Effects of the volatile anesthetic sevoflurane on tonic GABA currents in the mouse striatum during postnatal development

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Abstract

The volatile anesthetic sevoflurane, which is widely used in pediatric surgery, has proposed effects on GABA\A receptor-mediated extrasynaptic tonic inhibition. In the developing striatum, medium-sized spiny projection neurons have tonic GABA currents, which function in the excitatory/inhibitory balance and maturation of striatal neural circuits. In this study, we examined the effects of sevoflurane on the tonic GABA currents of medium spiny neurons in developing striatal slices. Sevoflurane strongly increased GABA\A receptor-mediated tonic conductance at postnatal days 3–35. The antagonist of the GABA transporter-1, 1-[2-[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride further increased tonic GABA conductance during the application of sevoflurane, thereby increasing the total magnitude of tonic currents. Both GABA (5 \mu M) and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride, the \(\delta\)-subunit-containing GABA\A receptor agonist, induced tonic GABA currents in medium spiny neurons but not in cholinergic neurons. However, sevoflurane additively potentiated the tonic GABA currents in both cells. Interestingly, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride-sensitive neurons made a large current response to sevoflurane, indicating the contribution of the \(\delta\)-subunit on sevoflurane-enhanced tonic GABA currents. Our findings suggest that sevoflurane can affect the tone of tonic GABA inhibition in a developing striatal neural network.

Introduction

Sevoflurane is a volatile anesthetic that is commonly used in pediatric surgery because of its fast action and very short recovery time (Mellon et al., 2007). As with other general anesthetics, sevoflurane has proposed effects on many targets, including GABA\A receptor (GABA\AR)-mediated tonic inhibition. GABA\ARs can mediate both synaptic phasic inhibition and extrasynaptic tonic forms of inhibition. GABA released from presynaptic terminals produces phasic inhibitory postsynaptic currents (IPSCs), and spillover of GABA from synaptic clefts can induce tonic GABA\A currents by activating persynaptic and extrasynaptic GABA\ARs. Alterations in the tonic GABA signaling are believed to be involved in disruptions of network dynamics associated with developmental diseases (Ben-Ari et al., 2007).

The striatum, the input stage of the basal ganglia, is one of the key structures of corticobasal ganglia circuits and is involved in emotional and cognitive functions as well as motor control. Accumulating evidence suggests that dysfunction of the frontostriatal system is involved in the pathophysiology of developmental disorders, including attention deficit hyperactivity disorder (ADHD) (Chudasama & Robbins, 2006). About 95% of striatal neurons are GABAergic medium-sized spiny projection neurons (MSNs). The maturation of GABAergic synaptic preceses that of glutamatergic excitatory inputs (Tepper et al., 1998; Uryu et al., 1999). This suggests that the disturbance of striatal GABAergic inhibition would have a large impact on the developing inhibitory network itself. GABA\ARs are pentameric anion-selective ion channels that assemble from 19 different subunits (Whiting et al., 1999). Tonic GABA\A currents mediated by \(\delta\)-subunit-containing GABA\ARs were observed in striatal MSNs (Ade et al., 2008; Janssen et al., 2011; Brickley & Mody, 2012; Egawa & Fukuda, 2013). Although anesthetics modulate sustained GABA\AR-mediated tonic conductance, which regulates the excitatory/inhibitory balance and maturation of striatal neural circuits, the effect of sevoflurane on tonic GABA conductance in the developing striatum has been little studied.

In this study, we examined the effects of sevoflurane on tonic GABA currents of MSNs in juvenile mouse striatum. We found that sevoflurane increased the tonic GABA\AR-mediated conductance on postnatal days (P)3–35. Blockade of the GABA transporter (GAT) further increased the sevoflurane-enhanced tonic GABA currents, suggesting that ambient GABA levels affect the efficacy of sevoflurane. Thus, by altering tonic inhibition, sevoflurane can exert its influence on the excitatory/inhibitory balance of the striatal network during pediatric surgery.
Materials and methods

Slice preparation

All experimental procedures were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology, and carried out in accordance with the Guideline for Animal Experimentation of the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the guidelines of the NIH in the USA. C57BL/6J mice (P3–35) were anesthetized with ether and decapitated. The brains were rapidly removed and put into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1 NaH2PO4, 1.2 MgCl2, 2.4 CaCl2 and 10 glucose, buffered to pH 7.4 with NaHCO3 (26 mmol) and saturated with 95% O2 and 5% CO2. Coronal brain slices (300 μm thick), including the striatum, were prepared with a Pro 7 Liner Microslicer (Dosaka, Kyoto, Japan) and incubated in ACSF at 32 °C for 30 min. The slices were maintained in ACSF at room temperature (21–25 °C).

