The Effect of Sevoflurane Inhalation on GABAergic Neurons Activation: Observation on the GAD67-GFP Knock-In Mouse

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ABSTRACT

The mechanisms underlying volatile anesthesia agents are not well elucidated. Emerging researches have focused on the participation of γ-aminobutyric acid (GABA) neurons but there still lacks morphological evidence. To elucidate the possible activation of GABAergic neurons by sevoflurane inhalation in morphology, Fos (as neuronal activity marker) and GABA neurons double labeling were observed on the brain of glutamic acid decarboxylase (GAD) 67-GFP knock-in mice after sevoflurane inhalation. Twenty GAD67-GFP knock-in mice were divided into three groups: S1 group: incomplete anesthesia state induced by sevoflurane; S2 group: complete anesthesia state induced by sevoflurane; control(C) group. Sevoflurane induced a significant increase of Fos expression in the dorsomedial hypothalamic nucleus (DM), periaqueductal grey (PAG), hippocampus (CA1, DG), paraventricular thalamic nucleus (PV), lateral septal nucleus (LS), and cingulate cortex (Cg1 and Cg2) in S1 group compared to C group, and increase of Fos expression in S2 group compared to S1 group. In S2 group, Fos was only expressed in the medial amygdaloid nucleus (MeA), Edinger–Westphal (E–W) nucleus, arcuate hypothalamic nucleus (Arc) and the ventral part of paraventricular hypothalamic nucleus (PaV). Double immunofluroscent staining indicated that in LS, almost all Fos were present in GABAergic neurons. In CA1, DG, DM, cg1, cg2, and PAG, Fos was expressed as well, but only few were present in GABAergic neurons. Fos expression was very high in thalamus, but no coexistence were found as no GABAergic neuron was detected in this area. Our results provided morphological evidence that GABAergic transmission in specific brain areas may participate in the sevoflurane-induced anesthesia. Anat Rec, 293:2114–2122, 2010.

Key words: sevoflurane; anesthesia; γ-aminobutyric acid (GABA); Fos; mouse

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As one of the inhaled anesthetics, sevoflurane has been extensively used in clinics. However, the mechanisms underlying sevoflurane anesthesia are not well elucidated and remain controversial (Perouansky et al., 2004). As lots of volatile anesthetic agents (including sevoflurane) have been shown to enhance the endogenous γ-aminobutyric acid (GABA)-mediated inhibition in the mammalian central nervous system (CNS) (Franks and Lieb, 1993), emerging researches have focused on GABAergic mechanisms of sevoflurane anesthesia. Neuropharmacological and electrophysiological methods have been used to clarify the effects of the sevoflurane on GABA-mediated synaptic transmission, and these studies indicate that there is an enhancement of $GABA_A$ receptor-mediated chloride currents (Banks and Pearce, 1999; Nishikawa and MacIver, 2001). It is much more interesting to investigate the participation of GABAergic neurons in the function of sevoflurane anesthetics. However, there is no morphological supports for the participation of GABAergic neurons in the brain because of difficulties in staining GABAergic neurons (Li et al., 1996), thus few morphological information is available concerning the sevoflurane on GABA neuronal activity.

In this study, we used transgenic mice coexpressing enhanced green fluorescent protein (GFP) and GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) 67 to characterize GABAergic neurons in CNS (Tamamaki et al., 2003). The GAD67-GFP knock-in mouse has been proved to be a very useful tool to observe the morphology of GABAergic neurons, which were revealed by GFP expression (Huang et al., 2008).

As a protooncogene, *c-fos*, and its protein Fos, have been shown to act as an inducible transcriptional factor and suggested to regulate the expression of the downstream response genes (Sassone-Corsi et al., 1988). It is considered as a marker of neuronal activation. Expression of Fos reflects an intracellular state of cells that varies primarily as a result of recent activation by intercellular signals (e.g., neurotransmitters, hormones, paracrine factors, and adhesion molecules) (Herdegen and Leah, 1998; VanElzakker et al., 2008). Even though it is nonspecific, when light, sound, and mechanical stimulation were avoided, increased Fos expression directly reflects increases in neuronal activity.

Sevoflurane has no irritative odour compared with other inhaled anesthetics, and the feature of less inhibitory to cardiovascular system. So, we performed the current study with sevoflurane. We hypothesized that sevoflurane exposure can activate GABAergic neurons as indicated by nuclei Fos expression, thus enhance inhibitory effect on neuronal transmission. This is considered to be a mechanism for sevoflurane anesthesia and has obtained its electrophysiological evidences (Gage and Robertson, 1985). Eletrophysiological studies demonstrate that inhibitory GABAergic neuron activity is enhanced by inhaled anesthetics, suggesting a mechanism by which sevoflurane induces anesthesia. To seek morphological evidence for this hypothesis, we performed this study on glutamic acid decarboxylase (GAD) 67-green fluorescent protein (GFP) knock-in mice with which GABAergic neurons can be easily visualized.

