Regular Article

Identification of Brain Regions Activated by Sevoflurane and Propofol and Regional Changes in Gene Expression

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General anesthetics have different efficacies and side effect incidences based on their mechanism of action. However, detailed comparative studies of anesthetics are incomplete. In this study, target brain regions and gene expression changes in these brain regions were determined for sevoflurane and propofol to understand the mechanisms that cause differences among anesthetics. Rats were anesthetized with sevoflurane or propofol for 1 hr, and brain regions with anesthesia-induced changes in neuronal activity were examined by immunohistochemistry and *in situ* hybridization for c-Fos. Among the identified target brain regions, gene expression analysis was performed in the habenula, the solitary nucleus and the medial vestibular nucleus from laser microdissected samples. Genes altered by sevoflurane and propofol were different and included genes involved in the incidence of postoperative nausea and vomiting and emergence agitation, such as *Egr1* and *Gad2*. GO enrichment analysis showed that the altered genes tended to be evenly distributed in all functional category. The detailed profiles of target brain regions and induced gene expression changes of sevoflurane and propofol in this study will provide a basis for analyzing the effects of each anesthetic agent and the risk of adverse events.

Key words: general anesthetics, propofol, sevoflurane, c-Fos, microarray profiling

I. Introduction

Although analgesia and sedation by general anesthesia are essential for surgical procedures, the basic mechanisms of action for general anesthesia are not fully understood. Anesthetic effects on gene expression changes in target organs and electrophysiological analysis in the brain have been analyzed [40]. In our previous study, the effects of sevoflurane on gene expression in a wide range of organs throughout the body were analyzed using expression arrays. We found that a group of clock genes controlling circadian rhythms was altered in the brain [40]. We also showed that sevoflurane anesthesia causes a phase shift of circadian behavioral rhythms through changes in the clock genes of the suprachiasmatic nucleus (SCN), which is the center of circadian rhythms in the brain. These results suggest that anesthesia-induced changes may contribute to circadian rhythm-related disorders, such as sleep disturbance, which is a postoperative side effect [21, 34]. However, these focused analyses could not elucidate the more general mechanisms of action common to multiple general anesthetics or the mechanisms which cause different side effect incidences. To elucidate the mechanisms of action and side effects of anesthetics, regions or neuronal nuclei in the brain affected by anesthetics in the short and long term should be considered.

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In this study, we aimed to compare the target brain regions of two frequently used general anesthetics, sevoflurane and propofol, and to compare the gene sets whose expression changes are induced in the target brain regions. We selected sevoflurane, a representative inhalational anesthetic, and propofol, an intravenous anesthetic for this study, since inhalational and intravenous anesthetics are known to have different mechanisms of action as well as different incidence of adverse effects. Several anesthetic target areas in the brain have been identified using changes in Fos as a marker of neural activation [4, 29, 42]. However, limited studies compare the brain activation regions of sevoflurane and propofol. Differences in the neural activation sites of different types of anesthetics and differences in gene expression changes in those brain regions may lead to differences in the incidence of side effects that persist for several hours after anesthesia, including postoperative nausea and vomiting (PONV) and emergence agitation [27, 44, 45]. In the present study, after identifying the target brain regions of sevoflurane and propofol, we performed gene expression analysis using DNA arrays in the identified regions that have been related to the mechanisms of anesthetic action and side effects in previous studies. Differences in the incidence of side effects among general anesthetics are discussed from the viewpoint of both the target brain regions of anesthesia and gene expression changes.

II.   Methods

Animals

Male Wistar rats (8–10 weeks old; Tokyo Laboratory Animals Science, Tokyo, Japan), were used in the present study. Rats were acclimated for 1 week prior to the experiments on a 14:10 hr light-dark cycle (lights on at 6 am) with ad libitum access to food and water. All experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Research in Nippon Medical School (Approval Number: 30–035).

Anesthesia

Rats were placed in separate clear plastic cages (32 × 22 × 13 cm) and exposed to 40% oxygen at a flow rate of 6 L/min during anesthetic treatment. The sevoflurane group was anesthetized with 2.4% sevoflurane (1 minimum alveolar concentration (MAC) in rats [6, 32], Pfizer Japan, Tokyo, Japan). In the propofol group, rats were temporarily placed in a rat holder (Natsume Seisakusyo, Tokyo, Japan) for the insertion of a 24-gauge plastic catheter needle (Terumo, Tokyo, Japan). The procedure from holding the rats to releasing them was completed within 5 min. Rats in the propofol group were randomly allocated to three groups: propofol (intravenous 36 mg/kg/hr after a 10 mg/kg bolus dose: based on previous studies [19, 36],

Pfizer Japan, Tokyo, Japan), intralipos (10% lipid, intravenous 36 mg/kg/hr after a 10 mg/kg bolus dose, Otsuka Pharmaceutical, Tokyo, Japan) and sham (plastic catheter placement only). Note that intralipos was used as one of the controls to adjust the intravenous caloric infusion because propofol is a lipid emulsion-based anesthetic [5]. Induction of anesthesia was started at 11:00 am and anesthesia was administered for 1 hr. Sample collection was performed immediately after the completion of anesthesia.