Electrophysiology

The slices were placed on a recording chamber, which was perfused with ACSF, and incubated with 95% O2 and 5% CO2 at a rate of 1–2 mL/min at 30 °C. A whole-cell patch-clamp technique was performed, using an EPC9/2 amplifier (HEKA Elektronik, Lamrecht/Pfalz, Germany). Patch pipettes (4–6 MΩ) were made from borosilicate glass capillaries (1.5 mm inner diameter, 1.17 mm outer diameter; Harvard Apparatus, Holliston, MA, USA) on a PC-10 puller (Narishige, Tokyo, Japan) and er (HEKA Elektronik, Lamrecht/Pfalz, Germany). Patch pipettes (4–6 MΩ) were made from borosilicate glass capillaries (1.5 mm inner diameter, 1.17 mm outer diameter; Harvard Apparatus, Holliston, MA, USA) on a PC-10 puller (Narishige, Tokyo, Japan) and filled with (in mm): 124 Cs-methanesulfonate, 11 KCl, 2 MgCl2, 10 HEPES, 4 Na2-ATP, 0.3 GTP, 0.1 spermine, 5 QX-314 and 0.5% biocytin, which was brought to 280 mOsm and pH 7.3 with CsOH. The pipette was attached to the cell, and suctioned after the sealing resistance increased over 1 GΩ and the whole-cell condition was achieved. Medium spiny neurons and cholinergic neurons in the striatum were identified according to their morphology using an infrared–differential interference contrast video microscope (BX50WI; Olympus, Tokyo, Japan and Dage-MTI, Michigan City, IN, USA). After electrical recordings, their morphology was further confirmed by histochemical procedures (see below).

Immediately after break in to the whole-cell configuration, the membrane capacitance was calculated and averaged from the currents induced by depolarising and hyperpolarising steps (10 pulses, ± 5 mV for 500 ms) at a holding potential of −70 mV (Lindau & Neher, 1988; Gentet et al., 2000). The cells were then held at 0 mV, and input resistance was measured every 15 s by a hyperpolarising pulse (−10 mV for 100 ms). The average resistances obtained at 5–10 min after break in were pooled and analysed. The experiment was discarded if the resistance changed by more than 15%. Signals were filtered at 5 kHz, digitised at 20 kHz and acquired using PULSE (HEKA Elektronik) and PowerLab (AD Instruments, Castle Hill, Australia).

For recordings of tonic GABA currents, the neurons were held at 0 mV in the presence of the N-methyl-D-aspartate receptor antagonist 2-amino-5-phosphonovarlic acid (25 μM), and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM) to block glutamate receptors. Tetrodotoxin (1 μM) was added to the ACSF to block voltage-gated Na+ channels. Tonic GABA currents were measured as the change in the holding currents before and after the application of the GABAA receptor antagonist picrotoxin (100 μM). Sevoflurane was perfused into the ACSF with 95% O2 and 5% CO2 at 2 minimum alveolar concentration (MAC) (4%) via Meratec (Senko Medical Instrument, Tokyo, Japan). Its concentration was monitored using a Life Scope (BSM-5132; Nihon Kohden, Japan) during recording (Oose et al., 2012). Franks & Lieb (1996) proposed that the mammalian MAC expressed as the free aqueous concentration in saline (1 MAC sevolurane was 0.33 mEq) was an appropriate indication for in vitro experience. In our recording chamber, the concentration of sevolurane measured using gas chromatography was 700 μM. All drugs were bath-applied and holding currents were evaluated for the last 30 s of each application.

Histochemical procedures

Slices containing biocytin-filled cells were stained as described previously (Suzuki et al., 2001; Miura et al., 2007). Briefly, the slices
were fixed in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer overnight at 4 °C, rinsed in phosphate buffer for 30 min, and incubated in phosphate buffer containing 0.5% H₂O₂ for 15 min to suppress endogenous peroxidase activity. The slices were incubated with avidin–biotin–peroxidase complex for 2 h at room temperature, and then stained using a 3,3’-diaminobenzidine tetrahydrochloride reagent kit following the manufacturer’s protocol (VECTASTAIN Elite ABC kit PK-6100, SK4100; Vector Laboratories, Burlingame, CA, USA). Based on previous experience with cholinergic neurons, slices were incubated overnight at 4 °C with a rabbit antibody against choline acetyltransferase (1 : 400, no. AB143; Millipore, Billerica, MA, USA). The slices were incubated at room temperature for 1 h with secondary anti-rabbit antibodies conjugated to Alexa555 (1 : 200, A21429; Invitrogen, Carlsbad, CA, USA). Alexa488-conjugated streptavidin (1 : 500, Invitrogen) was used for cell staining. Images were obtained with a fluorescence microscope (AF6000, Leica, Wetzlar, Germany).

**Drugs**

Sevoflurane was manufactured by Maruishi Pharmaceutical Co., Ltd (Osaka, Japan). 2-amino-5-phosphonovaleric acid and 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid ethyl ester (Ro15-4513) were obtained from Tocris Bioscience (Bristol, UK). Ethyl (S)-11,12,13,13a-tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5-a][1,4]benzodiazepine-1-carboxylate (L-655,708), 1-[2-[(diphenylmethylene)imino]oxy]ethyl-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP) and all other drugs were purchased from Sigma-Aldrich.