MATERIALS AND METHODS

Animals

The production of the GAD67-GFP knock-in mice has been already reported (Tamamaki et al., 2003). In this study, 20 male GAD67-GFP knock-in mice (heterozygote) aged 12 weeks were used. It has been confirmed that GAD67-GFP knock-in mice exhibit normal growth, normal reproductive behavior, and no abnormality in the brains at macroscopic level (Tamamaki et al., 2003). All experimental procedures were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, P. R. China).

Sevoflurane Inhalation

Animals were randomly divided into three groups: incomplete anesthesia group (S1 group, $n = 8$), complete anesthesia group (S2 group, $n = 8$) and normal control (C group $n = 4$). Mice were placed in a sealed plastic cage (L × W × H: 60 × 30 × 20 cm), and light, sound, and mechanical stimulation were avoided. It is known that body temperature is decreased with general anesthetics, but Fos expression is unaffected by body temperature decrease (Hargreaves et al., 2007). The sevoflurane (Abbott Lab., IL, USA) was delivered by vaporizers (Drager, Luebeck, Germany) driven by 100% oxygen pumps in S1 and S2 groups. Mice in C group were treated with 100% oxygen only instead of sevoflurane. The expired end-tidal concentration of agent was recorded. Vaporizer incrementally adjusted at 0.5%-step every 10–15 min. As the mice approached immobility, the righting reflex was tested every 3 min. Incomplete anesthesia state of mice in low dose group was indicated by loss of righting reflex (LORR) for no longer than 30 sec, and loss of the righting reflex exceeding 30 sec for three times was defined as complete anesthesia state (Sekine et al., 2006). The value of sevoflurane concentration were (1.7 ± 0.3)% and (2.9 ± 0.2)%, respectively. All mice in the experimental groups were maintained in the relative states (S1 or S2) for 1 hr.

Immunohistochemistry and Double Immunofluorescence

Mice were quickly sacrificed with lethal dose of aether, then perfused with 50 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 100 mL of 4% (wt/vol) paraformaldehyde in 0.2 M phosphate buffer (PB, pH 7.4). The brain was then removed and placed into the same fresh fixative for an additional 2–4 hr at 4°C. Subsequently the brain was placed into 30% (wt/vol) sucrose solution in 0.1 M PB (pH 7.4) overnight at 4°C, and then cut serially into 30 μm-thick coronal sections by using a freezing microtome (Kryostat 1720; Leitz, Mannheim, Germany). The sections were placed into four different dishes according to their numerical order. Each dish contained a complete serial section. Sections in the first dish were incubated at 4°C overnight with rabbit anti-Fos antibody (1:500; Abcam, UK); the incubation medium was prepared by using 0.05 M PBS (pH 7.4) containing 0.3% (vol/vol) Triton X-100, 0.25% (wt/vol)γ-carrageenan, 5% (vol/vol) normal donkey serum and 0.05% (wt/vol) NaN_3 (PBS-XCD). Sections in the second dish were incubated with a mixture of mouse anti-GFP antibody (1:500; Chemicon, USA) and rabbit anti-Fos antibody (1:500) overnight at 4°C. After a rinse with PBS, the sections in the first dish were incubated for 4 hr in PBS-XCD with the Biotinylated goat anti-rabbit IgG (1:200; Vector, USA) and for 1 hr in avidin-biotin-peroxidase complex (1:200; Vector). Sections in the