Tissue preparation for c-Fos expression

For immunohistochemistry (IHC) and *in situ* hybridization (ISH), rats were transcardially perfused with 50 ml of saline followed by 200 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) under the deep anesthesia with medetomidine hydrochloride (intraperitoneal 0.5 mg/kg: Nippon Zenyaku Kogyo, Fukushima, Japan) and pentobarbital (intraperitoneal 50–100 mg/kg: Kyoritsu Seiyaku, Tokyo, Japan). The perfusion fixation was started within 5 min after the intraperitoneal administration of medetomidine and pentobarbital. The brains were post-fixed with the fixative for 24 hr at 4°C, immersed in 20% sucrose for 48 hr at 4°C, and frozen at n-hexane at −80°C. Four series of continuous coronal brain sections were cut at 40 μm thickness using a cryostat, (Leica 3050; Leica, Heidelberg, German), and collected in phosphate buffered saline (PBS) (0.1 M phosphate buffer, 0.9% NaCl, pH 7.4).

Immunohistochemistry

Immunostaining for c-Fos was performed using a Histofine SAB-PO(R) kit (Nichirei Bioscience, Tokyo, Japan), following the manufacturer's instructions with some modifications as described previously [17, 37]. After washing a series of free-floating brain sections three times with 0.1 M PBS (pH 7.4) containing 0.3% Triton X-100 (PBST), sections were blocked for 30 min with a 5% goat normal serum in PBST. Sections were then treated with rabbit polyclonal anti-c-Fos antibody at a dilution of 1:1000 (226-003; synaptic systems, Göttingen, Germany) overnight at 4°C [13]. Sections were treated with biotin-conjugated secondary antibody for 90 min and then reacted with horseradish peroxidase conjugated streptavidin for 90 min at room temperature. Immunoreactivity for c-Fos were visualized with diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) as a chromogen in 50 mM Tris and 0.003% H_2O_2. Stained brain sections were mounted on glass slides and sealed with Permount (Fisher Scientific, Waltham, MA, USA). The specificity of the staining was confirmed by omitting the primary antibody as a negative control.

In situ hybridization

ISH was performed using a digoxigenin (DIG)-labelled antisense cRNA probe for *c-Fos* mRNA (position 308–1409 in NM_022197), as previously described [17,

37]. Briefly, free-floating brain sections were treated with 0.125 µg/mL proteinase K (Takara Bio, Otsu, Japan) in 10 mM Tris buffer (pH 7.4) and 10 mM EDTA for 25 min at 37°C. Sections were then acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. Sections were hybridized with 0.5 µg/ml DIG-labelled antisense probe for c-Fos in hybridization solution (Sigma-Aldrich, St Louis, MO, USA) containing 50% form-amide and 10% dextran sulphate for 16 hr at 60°C. Sections were incubated with RNaseA (20 µg/ml, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl) for 20 min at 37°C to remove excess probe, followed by stringency washes with decreasing salt concentrations. Sections were reacted with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics, Mannheim, Germany) at 1:1000 dilu-tion for 2 hr at 37°C. Visualization of DIG-labelled probes were performed with 20 µL/ml NBT/BCIP (Roche Diag-nostics, Basel, Switzerland) as a chromogen in Tris-based buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M MgCl$_2$) in the dark room for 2 hr. To confirm staining specificities, parallel staining was performed using a sense probe as a negative control. After mounting and drying, sections were enclosed with 90% glycerol.

c-Fos expressing cell counting

The stained brain sections were compared between groups and the corresponding control group. Brain regions showing changes in the number of c-Fos-positive cells were selected and micrographs of each region were acquired using a BX51 microscope (Olympus, Tokyo, Japan). C-Fos-positive regions were confirmed by cell counting. Cell counting was performed using ImageJ (version 1.52a NIH, Bethesda, MD, USA) in a blinded manner by randomizing the images of each brain region.