**Statistics**

All data are shown as mean ± SEM. The statistical significance of difference was assessed using Student’s t-test, one-way ANOVA and
Results

Developmental changes in membrane properties of medium spiny neurons

Experiments were performed on MSNs in the mouse striatum at different postnatal time points (P3, P7, P14, P21, P28 and P35). MSNs, identified as cells that had a small-to-medium-sized soma, were intracellularly injected with biocytin to confirm the cell types after electrical recordings. Figure 1A shows MSNs at P7, P14 and P21. Dendritic trees were extended and increased in number with development. First, we examined the developmental changes of membrane properties of MSNs. The membrane resistance and capacitance were measured from P3 to P35. The membrane resistance significantly decreased from P3 to P21, and remained stable after P21 (Fig. 1B) (P3, 467 ± 46 MΩ; P7, 437 ± 40 MΩ; P14, 213 ± 21 MΩ; P21, 158 ± 20 MΩ; P28, 149 ± 17 MΩ; P35, 160 ± 23 MΩ; ANOVA, F3,105 = 3.668, P < 0.01). The membrane capacitance gradually increased from P3 to P28, and peaked at P28 (Fig. 1C) (P3, 42 ± 5 pF; P7, 60 ± 6 pF; P14, 104 ± 5 pF; P21, 138 ± 12 pF; P28, 145 ± 10 pF; P35, 129 ± 11 pF; ANOVA, F3,110 = 2.357, P < 0.05). These neuronal membrane changes indicated that neuronal maturation occurred by P21-P28 in mouse striatum (Tepper et al., 1998; Uryu et al., 1999).

Sevoflurane enhances tonic GABA currents in developing medium spiny neurons

To evaluate the effects of sevoflurane on tonic GABA currents in developing MSNs, we performed whole-cell voltage-clamp recordings using a Cs⁺-based internal solution from MSNs at P3-P35. Figure 2A shows the typical current traces at a holding potential of 0 mV and Gaussian fits to all-points histograms at P7, P14 and P28. After taking recordings of whole-cell currents for at least 15 min (control), bath-applied sevoflurane (2 MAC) increased the holding currents (Fig. 2A1 and A2). GABA_A receptor-mediated currents were blocked when picrotoxin (100 μM) was applied at the end of the recordings.

The magnitude of tonic GABA currents increased during development, and this trend was also significant after normalising to membrane capacitance (Fig. 2B) (P3, 0.028 ± 0.067 pA/pF; P7, 0.18 ± 0.018 pA/pF; P14, 0.23 ± 0.058 pA/pF; P21, 0.33 ± 0.053 pA/pF; P28, 0.51 ± 0.075 pA/pF; P35, 0.50 ± 0.12 pA/pF). Sevoflurane (2 MAC) increased tonic GABA conductance in all age groups compared with the control (Fig. 2B) (P3, 0.43 ± 0.24 pA/pF, n = 8; P7, 0.40 ± 0.12 pA/pF, n = 9; P14, 0.89 ± 0.22 pA/pF, n = 5; P21, 0.87 ± 0.12 pA/pF, n = 11; P28, 1.11 ± 0.21 pA/pF, n = 6; P35, 0.95 ± 0.16 pA/pF, n = 5; two-way ANOVA, F1,38 = 45.56 vs. control, P < 0.01). The age-related difference in the amount of control and sevoflurane-enhanced tonic GABA conductance was confirmed by two-way ANOVA (F3,38 = 5.204 vs. age, P < 0.01). To compare the degree of enhancement by sevoflurane in each age group, the ratio of the tonic GABA currents recorded in the presence of sevoflurane to control is shown in Fig. 2C (P3, 347 ± 363%; P7, 236 ± 57%; P14, 595 ± 256%; P21, 286 ± 34%; P28, 299 ± 128%; P35, 209 ± 26%; ANOVA, F3,38 = 0.4145, NS). There was no significant difference among the age groups; however, sevoflurane enhanced tonic GABA conductance throughout the developmental period of MSNs. Although the increase in tonic GABA currents at P3 was significant (P < 0.01, paired t-test), the amount of sevoflurane-enhanced tonic currents was small (16.8 ± 9.9 pA, n = 8), and thus we did not investigate further at P3 in the later experiments.

As shown in Fig. 2A, the GABA_A receptor antagonist picrotoxin (100 μM) completely blocked sevoflurane-induced outward currents as well as tonic GABA currents, suggesting that the currents produced by sevoflurane were also mediated by GABA_ARs. To further confirm this, we measured the reversal potential of the sevoflurane-induced component of tonic GABA currents. Whole-cell currents were evoked by a slow ramp voltage command before and after the application of sevoflurane (Fig. 3A). Sevoflurane-induced currents, determined by the subtraction of each current (Fig. 3B), had a reversal potential of −50.5 ± 9.8 mV (n = 4), closely resembling the Cl⁻ equilibrium potential of our recording solutions (−57 mV).