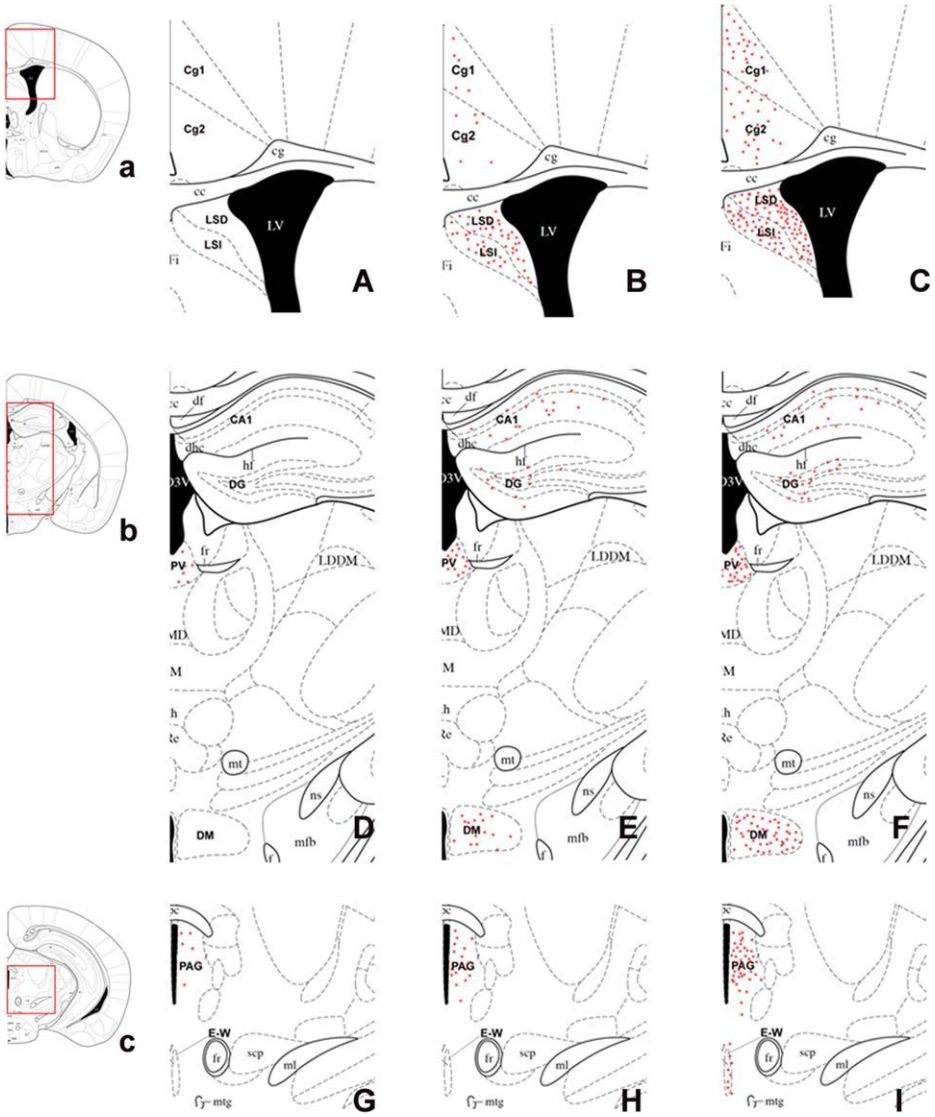

Fig. 1. Diagrams of brain sections at different levels showing Fos-IR neurons different brain regions of mice in control (**A**, **D**, **G**), S1 (**B**, **E**, **H**), and S2 (**C**, **F**, **I**) groups. Figures in [LS, Cg1, and Cg2 (A–C)] represented the red square in figure "a" magnified; Figures in [CA1, DG, DM, and PV (D–F)] represented the red square in figure "b" magnified; Figures in [PAG and E–W nucleus (G–I)] represented the red square in figure "c" magnified. Each dot represents one Fos-IR neuron.

second dish were incubated for 3 hr in PBS-XCD with Alexa 488-conjugated donkey anti-mouse IgG (1:500; Invitrogen, USA) and Alexa 594-conjugated donkey anti-rabbit IgG (1:500; Invitrogen). For immunohistochemistry, sections were placed into 50 mM Tris-HCl buffer (pH 7.6) by reaction with 0.02% (wt/vol) diaminobenzidine-4 HCl (DAB) and 0.003% (vol/vol) H_2O_2 for 15–20 min. Subsequently, the sections were mounted onto gelatin-coated glass slides. For immunofluorescence double-staining, the sections were mounted onto gelatinized glass slides and cover slipped for observation. The numbers of GABA-immunoreactive-like (IL), Fos-IL neurons, and Fos/GABA double-labeled neurons were counted and recorded in the third dish at the relative region according to the Nissl stained sections and the rat brain atlas (Paxinos and Watson, 1986). Sections in the third and forth dishes were stained for Nissle and negative control, respectively (data not shown).

Quantification and Statistical Analysis

Four nonadjacent sections from the complete serial section were selected randomly. The numbers of Fos-IL and/or GABA-IL cells in the same areas were counted under confocal visualization. The GABA-IL and total numbers of Fos-IL cells of the relative region were averaged across the four brain sections for each experimental group. Fos and GFP double-labeled neurons were counted and the proportion of double-labeling neurons to total GABA-IL neurons and total Fos-IL neurons were then calculated. Quantifications were performed in the

double-blind manner. The person scoring the Fos histology was blinded to groups.

The data were expressed as mean ± standard error mean (SEM). The results of C, S1 and S2 groups were analyzed with one-way ANOVA followed by a post hoc Bonferroni test. The difference between the numbers of Fos/GABA double-labeled neurons in S1 and S2 groups were analyzed by Student's t test and $P < 0.05$ was considered as a significant difference.