Microarray analysis of brain regions

For microarray analysis, rats were decapitated imme-diately after anesthesia, and unfixed brains were frozen in n-hexane at −80°C. Brains were sliced using a cryostat at a thickness of 30 µm and mounted on membrane slides (PEN-membrane 2.0 µm; Leica, Heidelberg, Germany) fol-lowed by Nissl staining. The medial habenular nucleus (MHb), the lateral habenular nucleus (LHb), the medial vestibular nucleus (MVe) and the solitary nucleus (Sol) were microdissected using an LMD7000 (Leica Microsys-tems, Heidelberg, Germany) and frozen at −80°C. RNA extraction was performed from tissue fragments using an RNeasy Micro kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A NanoDrop® ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the quantity and purity of total RNA. The integrity of total RNA was checked with an Agilent 2100 Bioana-lyzer using an Agilent RNA600 Pico kit (Agilent Technolo-gies, Santa Clara, CA, USA). Brain regions from five rats were combined into one sample, and samples that satisfied the criteria of total RNA > 100 ng and RNA integrity > 6.5

were used for microarray analysis. For the LHb of the intralipos group, five additional rats were used because of a slight lack of RNA.

Microarray preparation and expression profile acquisition

Microarray profiling was performed using a Clariom^{TM} S Array for Rat (Affymetrix, Santa Clara, CA, USA), following the manufacturer's instructions. Brief-ly, biotin-labelled cDNA was purified from 100 ng of total RNA using a GeneChip WT PLUS Reagent kit (Part# 902280 Applied Biosystems, Waltham, MA, USA). The cDNA was hybridized using GeneChip Hybridization Oven 640 (Applied Biosystems), washed and stained with GeneChip Fluidics Station 450 (Applied Biosys-tems). Images were acquired with a GeneChip Scanner 3000 7G System (Applied Biosystems), analyzed using the GeneChip Command Console Software ver. 3.2 and normalized with the GeneChip Expression Console Soft-ware ver. 1.3.0 (Applied Biosystems). The microarray data were analyzed using Transcriptome Viewer (Kurabo Industries, Osaka, Japan). The criteria for selecting genes that showed differential expression by anesthesia were set at fold change (FC) > 2 (upregulated) or FC < 0.5 (down-regulated). Fold changes were calculated as differences in the signals between the two groups. The gene ontology and GO enrichment analyses were performed using The PANTHER Classification System (Protein ANalysis THrough Evolutionary Relationships).

Statistical analyses

Quantitative results were expressed as mean ± stan-dard error. Student's t test were used to compare the sevoflurane group to the control group. One-way ANOVA followed by multiple comparisons using the Tukey's HDS test were used for the propofol groups to analyze the number of c-Fos positive cells. Statistical analyses were performed using IBM SPSS statistics version 26 (IBM, Armonk, NY, USA) to determine significant differences (P < 0.05). For GO enrichment analysis, a Fisher's exact test was performed and the false discovery rate was calculated for correction using The PANTHER Classification System version 16.0.

III. Results

Histological identification of brain regions activated by sevoflurane

Changes in neural activity in brain regions induced by sevoflurane were identified using c-Fos as a marker. For the visualization of c-Fos, both mRNA detection by ISH and protein detection by IHC were performed. We identified eight brain regions activated by sevoflurane. Representative photomicrographs and quantitative results are shown in Fig. 1 and Supplemental Fig. 1, and the data are summarized in Table 1. The ISH and IHC results were similar in all regions except for the supraoptic nucleus.

Fig. 1. Brain regions with changes in c-Fos expression after sevoflurane anesthesia. Photomicrographs of representative brain regions that showed increased c-Fos expression by sevoflurane in immunohistochemistry and *in situ* hybridization analyses (left panels), and quantification of c-Fos positive cells in each region (right panels). **A**: Habenular nucleus. The white dashed line is the boundary between the lateral and medial habenular nucleus. **B**: Medial vestibular nucleus. **C**: Solitary nucleus. Brain regions not included in the subsequent microarray analysis are shown in Supplemental Fig. 1. Bars = 200 μm. Values are shown as mean ± SE (n = 4). *, $P < 0.05$ (Student's t test); Sev, Sevoflurane; MHb, Medial habenular nucleus; LHb, Lateral habenular nucleus.

Table 1. *Regions with changes in c-Fos expression after administration of the two anesthetics*

