immediately immersed in a cold (1–4 °C) modified Ringer solution, comprised of 234 mM sucrose; 2.5 mM KCl; 1.25 mM NaH_2PO_4; 10 mM $MgSO_4$; 0.5 mM $CaCl_2$; 26 mM $NaHCO_3$; and 11 mM glucose saturated with 95% O_2 and 5% CO_2. A block of tissue