Ambient GABA affects the magnitude of the tonic GABA current in the presence of sevoflurane

Next we tested for the effect of ambient GABA on the tonic GABA current during the application of sevoflurane. The amplitude of the tonic GABA currents depends on the concentration of ambient GABA, which is affected by the activity of the GAT. Blockade of GAT would increase the concentration of ambient GABA, which might activate, and thus desensitise, extrasynaptic GABA_ARs. Previous reports showed that tonic GABA currents were increased by a GAT-1 antagonist (Kirmse et al., 2008), but not by a GAT-2/3 antagonist (Kirmse et al., 2009). However, it is not clear whether the blockade of GAT-1 will further increase tonic GABA currents in

FIG. 3. Reversal potential of the sevoflurane-induced current is close to the equilibrium potential of chloride. (A) Whole-cell currents induced by a slow voltage-ramp command (−30 to −90 mV, 4 s) in the absence (thick trace) or presence (bold trace) of sevoflurane. (B) The sevoflurane-induced current obtained from subtraction between the two traces shown in A. This cell had a reversal potential of −52 mV, which is close to the calculated equilibrium potential of our internal solutions.
the presence of sevoflurane. To block GABA uptake, the GAT-1 antagonist (10 μM NO-711) was added in the presence of sevoflurane (2 MAC). Figure 4A1 and A2 shows representative traces of NO-711 application at P7, P14 and P28. NO-711 significantly enhanced the tonic GABA currents during the application of sevoflurane (Fig. 4B) (P7: sevoflurane, 0.23 ± 0.02 pA/pF; NO-711, 0.39 ± 0.13 pA/pF; P14-P21: sevoflurane, 0.87 ± 0.17 pA/pF; NO-711, 1.51 ± 0.33 pA/pF; P28-P35: sevoflurane, 0.96 ± 0.20 pA/pF; NO-711, 1.4 ± 0.35 pA/pF; two-way ANOVA, F1,18 = 13.99 vs. sevoflurane, P < 0.01). The amount of tonic GABA conductance recorded with and without NO-711 was larger at P14-P21 and P28-P35 compared with P7 (Fig. 4B); however, the ratio of the enhancement by NO-711 was not significantly different among age groups (Fig. 4C) (P7, 163 ± 62%; P14-P21, 173 ± 12%; P28-P35, 152 ± 24%; ANOVA, F2,18 = 0.09935 vs. age, NS).

Continuous application of GABA would result in the desensitisation of GABAARs and may change the magnitude of tonic GABA currents. We then investigated whether continuously applied GABA (5–150 μM) altered the effect of sevoflurane, because surgical procedures requiring general anesthesia take several hours. Sevoflurane increased tonic GABA currents after 10 min of exposure to GABA (Fig. 5A and B) (two-way ANOVA, F1,31 = 31.24 vs. GABA, P < 0.01). In the absence and presence of sevoflurane, steady-state currents increased with increasing concentrations of exogenously applied GABA (Fig. 5B) (0 μM GABA: control, 34.8 ± 4.6 pA; sevoflurane, 99.6 ± 11.9 pA, n = 19; 5 μM GABA: control, 60.7 ± 10.3 pA; sevoflurane, 106 ± 11.7 pA, n = 7; 20 μM GABA: control, 79.2 ± 20.0 pA; sevoflurane, 116.0 ± 37.5 pA, n = 5; 150 μM GABA: control, 105.8 ± 24.3 pA; sevoflurane, 154.0 ± 7.5 pA, n = 4; two-way ANOVA, F3,31 = 3.747 vs. concentration, P < 0.05).

**Fig. 4.** The activity of the GAT affects the amplitude of tonic GABA currents during the application of sevoflurane. (A1) Representative continuous recordings of whole-cell current traces of NO-711 application at P7, P14 and P28. (A2) Expanded traces of tonic currents shown in A1. Sevoflurane shifted holding currents and the GAT-1 antagonist NO-711 caused an additional shift to more positive in these groups. The right panel shows Gaussian fits to all-points histograms derived from 30 s recording periods (control, sevoflurane, NO-711 and picrotoxin). (B) The mean tonic conductance [change in the holding currents (ΔIhold)/capacitance] of sevoflurane (gray bars) and NO-711 application (black bars). NO-711 application increased the sevoflurane current (two-way ANOVA, P < 0.01), and the difference at P14-P21 was especially significant (**P < 0.01 vs. sevoflurane, posthoc test). (C) NO-711-induced currents normalised to tonic GABA currents with sevoflurane (NO-711/sevoflurane). There was no developmental change (ANOVA). The number of experiments is indicated in the parentheses.
Recent studies had shown that GABA<sub>A</sub>R δ-subunit- or α<sub>2</sub>-subunit-mediated tonic GABAergic inhibition in striatal MSNs and the contribution of the subunits to tonic GABA currents changed developmentally (Ade et al., 2008; Santhakumar et al., 2010). We examined the contribution of the GABA<sub>A</sub>R subunit to the effect of sevoflurane on the striatal tonic GABA current during development. To standardise conditions, GABA (5 μM) was added to the ACSF in this experiment. First, we examined whether THIP, the δ-subunit-containing GABA<sub>A</sub>R agonist, occluded or synergistically enhanced the effect of sevoflurane on tonic GABA currents. THIP (1 μM) increased tonic GABA currents (Fig. 6A1). In the presence of THIP, sevoflurane (2 MAC) further enhanced tonic GABA currents in all age groups (sevoflurane: P<sub>7</sub>, 79.3 ± 24.0 pA, n = 6; P<sub>14-P21</sub>, 173.8 ± 34.8 pA, n = 9; P<sub>28-P35</sub>, 269 ± 41.5 pA, n = 6). To compare the changes in tonic GABA currents with THIP and sevoflurane, the ratio of each current to control is shown in Fig. 6A2. THIP application increased the tonic GABA currents and 2 MAC of sevoflurane further increased them (Fig. 6A2) (P<sub>7</sub>: control, 100 ± 34%; THIP, 147 ± 52%; sevoflurane, 306 ± 103%; P<sub>14-P21</sub>: control, 100 ± 16%; THIP, 133 ± 29%; sevoflurane, 248 ± 52%; P<sub>28-P35</sub>: control, 100 ± 20%; THIP, 202 ± 30 pA; sevoflurane, 475 ± 78%). Although the increases in THIP and sevoflurane were especially significant in each age group (Fig. 6A2) (two-way ANOVA, F<sub>2,42</sub> = 34.69, P < 0.01 vs. control), there was no difference among age groups. However, the degree of the changes varied in each cell. Figure 6A3 shows scatter plots of the data shown in Fig. 6A2. The percentage of increase by sevoflurane was plotted against the percentage of change by THIP application. The enhancement of tonic GABA currents by sevoflurane was well-correlated with the changes with THIP (R<sup>2</sup> = 0.7317).