RESULTS

Immunohistochemistry

Fos immunohistochemical staining showed that very few Fos-LI neurons were observed in the brain in the control group (Figs. 1, 2). The distribution of Fos-LI neurons in the brain in S1 and S2 groups were mainly in following seven regions: cingulate cortex (Cg1 and Cg2), dorsomedial hypothalamic nucleus (DM), periaqueductal grey (PAG), CA1 region of hippocampus (CA1), dentate gyrus (DG), paraventricular thalamic nucleus (PV), and lateral septal nucleus (LS) (Figs. 1, 2). Representative photomicrographs of Fos staining and statistically analysis between groups were presented in Fig. 3. One-way ANOVA showed significant differences among these groups. Post hoc analysis revealed a significantly increase in S2 group in comparison to S1 group ($P < 0.01$), and increase in S1 group in comparison to C group ($P < 0.01$). The number of Fos-IL neurons per mm^2 in theses regions were as follows: Cg1, Cg2 (S1: 10.3 ± 6.2; S2: 27.3 ± 5.1), DM (S1: 10.2 ± 3.9; S2: 19.8 ± 7.2), PAG (S1: 12.3 ± 4.6; S2: 18.9 ± 7.7), CA1 (S1: 3.8 ± 1.1; S2: 6.0 ± 2.8), DG (S1:4.2 ± 2.2; S2:7.8 ± 2.1), PV (S1: 9.7 ± 2.1; S2: 21.5 ± 5.6), LS (S1: 25.1 ± 7.8; S2: 47.4 ± 10.1). Besides the seven areas mentioned earlier, Fos expression was also detected in the following areas of S2 group, but not in those of S1 group: medial amygdaloid nucleus (MeA) (16.3 ± 9.8), Edinger–Westphal (E–W) nucleus (17.8 ± 7.3), arcuate hypothalamic nucleus (Arc) (21.7 ± 7.3), the ventral part of paraventricular hypothalamic nucleus (PaV) (18.9 ± 7.1).

Double Immunofluorescence

We further performed GFP and Fos double immunofluorescent histochemistry to investigate the coexistence of Fos in GABAergic neurons. In LS, numerous Fos/GABA double-labeled neurons were observed, and almost all Fos positive neurons were also positive for GABA [S1 group: (95.8 ± 3.8)%, S2 group: (94.4 ± 3.6)%] (the percentage is Fos/GABA double-labeled neurons to Fos-IL neurons) (Fig. 4). Though Fos expressions were also very high in CA1, DG, DM, cg1, cg2, and PAG, only a few Fos-IL neurons were positive for GABA (Table 1). Representative photomicrographs were shown in Fig. 5. In addition, Fos expression in medial amygdaloid nucleus, E–W nucleus, arcuate hypothalamic nucleus (Arc), paraventricular hypothalamic nucleus and ventral part (PaV) in S2 group were scarcely coexistent with GABA. Especially, noncoexistence of Fos and GABA was found in PV as GABA-IL cells were not observed in this area (Fig. 5 E, E′; Table 1).

We also observed that, as the concentration of sevoflurane increased, not only Fos-IL but also the Fos and GABA coexistent neurons exhibited an increased tendency in LS [S1 group: (16.3 ± 4.5)%, S2 group: (25.4 ± 9.1)%], CA1 [S1 group: (9.6 ± 3.3)%, S2 group: (17.1 ± 1.3)%], DG [S1 group: (13.1 ± 7.4)%, S2 group: (21.9 ± 11.2)%], Cg1 and Cg2 [S1 group: (2.9 ± 1.4)%, S2 group: (6.1 ± 2.0)%], DM [S1 group: (7.1 ± 2.2)%, S2 group: (12.2 ± 3.1)%], PAG [S1 group: (17.7 ± 9.9)%, S2 group: (24.8 ± 7.1)%]. Student's t test revealed significant differences between S2 and S1 groups in the Fos and GABA coexistent neurons in LS, CA1, DG, Cg1, Cg2, DM, and PAG ($P < 0.01$).