|		Sevoflurane					Propofol			
	treat	IHC		ISH		treat	IHC		ISH	
Islands of Calleja	Sev	174 ± 59.4		79.3 ± 19.7		Prop	7.9 ± 0.8		1.0 ± 0.6	
	O$_2$	16.6 ± 1.7	P = 0.038	5.5 ± 2.0	P = 0.01	Intra	9.13 ± 1.01	n.s.	5.6 ± 2.7	n.s.
						Sham	13.0 ± 2.5	n.s.	5.4 ± 2.4	n.s.
Central Amygdala	Sev	147 ± 16.0		76.7 ± 20.5		Prop	51.4 ± 7.3		14.9 ± 3.5	
	O$_2$	36.3 ± 10.5	P = 0.001	6.3 ± 2.9	P = 0.015	Intra	77.4 ± 16.4	n.s.	30.3 ± 8.0	n.s.
						Sham	53.6 ± 6.8	n.s.	4.4 ± 3.4	n.s.
Suprachiasmatic nucleus	Sev	39.5 ± 13.4		1.9 ± 0.6		Prop	38.6 ± 4.6		1.6 ± 0.3	
	O$_2$	45.4 ± 9.1	n.s.	3.4 ± 0.7	n.s.	Intra	33.1 ± 7.2	n.s.	6.5 ± 1.1	P = 0.016
						Sham	30.6 ± 7.3	n.s.	7.5 ± 1.3	P = 0.006
Supraoptic nucleus	Sev	19.0 ± 3.2		21.4 ± 8.1		Prop	24.9 ± 11.4		10.8 ± 5.8	
	O$_2$	4.0 ± 1.5	P = 0.006	35.3 ± 30.8	n.s.	Intra	41.4 ± 22.4	n.s.	25.3 ± 12.5	n.s.
						Sham	11.1 ± 3.1	n.s.	19.9 ± 8.6	n.s.
Paraventricular nucleus	Sev	103 ± 31.6		56.4 ± 10.4		Prop	234 ± 23.8	*	57.0 ± 22.6	
	O$_2$	143 ± 46.1	n.s.	45.4 ± 18.0	n.s.	Intra	438 ± 52.6	P = 0.041	140 ± 32.9	n.s.
						Sham	212 ± 63.0	n.s.	80.0 ± 25.3	n.s.
Medial Habenular nucleus	Sev	69.1 ± 7.9		36.6 ± 4.0		Prop	6.3 ± 1.0		2.0 ± 0.5	
	O$_2$	5.9 ± 1.9	P = 0.000	0.5 ± 0.2	P = 0.000	Intra	5.5 ± 2.0	n.s.	1.9 ± 0.7	n.s.
						Sham	6.3 ± 1.3	n.s.	1.8 ± 0.8	n.s.
Lateral Habenular nucleus	Sev	6.8 ± 2.1		14.4 ± 3.8		Prop	100 ± 14.1		68.1 ± 6.9	
	O$_2$	37.1 ± 12.2	n.s.	22.1 ± 10.5	n.s.	Intra	34.8 ± 10.4	P = 0.007	20.9 ± 6.8	P = 0.001
						Sham	29.5 ± 9.3	P = 0.005	24.0 ± 3.3	P = 0.001
Interpeduncular nucleus	Sev	46.6 ± 5.7		43.6 ± 11.3		Prop	2.4 ± 0.6		2.3 ± 1.1	
	O$_2$	1.8 ± 0.3	P = 0.000	1.5 ± 0.7	P = 0.000	Intra	3.3 ± 1.1	n.s.	1.1 ± 0.4	n.s.
						Sham	5.4 ± 1.1	n.s.	1.6 ± 0.2	n.s.
Medial vestibular nucleus	Sev	87.0 ± 22.7		155 ± 37.8		Prop	97.0 ± 19.2		104 ± 40.9	
	O$_2$	4.1 ± 1.4	P = 0.000	2.0 ± 1.2	P = 0.000	Intra	7.6 ± 2.9	P = 0.001	1.4 ± 0.9	P = 0.032
						Sham	6.8 ± 2.3	P = 0.001	3.4 ± 1.0	P = 0.035
Solitary nucleus	Sev	27.0 ± 2.8		27.8 ± 1.9		Prop	10.6 ± 2.4		14.1 ± 2.6	
	O$_2$	6.4 ± 2.4	P = 0.001	5.8 ± 1.2	P = 0.000	Intra	16.6 ± 9.1	n.s.	27.3 ± 5.5	n.s.
						Sham	19.3 ± 6.0	n.s.	13.6 ± 4.8	n.s.
Inferior olivary nucleus	Sev	28.4 ± 3.0		36.0 ± 7.4		Prop	37.1 ± 2.3		23.1 ± 4.2	
	O$_2$	0.1 ± 0.1	P = 0.000	0 ± 0	P = 0.003	Intra	0 ± 0	P = 0.000	0.3 ± 0.3	P = 0.000
						Sham	0 ± 0	P = 0.000	0.1 ± 0.1	P = 0.000

Four rats in each group. The numbers of c-Fos positive cells are shown as mean ± SE. In the propofol group, the P values are compared to the intralipos group or sham respectively. P < 0.05 was considered a significant difference. n.s., No significant difference to control; *, Propofol group had significantly lower expression compared to the intralipos group; Sev, Sevoflurane; Prop, Propofol; Intra, Intralipos.

This differences in ISH and IHC results may be due to the time lag between mRNA changes and protein changes. Additionally, we examined the neurochemical properties of neurons activated by sevoflurane in the medial habenula, where the connection between neurochemical properties and function has been studied [2, 26]. Double immunofluorescence revealed that a portion of the c-Fos positive cells overlapped with substance P immunopositive neurons on the dorsal MHb, but hardly overlapped with cholinergic neurons in the ventral MHb (Supplemental Fig. 2). Staining without the primary antibody as the negative control for IHC showed no specific signal. Similarly, no specific signal was observed in staining with the sense probe as the negative control for ISH (Supplemental Fig. 3).