Next we examined whether the α<sub>2</sub>-subunit antagonist L-655,708 reduced tonic GABA currents and then attenuated the enhancement by sevoflurane (Fig. 6B1). Whereas L-655,708 (200 nm) decreased the tonic GABA currents, sevoflurane applied with L-655,708 increased the tonic currents (sevoflurane: P<sub>7</sub>, 35.3 ± 13.2 pA, n = 3; P<sub>14-P21</sub>, 122.7 ± 13.9 pA, n = 10; P<sub>28-P35</sub>, 102.1 ± 13.2 pA, n = 7). The degree of changes by L-655,708 and sevoflurane was significant in all age groups (Fig. 6B2) (P<sub>7</sub>: control, 100 ± 36%; L-655,708, 65 ± 23%; sevoflurane, 192 ± 88%; P<sub>14-P21</sub>: control, 100 ± 12%; L-655,708, 75 ± 9%; sevoflurane, 135 ± 16%; P<sub>28-P35</sub>: control, 100 ± 17%; L-655,708, 78 ± 15%; sevoflurane, 157 ± 22%; two-way ANOVA, F<sub>2,34</sub> = 33.89, P < 0.01 vs. control); however, a significant difference was not seen among age groups. The percentage increase by sevoflurane was poorly correlated with the inhibition by L-655,708 (Fig. 6B3) (R<sup>2</sup> = 0.007605).

We also used Ro15-4513, an inverse agonist at benzodiazepine-sensitive α<sub>1</sub>-, α<sub>2</sub>-subunit- and α<sub>3</sub>-subunit-containing receptors and a partial agonist of α<sub>2</sub>-subunit- and α<sub>6</sub>-subunit-containing receptors (Fig. 6C1). Ro15-4513 is less selective for GABA<sub>A</sub>R subunits compared with L-655,708; however, it may have a similar effect to L-655,708 by acting as an inverse agonist to α<sub>2</sub>-subunit-containing GABA<sub>A</sub>Rs. The application of Ro15-4513 (300 nm) slightly decreased the tonic GABA currents and co-applied sevoflurane increased the current amplitude (sevoflurane: P<sub>7</sub>, 27.6 ± 9.7 pA, n = 3; P<sub>14-P21</sub>, 132.8 ± 23.9 pA, n = 7; P<sub>28-P35</sub>, 163.8 ± 29.4 pA, n = 4). Ro15-4513 had the same effect as the application of L-655,708 (Fig. 6C2) (P<sub>7</sub>: control, 100 ± 28%; Ro15-4513, 38 ± 11%; sevoflurane, 101 ± 43%; P<sub>14-P21</sub>: control, 100 ± 18%; Ro15-4513, 80 ± 14%; sevoflurane, 190 ± 37%; P<sub>28-P35</sub>: control, 100 ± 9%; Ro15-4513, 79 ± 14%; sevoflurane, 228 ± 47%; two-way ANOVA, F<sub>2,22</sub> = 15.84, P < 0.01 vs. control). There was no significant difference among age groups, and the fitting line of Ro15-4513 had a small correlation (Fig. 6C3) (R<sup>2</sup> = 0.1050).