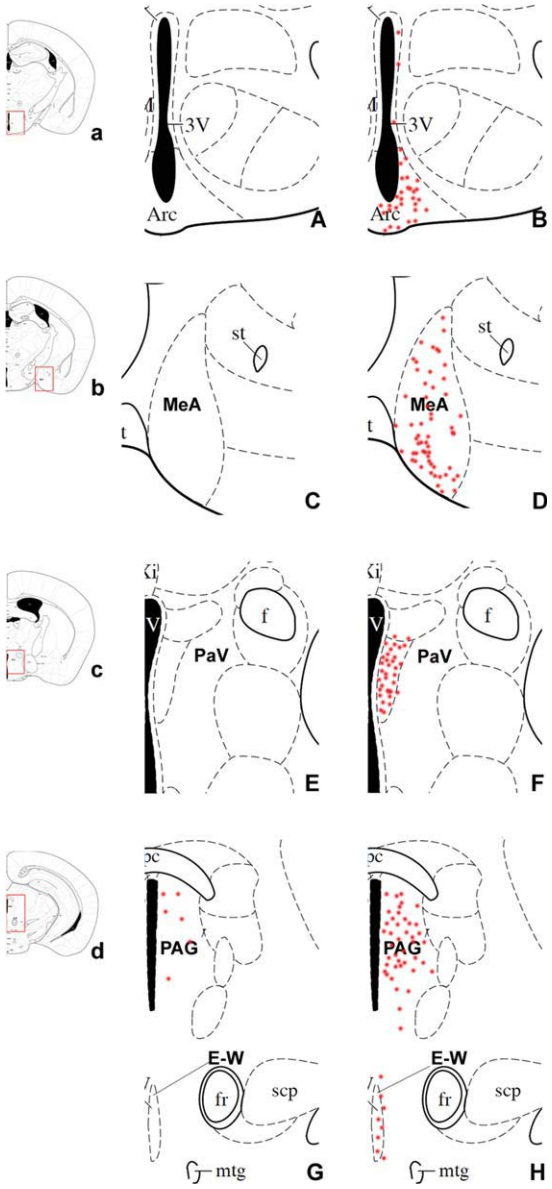

Fig. 2. Diagrams of brain sections at different levels showing Fos-IR neurons in different brain regions of mice in control (**A**, **C**, **E**, **G**) and S2(**B**, **D**, **F**, **H**) groups. Figures in Arc (A, B) represented the red square in figure "a" magnified; Figures in MeA (C, D) represented the red square in figure "b" magnified; Figures in PaV (E, F) represented the red square in figure "c" magnified; Figures in E–W nucleus (G, H) represented the red square in figure "d" magnified. Each dot represents one Fos-IR neuron.

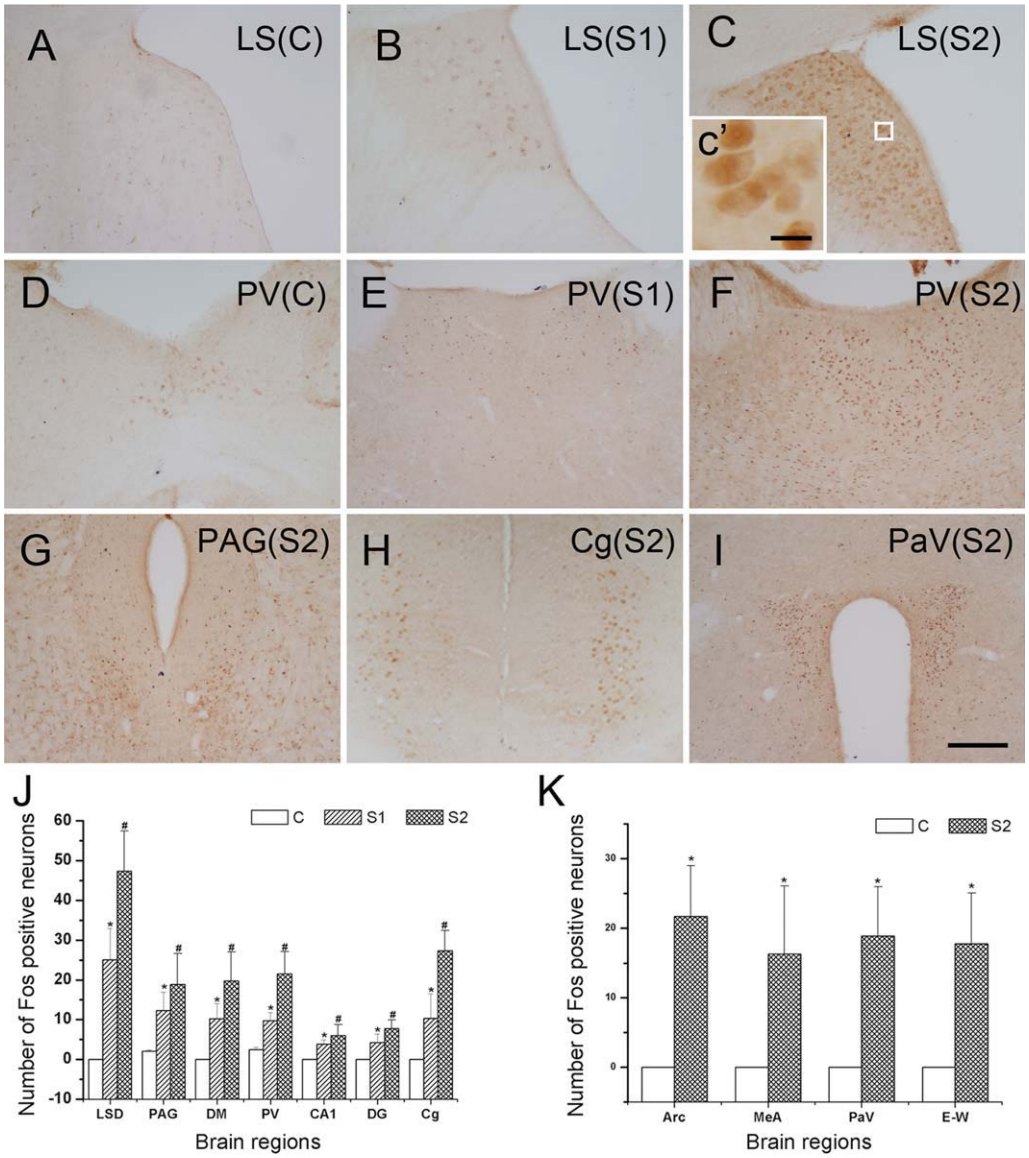

Fig. 3. Representative photomicrographs of brain sections showing Fos-IR neurons in different brain regions of mice in control (A, D), S1 (B, E), and S2 (C, F-I) groups. The magnified image of the rectangle indicated in the panel in C is shown in C′. J-K showing the statistical analysis of Fos-IR neurons in different groups. Complete anesthesia (S2) induced by sevoflurane inhalation produced much higher expressions of Fos than that of control groups in LS (A–C), PV (D–F). Besides, sevoflurane inhalation induced Fos upregulation in PAG (G), Cg (H), and PaV (I). Scale bar = 200 μm in I for A–I; 20 μm in C′. *differ from C; #differ from S1. $^{*,\#}P < 0.01$.