Histological identification of brain regions activated by propofol

ISH and IHC for c-Fos identified three brain regions with increased neuronal activity and one brain region with decreased activity induced by propofol administration (Fig. 2 and Supplemental Fig. 4). The MVe and the inferior olivary nucleus (ION) were commonly activated by sevoflurane and propofol, while all other identified regions were activated by only one of the two anesthetics (Table 1).
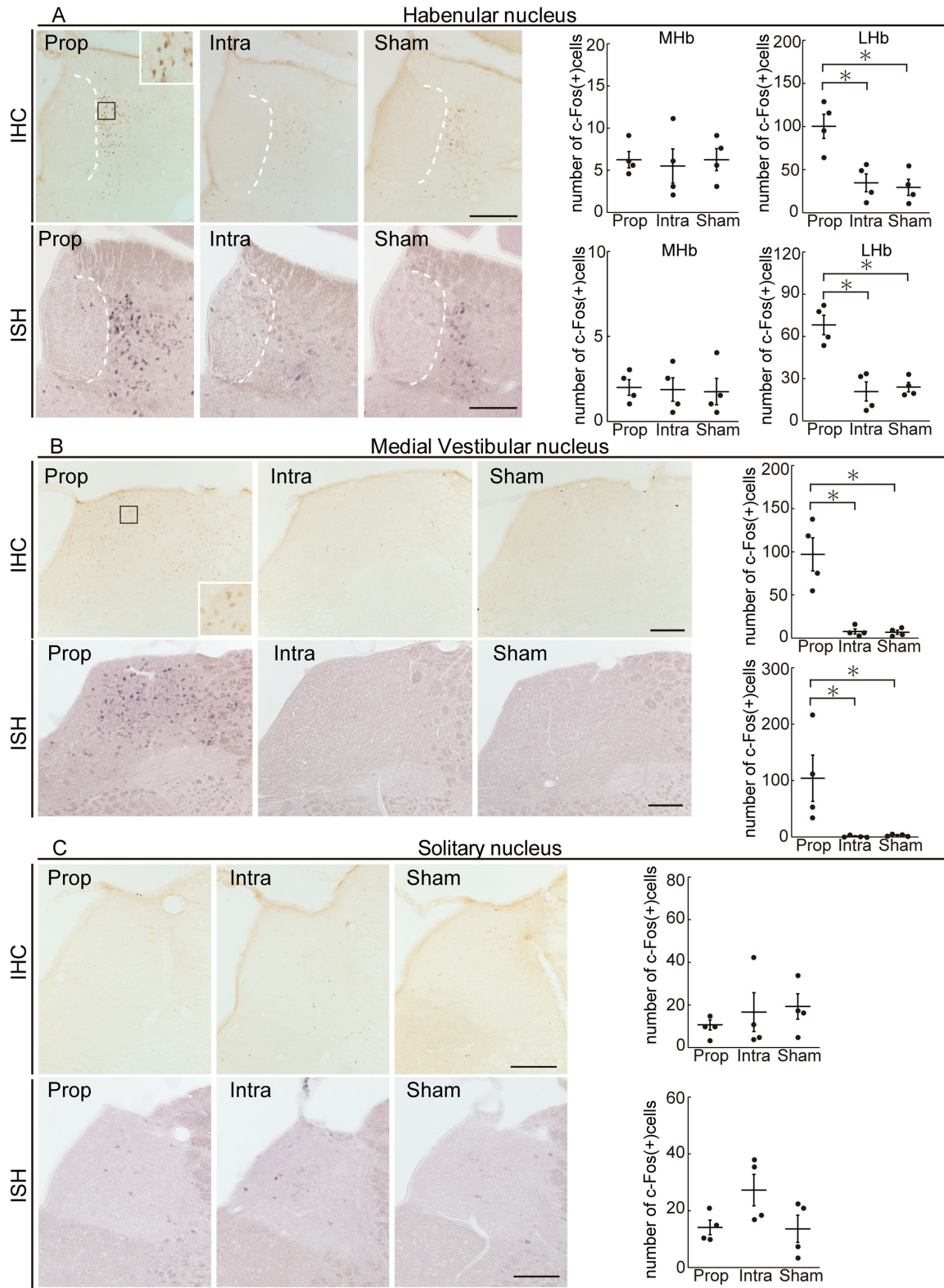
Fig. 2. Brain regions with changes in c-Fos expression after propofol anesthesia. Photomicrographs of representative brain regions (left panels) and quantification of c-Fos positive cells in each region (right panels). **A**: Habenular nucleus. The white dashed line is the boundary between the lateral and medial habenular nucleus. **B**: Medial vestibular nucleus. **C**: Solitary nucleus. c-Fos was not induced by propofol in the solitary nucleus, in contrast to sevoflurane anaesthesia. Brain regions not included in the subsequent microarray analysis are shown in Supplemental Fig. 4. Bars = 200 μm. Values are shown as mean ± SE (n = 4). *, P < 0.05 (one-way ANOVA followed by multiple comparisons using the Tukey's HDS test); Prop, Propofol; Intra, Intralipos; MHb, Medial habenular nucleus; LHb, Lateral habenular nucleus.
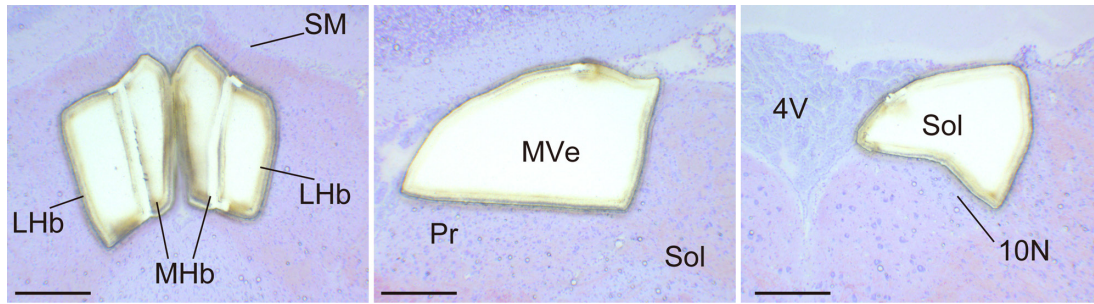
Fig. 3. Brain regions acquired by laser microdissection. Representative photomicrographs of brain slices after laser microdissection for microarray analysis sampling. Brain slices were Nissl-stained to identify microdissection regions. MHb, Medial habenular nucleus; LHb, Lateral habenular nucleus; SM, nucleus of the stria medullaries; MVe, Medial vestibular nucleus; Pr, Prepositus nucleus; Sol, Solitary nucleus; 4V, Fourth ventricle; 10N, Vagus nerve. Bars = 320 μm.