**Cell type specificity of the effects of sevoflurane on tonic GABA currents**

Striatal neurons contain 2–3% of cholinergic interneurons, which are morphologically and neurochemically distinguished from MSNs (Kawaguchi et al., 1995; Bolam et al., 2000; Suzuki et al., 2001). Acetylcholine released from cholinergic neurons is quintessential for striatal functions (Aosaki et al., 2008; Santhakumar et al., 2010), and therefore we investigated whether GABA, THIP and sevoflurane increase or decrease tonic GABA currents in cholinergic neurons. Cholinergic neurons had large soma (> 20 μm), and were easily identified under the infra-red-differential interference contrast microscope. After electrical recordings, cell types were further confirmed by immunostaining against choline acetyltransferase (Fig. 7A and B). The application of GABA (5 μM) increased the tonic GABA current in MSNs, but this change was not seen in cholinergic neurons (Fig. 7C) (MSNs, P<sub>14-P28</sub>, n = 38: control, 55.9 ± 5.1 pA; GABA, 71.4 ± 5.9 pA, P < 0.01; cholinergic neurons, P<sub>14-P28</sub>, n = 16: control, 56.2 ± 9.6 pA; GABA, 51.4 ± 11.9 pA, NS, paired t-test). In contrast to MSNs, THIP failed to increase the tonic GABA currents in cholinergic neurons. Sevoflurane enhanced tonic GABA currents in both cell types (Fig. 7D) (MSNs: GABA, 69.3 ± 8.7 pA; THIP, 104.3 ± 14.1 pA; sevoflurane, 212.3 ± 27.6 pA; cholinergic neurons: GABA, 44.0 ± 7.3 pA; THIP, 28.0 ± 5.9 pA; sevoflurane, 71.5 ± 10.5 pA; two-way ANOVA, F<sub>2,64</sub> = 29.95 vs. GABA, P < 0.01). The degree of enhancement was relatively small in cholinergic neurons despite their large cell bodies (two-way ANOVA, F<sub>1,32</sub> = 21.88 vs. MSN, P < 0.01). These results indicate that the regulation of the GABA<sub>A</sub>R subunit,
contributing to tonic GABA currents, was different between medium-sized spiny (MS) projection neurons and cholinergic interneurons.

Sevoflurane dose-dependently affects tonic and phasic GABA currents in striatal medium-sized spiny projection neurons

Finally, we evaluated the dose-dependency of the effect of sevoflurane on tonic and phasic GABA currents in MSNs (P14-P21). Clinically used concentrations of sevoflurane (1, 2 and 4%) increased tonic GABA currents in a dose-dependent manner (Fig. 8A and B) (1%: control, 0.27 ± 0.092 pA/pF; sevoflurane, 0.30 ± 0.091 pA/pF, n = 6; 2%: control, 0.25 ± 0.022 pA/pF; sevoflurane, 0.46 ± 0.057 pA/pF, n = 12; 4%: control, 0.35 ± 0.049 pA/pF; sevoflurane, 0.96 ± 0.12 pA/pF, n = 16; two-way ANOVA, $F_{2,31} = 11.11$ vs. concentration, $P < 0.01$, $F_{1,31} = 19.53$ vs. control, $P < 0.01$). The ratio of sevoflurane-enhanced tonic GABA conductance increased with the concentration (Fig. 8C) (1%, 122 ± 12%, $n = 6$; 2%, 195 ± 33%, $n = 12$; 4%, 327 ± 51%, $n = 16$; ANOVA, $F_{2,31} = 4.587$, $P < 0.05$).

In contrast, sevoflurane slightly but significantly suppressed the amplitude and frequency of phasic GABA currents. We recorded miniature IPSCs for 20–30 min (control), and then applied sevoflurane (1, 2 and 4%) for 10 min in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione, 2-amino-5-phosphonovaleric acid and tetrodotoxin (Fig. 8D). Sevoflurane decreased the amplitude (Fig. 8E) (1%: control, 9.9 ± 0.93 pA; sevoflurane, 10.5 ± 0.90 pA, $n = 4$; 2%: control, 13.3 ± 0.89 pA; sevoflurane, 12.5 ± 0.76 pA, $n = 9$; 4%: control, 11.2 ± 1.3 pA; sevoflurane, 9.1 ± 1.0 pA, $n = 7$; two-way ANOVA, $F_{1,17} = 7.053$ vs. control, $P < 0.05$) and frequency (Fig. 8G) (1%: control, 3.6 ± 0.37 Hz; sevoflurane, 4.0 ± 0.19 Hz, $n = 4$; 2%: control, 3.8 ± 0.68 Hz; sevoflurane, 3.6 ± 0.63 Hz, $n = 9$; 4%: control, 4.1 ± 0.93 Hz; sevoflurane, 3.2 ± 0.67 Hz, $n = 7$; two-way ANOVA, $F_{1,17} = 8.693$ vs. control,

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Developmental changes of membrane properties in medium-sized spiny projection neurons and the effects of the volatile anesthetic sevoflurane on tonic GABA conductance

In the striatum, about 95% of neurons are GABAergic medium-sized projection neurons (MSNs). They receive GABAergic inhibitory inputs from neighboring MSNs and GABAergic interneurons, whereas glutamatergic excitatory afferents come from the cerebral cortex and thalamus. Tepper et al. (1998) demonstrated that the density of axodendritic and axospinous GABAergic synapses was at a maximum at P15 and remained elevated until adulthood. However, synaptogenesis of excitatory synapses follows the maturation of inhibitory synapses. At P21, the density of excitatory synapses is close to that of an adult. Thus, the total maturation of striatal neural circuits would occur at P21-P28. In our study, we used mice of 3–35 days old, as this age bracket in mice is considered to be an important stage for neuronal maturation. As with previous studies using rats (Tepper et al., 1998; Uryu et al., 1999), we confirmed that the membrane properties (input resistance and capacitance) of MSNs matured until P21–P28 in C57BL/6J mice. Tonic GABA conductance of MSNs was clearly observed and increased during P3–P35. Because synaptogenesis induces the growth of presynaptic and postsynaptic elements, the increase of tonic currents might, at least in part, reflect the maturation of the GABAergic system.