We calculated the percentages of Fos/GABA double-labeled neurons to GABA-IL neurons and to Fos-IL neurons respectively and found the percentage of Fos/GABA double-labeled neurons to GABA-IL neurons were also much higher in S2 group (LS, CA1, DG, Cg1, Cg2, DM, and PAG) compared with that in S1 group ($P < 0.01$). However, the percentage of Fos/GABA double-labeled neurons to Fos-IL neurons was no significant difference between S1 and S2 groups ($P > 0.01$) (Table 1).

DISCUSSION

In this study, we observed that sevoflurane inhalation induced neuronal activation in several different brain regions, like CA1 and DG of hippocampus, DM, LS, Cg1, and Cg2 of cortex, PV, PAG, Arc, MeA, PaV, and E–W nucleus. Additionally, in some brain regions, especially in the LS, most of the activated neurons were GABAergic. Furthermore, the number of activated GABAergic neurons increased as the concentration of sevoflurane increased. Our results suggested that activation of GABAergic neurons in the brain may play a pivotal role during sevoflurane-induced anesthesia.

GAD67-GFP knock-in mice were used in the present study to better show GABAergic neurons, and GABA contents had no difference with that of wild-type mouse after 7-weeks-old. The mice we used in our experiment were aged 12 weeks, when there was no significant

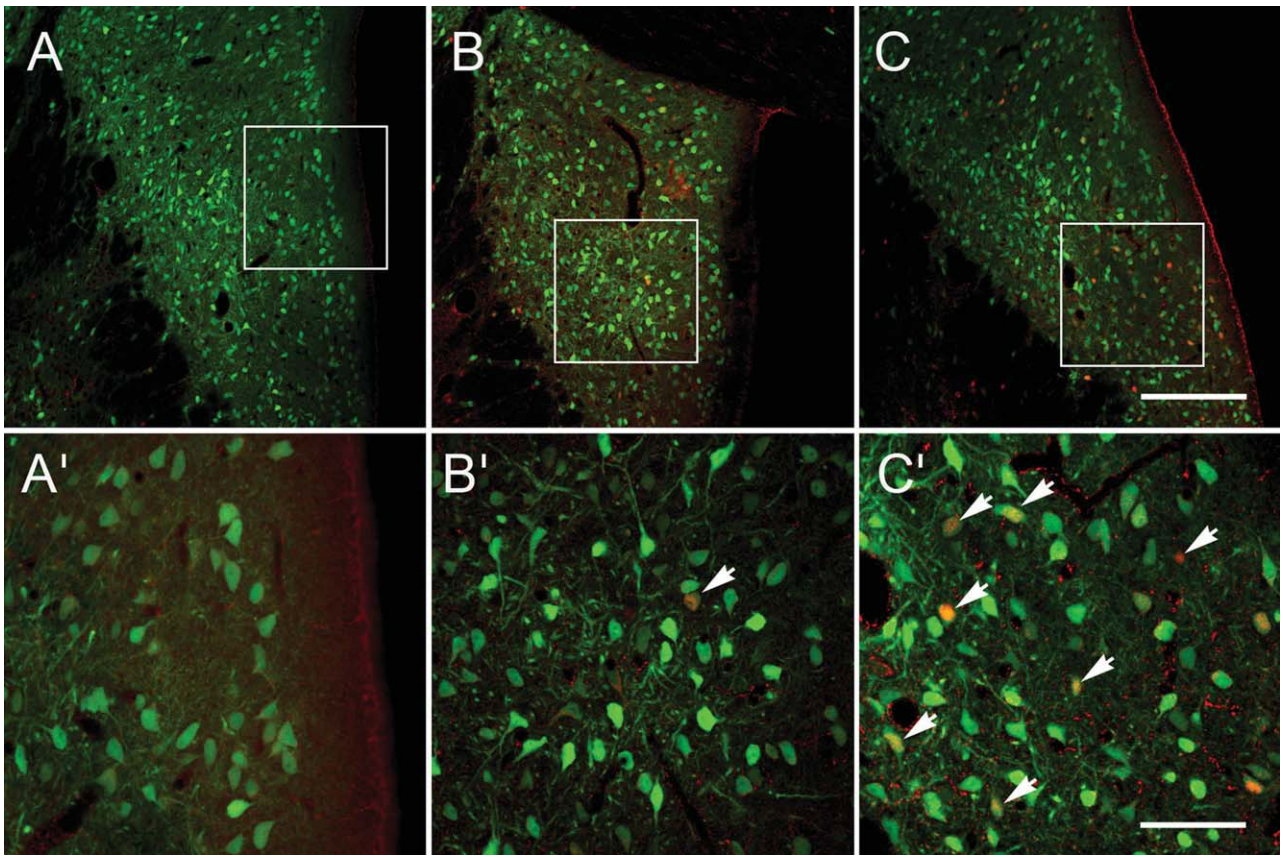

Fig. 4. Immunofluorescent double-staining for GABA (green) and Fos (red) in LS in control (**A**, **A**′), S1 (**B**, **B**′), and S2 groups (**C**, **C**′). A′–C′ are the magnified images of the rectangles indicated in A–C, respectively. White arrows indicated the Fos/GABA double-labeled neurons. Scale bar = 300 μm in C for A–C; 100 μm in C′ for A′–C′.