The two control groups for propofol treatment (intralipos and sham) showed similar trends in most brain regions; however, in the paraventricular nucleus, elevated c-Fos was observed, probably due to vein irritation caused by continuous intralipos administration under non-anesthetic conditions [20, 39].

Gene expression analysis in brain regions activated by anesthesia

Among the activated brain regions identified above, we focused on the Sol, MVe and habenula for gene expression analyses because these regions may be related to the mechanism of anesthesia and its side effects. Sol and the vestibular nucleus may be involved in PONV and the LHb is involved in propofol-induced sedation via glutamatergic output [13, 18]. The habenula was divided into the MHb and LHb subregions for gene expression analysis because of the anesthetic-dependent differences in the induction of c-Fos (Figs. 1 and 2). Representative images of these regions collected by laser microdissection are shown in Fig. 3.

In the expression array, 23188 genes were analyzed; the list of genes with FC > 2 or FC < 0.5 for each anesthetic was registered in the GEO of NCBI (registration number: GSE192399). In the sevoflurane group, 1.55% of the genes in the MHb, 1.05% in the LHb, 4.12% in the MVe and 1.38% in the Sol were altered. In the propofol group, 2.29% in the MHb, 1.60% in the LHb, 1.87% in the MVe and 2.44% in the Sol were altered. Thirty common genes were altered by both anesthetics in the MVe, where c-Fos induction was similar between the two anesthetics. Relatively few genes, (9, 6 and 7 genes, respectively) were altered by both anesthetics in the MHb, LHb and Sol, where c-Fos induction was different (Supplemental Table 1). Among these genes, none were commonly altered in all regions. In the brain regions that showed significant anesthesia-induced increases in c-Fos by IHC and ISH, the same c-Fos increases were observed by expression array analysis, confirming the validity of the two methods (Table 2).

GO enrichment analysis showed that genes induced

Table 2. *Fos changes due to anesthetics in the gene expression analysis*

|	MHb	LHb	MVe	Sol
Sevoflurane	2.70	−0.03*	4.47	2.46
Propofol	0.49*	1.84	2.32	0.25*

The numbers indicate the change due to anaesthetics in log FC. In the brain regions where c-Fos was upregulated by anaesthesia in the immunohistochemistry (IHC) and *in situ* hybridization (ISH) analyses, the same upregulation of Fos was observed in the microarray analysis. Note that the three regions marked with asterisks are regions where no anesthetic induction of c-Fos was observed by IHC and ISH analysis. MHb, Medial habenular nucleus; LHb, Lateral habenular nucleus; MVe, Medial vestibular nucleus; Sol, Solitary nucleus.

in the brain by the two general anesthetics were not biased toward any particular functional annotation, but were equally represented in each functional region (Fig. 4). The exception to this was an increase in the number of genes with neuropeptide receptor binding annotation in the LHb of the propofol group (Fold enrichment = 13.75, raw p value = 1.97E-06, False Discovery Rate = 9.34E-03).