Volatile anesthetics (halothane, enflurane, isoflurane and sevoflurane) all modulate GABA responses, but each of them has rather different effects on synaptic and extrasynaptic GABA Rs-mediated currents. Isoflurane increases the amplitude of tonic GABA currents in thalamocortical neurons in the thalamus (Jia et al., 2008) and CA1 pyramidal neurons in the hippocampus (Caraiscos et al., 2004). However, a low concentration of sevoflurane (0.23 mM) does not change tonic GABA currents in layer V pyramidal neurons in the cortex, although an enhancement of phasic IPSCs and decrease of cell excitability were clearly observed (Nishikawa et al., 2011). However, sevoflurane (0.3–1.0 mM) enhances GABA-induced currents in isolated hippocampal CA1 neurons. Until now, it has been unclear whether sevoflurane affects tonic GABA currents in MSNs in the striatal slice preparations. In our study, sevoflurane (2 MAC, 0.7 mM) increased tonic GABA conductance at all ages tested (Fig. 2). The magnitude of sevoflurane-enhanced tonic currents increased with development. By increasing the tonic GABA conductance, sevoflurane could affect the excitatory/inhibitory balance and/or shunt the synaptic inputs to the dendritic tree of MSNs. Nevertheless, our results indicated that sevoflurane had a certain effect on tonic GABA inhibition in MSNs during early developmental stages.

Ambient GABA modulates the magnitude of sevoflurane-enhanced tonic GABA currents

Houston and coworkers reported that the potentiation of tonic GABA conductance by anesthetics is dependent on the concentration of GABA. The intravenous anesthetic propofol-induced current shift is masked by the presence of 1 μM of GABA (Houston et al., 2012). The concentration of ambient GABA is regulated by neuronal and glial GATs, and the spillover of GABA from synaptic clefts

References

Kotani & Akaike, 2013; Uryu et al., 1999; Tepper et al., 1998; Jia et al., 2008; Caraiscos et al., 2004; Nishikawa et al., 2011; Houston et al., 2012.
can activate neighboring extrasynaptic GABA_A Rs. Sebe et al. (2010) showed that the tonic GABA current, in mouse neocortical pyramidal cells, developmentally increased when GABA uptake was blocked using a GAT-1 and GAT-2/3 blocker. However, in the striatum, tonic GABA currents were increased by blockade of a GAT-1 but not GAT-2/3 antagonist (Kirmse et al., 2008, 2009). In our results, the sevoflurane-enhanced tonic GABA currents again increased when GAT-1 was blocked. After the application of 5–150 μM of GABA for 10 min, sevoflurane further induced the additional current shift (Fig. 5B). Considering the observation that sevoflurane-induced tonic GABA currents were not occluded by co-applied GABA, the superimposed currents by NO-711 could be due to the increase of tonic currents by elevated GABA concentration. The result indicates that the ambient level of GABA can increase and decrease the magnitude of tonic currents during the application of sevoflurane.

### Contributions of the GABA_A receptor subunit to sevoflurane-enhanced tonic GABA currents

The GABA_A α5-subunit and δ-subunit, which mediate the tonic GABA current, are expressed in the developing striatum (Laurie et al., 2012). Therefore, the contribution of these subunits to sevoflurane-enhanced tonic GABA currents was examined.
et al., 1992). It is reported that the most abundant subunit combination of the extrasynaptic GABA receptor is α5β3γ2 (McKernan & Whitington, 1996). In addition to δ-subunit-containing GABAARs, the α2-subunit and α6-subunit mediate the tonic GABA current in other parts of the brain (Egawa & Fukuda, 2013). In the striatum, MSNs are divided into two cell types, i.e. MSNs expressing the dopamine D1 receptors (D1+ MSN) and dopamine D2 receptors (D2+ MSN) (Gerfen & Surmeier, 2011). Ade et al. (2008) examined young mice (P16-P25) and demonstrated that D2+ MSNs with a higher expression of the α2-subunit had a larger tonic inhibition than D1+ MSNs. Santhakumar et al. (2010) showed that the developmental change of GABAAR subunit expression made the difference in the tonic GABA current between young and adult mice. Adult mice (>P30) have a larger magnitude of tonic GABA currents in D1+ than observed in D2+ MSNs (Santhakumar et al., 2010). As with previous studies, the δ-subunit-containing GABAAR antagonist flurane (Fig. 6) The contributions of the GABAAR δ-subunit could be masked in the averaged data obtained from both D1+ MSNs and D2+ MSNs, although we could not divide the MSNs into two types by the effects of L-655,708. The results could be due to the incomplete blockade of δ-subunit-containing GABAARs and/or the presence of another site of sevoflurane action in addition to the GABAAR δ-subunit. To elucidate the contribution of the GABAAR δ-subunit to the effects of sevoflurane, cell-type-specific recordings using genetically-modified animals might be appropriate for further studies (Ade et al., 2008; Santhakumar et al., 2010).