difference of GABA contents in the transgenic and wild-type mice (Tamamaki et al., 2003). The neurologic function is not changed in GAD67-GFP knock-in mice compared with wild-type ones. *c-fos* mRNA expression induced by sevoflurane in the brain did not show significant difference between wild-type mice and GAD67-GFP knock-in mice (Supporting Information Figure), suggesting the reliability and specificity of the knock-in mice.

Although there is no definitive evidence that specific regions of the brain are targets of inhaled anesthetics, attention has been focused on structures with roles in anesthetic-sensitive functions. Studies from PET and electrophysiology indicated that in some brain regions, such as thalamus, hippocampus, inhalation anesthetic agents induced changes in glucose metabolic rate or postsynaptic potential (Gage and Robertson, 1985; Alkire et al., 2000), where anesthetic actions in these brain regions are known occur. However, there is still no morphological evidence about the functional targets of these inhalation anesthetic. So actually, we provided here some morphological supports. The reticular-activating system, hippocampus, cortex and hypothalamus were found to be involved in memory, learning and sleep, and sensitive to inhalation anesthetic agents (Angel, 1993; Wakasugi et al., 1999; Heinke and Schwarzbauer, 2002). In addition, researchers founded that volatile anesthetics differentially enhanced $GABA_A$ receptor-mediated synaptic inhibition in rat hippocampal interneurons (Nishikawa and MacIver, 2001).

Morphological studies have demonstrated that LS-hippocampus-hypothalamus-midbrain consisted limbic midbrain loop is an important pathway to hippocampus and act on regulating excitability of hippocampal neurons (Kendig et al., 1991; Franks and Lieb, 1994), and septo-hippocampal pathway has connection with anesthesia and amnesia (Savage et al., 2007). In our study, we observed that Fos expression was detected in the LS, CA1, DG, DM, Cg1, Cg2, PAG, and PV under incomplete anesthesia induced by sevoflurane, and more regions were detected under complete anesthesia. Fos expression was increased as the concentration of sevoflurane increased.

GABAergic neurons are the major inhibitory neurons which release inhibitory transmitter in the mammalian brain (Fonnum, 1987). Recent studies have demonstrated that various concentrations of sevoflurane has inhibitory effects on long-term potentiation (LTP) of hippocampal CA1 neurons through GABA-mediated mechanisms, and thus contributed to sevoflurane-induced anesthesia and the loss of recall (Ishizeki et al., 2008). Consistent with this finding, we observed in this study that almost all Fos expressions were present in GABAergic neurons in LS after sevoflurane inhalation, suggesting that GABAergic neurons in this nucleus are key

TABLE 1. Number of GABA-, fos-immunoreactive neurons, and Fos/GABA double-labeled neurons in the different brain regions