IV. Discussion

In the present study, we performed a histological analysis of the effects of two general anesthetics, sevoflurane and propofol, on neural activity in the brain at the level of the neuronal nuclei. Although these two anesthetics differ in administration and mechanism of action, we identified the ION and MVe as common regions activated by both anesthetics. The brain regions commonly activated by both anesthetics may be associated with the sedative and analgesic effects common to anesthetics, including suppression of ascending sensory pathways. We identified multiple brain regions that were activated differently by each anesthetic. The brain regions differentially activated by the two anesthetics may be linked to differences in the characteristics of the anesthetics, such as the incidence of side effects [3, 23].

The incidence of PONV is associated with the type of general anesthetic. The use of volatile anesthetics like sevoflurane is a risk factor for PONV [3], and the use of
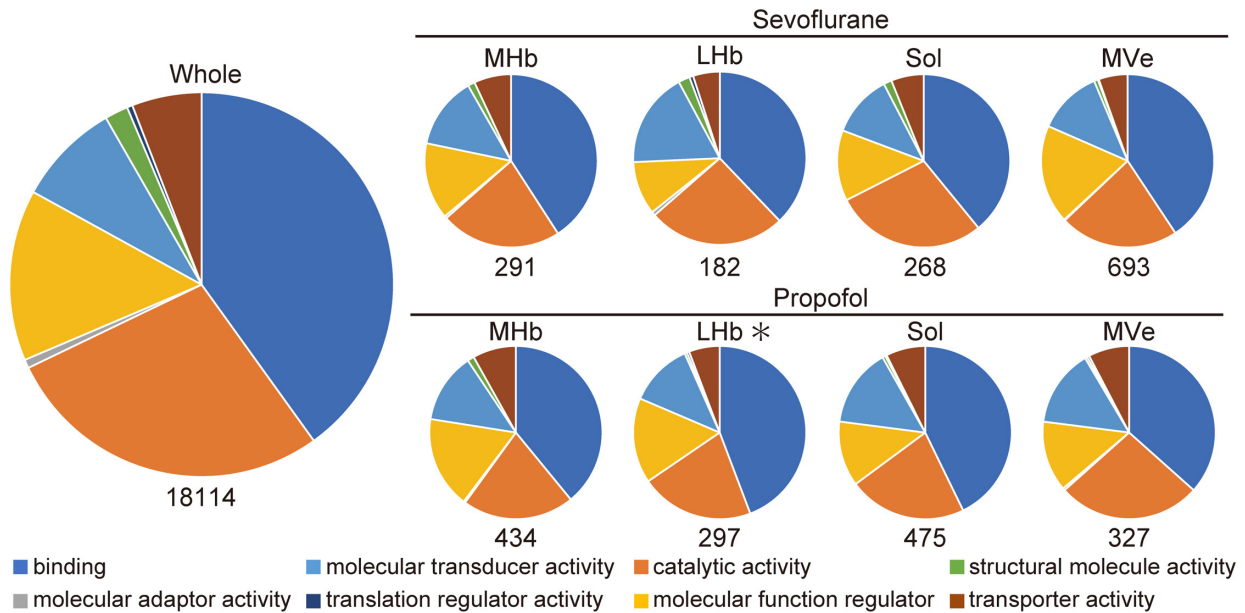
Fig. 4. Molecular functions of genes altered by anaesthetics in each brain region. GO analysis was performed on the genes annotated with molecular functions among the genes analyzed by microarray. The far-left pie chart shows the distribution of molecular functions for all genes analyzed, and the remaining pie-charts show the distribution of molecular functions of genes altered by anaesthetics in each region of the brain. The number below each pie chart indicates the number of genes. In the GO enrichment analysis, only the LHb in the propofol group showed significant enrichment of genes annotated with neuropeptide receptor binding (Fold enrichment = 13.75, raw p value = 1.97E-06, False Discovery Rate = 9.34E-03). MHb, Medial habenular nucleus; LHb, Lateral habenular nucleus; Sol, Solitary nucleus; MVe, Medial vestibular nucleus.

propofol, an intravenous anesthetic, reduces the incidence of PONV [12, 41]. The vestibular nucleus and Sol are involved in the vomiting reflex [11, 18, 46]; expression of c-Fos was increased in the Sol when nausea was induced in rats using cisplatin [8]. In addition, increased expression of *Egr1*, one of the immediate early genes [25, 30], and decreased expression of the dopamine β-hydroxylase gene in the catecholamine biosynthetic pathway in the Sol may be involved in the development of PONV [43]. In the present study, c-Fos expression was increased only with sevoflurane, and gene expression analysis revealed that dopamine β-hydroxylase was decreased only with sevoflurane (FC 0.44). This suggests that differences in the neural activation of the Sol contribute to the differences in PONV development between sevoflurane and propofol.