Although the δ-subunit-containing GABAAR agonist THIP increases tonic GABA currents in both D1+ MSNs and D2+ MSNs, the genetic depletion of the δ-subunit decreases the amplitude of tonic GABA currents in adult (Santhakumar et al., 2010) but not young (P16-P25) mice (Janssen et al., 2011), suggesting the partial contribution of the GABAAR δ-subunit to tonic GABA currents. In this study, the δ-subunit-containing GABAAR agonist THIP increased tonic GABA currents, and sevoflurane induced the additive enhancement in all age groups. Significant differences in the efficacy of sevoflurane were not observed among age groups; however, differences between drugs varied by each cell type. The amount of the THIP increase was highly correlated with the effect of sevoflurane on tonic GABA inhibition. This result indicates that THIP-sensitive MSNs are also sensitive to sevoflurane, and that sevoflurane-enhanced tonic GABA currents might be further enhanced by co-applied drugs acting on δ-subunit-containing GABAARs.

Cholinergic neurons, which have different neuronal characteristics from MSNs, critically modulate the activity of MSNs (Tepper & Bolam, 2004; Aosaki et al., 2010; Inoue et al., 2012). We therefore examined cholinergic neurons and found that exogenous GABA (5 μM) failed to increase tonic GABA currents in contrast to the results of MSNs. THIP decreased tonic GABA currents and the enhancement by co-applied sevoflurane is relatively small in cholinergic neurons. In newborn cortical neurons, THIP decreases the tonic GABA currents in the presence of 5 μM of GABA (Sebe et al., 2010). The differences would be due to the distinct expression of the GABAAR subunit in different ages and cell types, suggesting that the regulation of the GABAAR subunit, contributing to tonic GABA currents, is different between MS projection neurons and cholinergic interneurons.

Anesthetic effect on the striatum in the developing brain

The striatum is the major nucleus of the basal ganglia and plays a key part, related to motor behavior and habit learning. Dysfunction of the frontostriatal system may underlie the behavioral symptoms observed in developmental disorders, including ADHD (Chudasama & Robbins, 2006). Although early exposure to general anesthesia disturbs the development of neuronal networks (Satomo et al., 2009; Jevtovic-Todorovic et al., 2012; Sprung et al., 2012), little interest has been shown in the effect of anesthetics on tonic GABA inhibitions in the striatum. In this study, we examined mouse striatum to observe the anesthetic effect on tonic GABA currents during development. The application of sevoflurane has an effect on tonic GABA-induced conductance in the striatum during early developmental stages (P3-P35). Sevoflurane further increased the amplitude of tonic GABA currents in the presence of THIP, a δ-subunit-containing GABAAR agonist. At present, it is still unclear how anesthetic-induced GABA-mediated responses affect the developing brain, and thus knowledge of the effects of anesthetics would be meaningful for animal studies and clinical use. A previous study indicates that 2 MAC is an appropriate concentration to produce general anesthesia when sevoflurane is administered as a sole agent (Kimura et al., 1994). Usually, sevoflurane is used with other anesthetics to decrease the concentration of sevoflurane and the incidence of side-effects. In that case, drugs acting with δ-subunit-containing GABAAR should be used cautiously.

Although GABAergic mechanisms might be involved to some extent in the hypothalamic actions of volatile anesthetics (Karakosta et al., 2010; Kim et al., 2012; Liang et al.; but see Nishikawa et al., 2011), it is still unclear whether enhanced tonic GABA currents contribute to the hypothalamic action of sevoflurane. The physiological and pathological roles of sevoflurane-enhanced tonic conductance remain to be elucidated.

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Abbreviations

ACSF, artificial cerebrospinal fluid; D1+ MSN, medium-sized spiny projection neuron expressing the dopamine D1 receptor; D2+ MSN, medium-sized spiny projection neuron expressing the dopamine D2 receptor; GABAAR, GABA receptor; GAT, GABA transporter; IPSC, inhibitory postsynaptic current; L-655,708, ethyl (S)-11,12,13,14-tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5-a][2,1-c][1,4]benzodiazepine-1-carboxylate; MAC, minimum alveolar concentration; MSN, medium-sized spiny projection neuron; NO-711, 1-[(diethylamino)methyl]imidazo[1,2-b][1,4]benzodiazepine-3-carboxylate ethyl ester; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-carboxylic acid hydrochloride; P, postnatal day; Ro15-4513, 8-azido-5,6-dihydro-5-oxo-4H-imidazol[1,5-a][1,4]benzodiazepine-3-carboxylic acid ethyl ester; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-carboxylic acid hydrochloride.

References


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