Regions		LS	CA1	DG	Cg1, 2	DM	PAG	PV	Arc	MeA	PaV	E–W
C group	GABA-IL	76.3 ± 7.2	32.2 ± 5.4	34.8 ± 5.5	77.8 ± 8.9	36.8 ± 4.7	35.5 ± 7.3	0.0 ± 0.0	28.3 ± 5.5	21.7 ± 5.6	19.9 ± 8.9	26.5 ± 7.7
	Fos-IL	1.4 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Fos/GABA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/Fos-IL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/GABA-IL	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S1 group (low dose)	GABA-IL	78.8 ± 5.5	33.7 ± 2.1	32.3 ± 3.9	72.9 ± 7.2	37.1 ± 7.1	36.1 ± 5.6	0.0 ± 0.0	31.7 ± 6.8	24.5 ± 6.7	22.6 ± 8.2	23.7 ± 6.3
	Fos-IL	17.7 ± 3.3	8.8 ± 1.7	11.5 ± 3.2	12.2 ± 3.9	14.4 ± 5.7	20.5 ± 3.9	21.3 ± 3.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Fos/GABA	13.2 ± 2.3	3.3 ± 1.1	4.7 ± 2.3	1.8 ± 0.7	2.2 ± 1.5	5.4 ± 2.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/Fos-IL	95.8 ± 3.8	33.7 ± 10.5	35.6 ± 13.3	12.2 ± 8.7	8.8 ± 4.6	20.3 ± 9.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/GABA-IL	16.3 ± 4.5	9.6 ± 3.3	13.1 ± 7.4	2.9 ± 1.4	7.1 ± 2.2	17.7 ± 9.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
S2 group (high dose)	GABA-IL	75.2 ± 6.9	35.0 ± 4.7	33.7 ± 5.1	73.9 ± 4.1	34.9 ± 6.3	40.2 ± 7.6	0.0 ± 0.0	32.7 ± 6.3	22.7 ± 5.8	23.1 ± 7.5	25.8 ± 6.1
	Fos-IL	24.7 ± 5.2	12.7 ± 1.2	17.4 ± 4.8	20.4 ± 3.5	25.6 ± 7.8	37.8 ± 8.4	39.2 ± 5.8	16.7 ± 4.3	10.8 ± 3.8	11.2 ± 4.2	13.7 ± 6.6
	Fos/GABA	20.5 ± 3.1*	5.4 ± 2.9*	7.1 ± 2.5*	4.4 ± 1.9*	3.9 ± 1.0*	9.2 ± 2.8*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/Fos-IL	94.4 ± 3.6	30.5 ± 7.9	32.4 ± 10.3	18.7 ± 7.1	11.1 ± 4.4	18.0 ± 8.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	%/GABA-IL	25.4 ± 9.1*	17.1 ± 1.3*	21.9 ± 11.2*	6.1 ± 2.0*	12.2 ± 3.1*	24.8 ± 7.1*	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%/Fos-IL, percentage of Fos/GABA double-labeled neurons to Fos-IL neurons. %/GABA-IL, percentage of Fos/GABA double-labeled neurons to GABA-IL neurons.
Data are shown as means ± S.E.M.
*$P < 0.01$, compared with S1 group.

Fig. 5. Immunofluorescent double-staining for GABA (green) and Fos (red) in DG (**A**, **A′**), CA1 (**B**, **B′**), PAG (**C**, **C′**), Cg (**D**, **D′**), and PV (**E**, **E′**) after sevoflurane induced complete anesthesia (S2 group). A′–E′ are the magnified images of the rectangles indicated in A–E, respectively. Single arrows or arrow heads indicated the GABA or Fos-positive neurons, respectively double arrows indicated GABA/Fos double-labeled neurons. Scale bar = 150 μm in E for A–E; 50 μm in E′ for A′–E′.

neurons involved in sevoflurane-induced anesthesia. In CA1, DG in hippocampus, DM, Cg1 and Cg2, a few number of GABAergic neurons were also expressed Fos after sevoflurane treatment, but more Fos expressed

outside GABA neurons. It was suggested that GABA neurons are not the unique neurons activated by sevoflurane. Probably other kinds of neurons also are activated and participate in sevoflurane anesthesia. In addition, neocortex is also the major target of volatile anesthetics through increase of $GABA_A$ receptor-mediated inhibition (Hentschke et al., 2005).

It is well known that mild concentration of inhaled anesthetic produces light sedation (Heinke and Schwarzbauer, 2002), whereas complete anesthesia induces deep sedation and ablation of movement in response to pain (Lydic and Baghdoyan, 1997). In our study, we found almost all Fos coexisted with GABA neurons in LS, and as the concentration of sevoflurane increased, coexistent neurons also increased in this region. We presumed that almost all GABAergic neurons in LS participate in sevoflurane-induced amnesia and with the concentration increase, more GABAergic neurons were enrolled. In this study, we observed that Fos expression was only detected in the amygdala, Arc, E–W nucleus and PaV under complete anesthesia state. It has been demonstrated that the amygdala, PAG, Arc, and PaV nucleus play important roles in analgesia (Du et al., 1994; Yu et al., 2007; Lu et al., 2008). As complete anesthesia can produce analgesic effect besides sedation, We presumed that the aforementioned nucleus may participate in sevoflurane-induced analgesia, but we need further evidence to verify it. In these nucleuses (amygdala, Arc nucleus, E–W nucleus, and PaV nucleus), none or very few Fos-expressing neurons were GABAergic, suggesting that the effect of sevoflurane-induced analgesia may not be involved as considerably as GABA-mediated mechanisms.

In addition, previous studies have suggested that the primary basis for anesthesia may be the blocking or disruption of sensory information processing through the thalamus (Angel, 1993), and assessment of regional uptake of glucose in deep anesthesia indicates that thalamus are more depressed (Alkire et al., 2000). But, in our study, it was very interesting that Fos was expressed in the PV, whereas there was no coexistence between Fos and GABA in this area because of the absence of GABAergic neurons. So we presumed that PV was one of target regions through which sevoflurane induces anesthesia, but the activated neurons were non-GABAergic neurons.

Furthermore, although these results cannot rule out the possibility that the Fos expression that we detected actually direct activation by sevoflurane, its expression is correlated with regions which related with anesthesia, and most of the Fos are expressed in certain brain regions containing GABAergic neurons. Our results provide the morphological evidence, which suggest the possible GABA-mediated mechanisms of sevoflurane-induced anesthesia, but more functional studies are needed to explore the anesthesia mechanism of GABAergic neurons in certain brain regions.

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