In the present study, sevoflurane and propofol increased c-Fos in the MHb and LHb, respectively. The habenular nucleus plays a variety of physiological roles, including arousal, anxiety and aggressive behavior [38]. The MHb is composed of multiple neurons with different neurochemical properties, the major ones being ventral cholinergic neurons and dorsal substance P-ergic neurons, both projecting to the interpeduncular nucleus [2, 7, 26]. In the present study, c-Fos induction by sevoflurane was found in substance P-ergic neurons, which may play a role in the sedative effects of sevoflurane, since it has been reported that this neuron is required for atonia during REM sleep [14]. There are also several reports suggesting the importance of sevoflurane action via the MHb, such as the

report that nitric oxide (NO) signaling via guanylyl cyclase in the MHb facilitates the effects of sevoflurane [31]. As for the LHb, it is important for propofol-induced sedation [13], and various other sedatives (ketamine, ethanol, pentobarbital, muscimol and chloral hydrate) have been reported to increase c-Fos expression in the LHb [1, 29]. The LHb consists mainly of glutamatergic neurons, with the medial part projecting to the raphe nucleus and the lateral part to the reticular formation [2, 13, 15]. In the current study, c-Fos expression was found to be biased toward the medial part of LHb in the propofol group (Fig. 2). Given that serotonergic neurons in the raphe nucleus are associated with sleep [35], it is likely that the LHb is involved in the sedative effects of propofol. In addition, gene expression analysis in this study showed that *Gad2* expression in the LHb was suppressed by propofol (FC 0.36). Considering the reports that activation of GABAergic neurons in the LHb promotes aggression [10], and propofol has a lower risk of emergence agitation than volatile anesthetics such as sevoflurane [24, 45], perturbation of GABAergic neurons in the LHb by anesthetics may be involved in the risk of emergence agitation.

Sevoflurane and propofol both increased the expression of c-Fos in the MVe and ION. The MVe integrates vestibular sensation with deep sensation of joints and muscles for postural control [16], and the ION is involved in cerebellar-mediated motor regulation [9]. Therefore, the activation of these two regions may be common in anesthesia. In addition, gene expression analysis in the MVe

revealed 30 genes that were altered in the same direction. Among them, *Grin2d*, a subunit of the NMDA receptor, and *Npas4*, which plays an important role in synaptogenesis [22], were strongly upregulated and immediate early genes, including *Fos*, *Jun* and *Egr1* [30], were upregulated. The anesthesia-induced upregulation of these genes responsible for synaptic plasticity may be involved in the side effects that persist for hours to days or longer after awakening from anesthesia, such as postoperative shivering and postoperative delirium [28, 33].

This study has a limitation in terms of research methodology; in the evaluation of neural activity using c-Fos, the identification of regions with reduced neural activity was difficult, except for regions with a certain degree of neural activity and the presence of c-Fos under normal conditions. In fact, suppression of neural activity by anesthesia was observed only in the SCN of the propofol-treated group. Additional studies using electrophysiological methods may be necessary to determine the regions where anesthetics suppress neural activity. We focused on four regions for expression array analysis based on the relevance of the effects and side effects of anesthetics. Activity in these regions was compared between the two types of anesthesia, but further research on the remaining regions where neural activity changed and comparison with other anesthetics may be needed. The relationship between differences in target brain regions and genetic alterations for each anesthetic and actual differences in the incidence of adverse effects for each anesthetic needs to be confirmed by behavioral experiments using animals. However, at present, with a few exceptions [25], there are few well-accepted animal behavioral indices that correspond to adverse effects of anesthetics in humans. Therefore, an appropriate animal model needs to be established.

In conclusion, our results suggest that differences in the target brain regions between sevoflurane and propofol anesthesia and gene expression changes in these regions may provide the neural basis for differences in the risk of adverse events between anesthetics. The results and research perspective from this study will be useful for future studies on the safety of general anesthetics.

V. Author Contributions

All authors discussed the results and contributed to the manuscript. Detailed contributions were as follows: NK and SH: conceptualization; NK, SH and TM: methodology; NK, TM and KM: investigation; NK: writing—original draft preparation; NK, SH, HO and AS: writing—review and editing; SH, HO and SA: supervision; HO and KM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

VI. Conflicts of Interest

There are no conflicts of interest to declare.

VII. Funding

VIII. References

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