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Determination of the Effects of Sevoflurane Anesthesia in Different Maturing Stages of the Mouse Hippocampus by Transcriptome Analysis

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Abstract

Purpose: Postoperative cognitive dysfunction (POCD) is a serious complication after general anesthesia. POCD is more likely to occur in elderly patients, but the mechanism of POCD has not been fully elucidated. We hypothesized that the difference of mRNA expression profile in the brain depending on the maturing stage causes the difference in the effect of sevoflurane anesthesia. We investigated the mRNA expression profile of hippocampal cells in young mice and in aged mice under sevoflurane anesthesia using transcriptome analysis.

Methods: This study was conducted after approval from our institutional animal ethics committee, the Animal Research Center of Sapporo Medical University School of Medicine (project number: 12-033). Eight mice were assigned to two groups: a young group and an aged group. Each of the 4 mice in the two groups was anesthetized with 3.5% sevoflurane for 1 hour. Subsequently, mRNA was isolated from hippocampal cells and RNA sequencing was performed on an Illumina HiSeq 2500 platform. Mapping of the quality-controlled, filter paired-end reads to mouse genomes and quantification of the expression level of each gene were performed using R software.

Results: The *Lhx9* gene, which is thought to be associated with neuronal inflammation, was the most highly up-regulated gene in aged mice. The *Epyc* gene, which encodes a protein related to the phospholipase-C pathway and ERK signaling, was the most down-regulated gene in aged mice.

Conclusions: The findings suggest that sevoflurane anesthesia induces neuronal inflammation *via* a LIM-homeodomain family related gene in aged mice and causes POCD.

Keywords: Transcriptome analysis; Hippocampus; Postoperative cognitive dysfunction

Introduction

Postoperative cognitive dysfunction (POCD) is a frequent and serious complication after general anesthesia [1]. POCD is known to have a negative impact on the quality of life in affected patients [2]. Despite the high prevalence of POCD, the mechanism of POCD has not been fully elucidated. Recent studies have revealed that clinical risk factors of POCD are frontal cortex function, lifestyle, medication, and age [3-6]. General anesthesia might cause neuroinflammation in the developing brain [7], but it is difficult to determine cognitive changes caused by the anesthetic agent *per se*. POCD is usually transient, and it is difficult to establish clear diagnostic criteria for POCD [1,8]. Elucidation of the biological mechanism of POCD would be useful for improving the diagnosis and prevention of POCD. It is known that the requirement of volatile anesthetics is decreased with advance of age [9]. This suggests that volatile anesthetic agents cause different biological changes depending on the brain maturing stage.

We previously reported that exposure to sevoflurane changes mRNA profile in the juvenile mouse hippocampus by transcriptome analysis. In the juvenile mouse, the *Lhx9* gene was highly down-regulated by sevoflurane exposure, while the *Rtn4rl2* gene was highly up-regulated [10]. The *Lhx9* gene encodes a LIM-homeodomain factor, which is essential for the development of thalamic neurons [11]. The *Rtn4rl2* gene encodes the Nogo receptor, which is involved in the adhesion of dendritic cells to myelin in the central nervous system [12]. These findings suggest that sevoflurane anesthesia induces neuroinflammation in juvenile mice, but data for aged mice have not been shown. Surgical stress induces systemic inflammation and increases levels of cytokines such as TNF-alpha. After transition of inflammatory cytokines to the blood-brain barrier, they activate glial cells, which cause neuroinflammation. Cholinergic neurons alter the activation of glial cells, but the alteration is affected by aging. Subsequently, the aging of cholinergic neurons is thought to be a potential biological mechanism of POCD and the reason why POCD is likely to occur in elderly patients [13].

Is general anesthesia itself harmful for the aged brain? [13] Is the anesthetic agent itself likely to cause neuroinflammation in the aged brain? Alternatively, the anesthetic agent might activate unknown pathways that lead to the occurrence of POCD. We hypothesized that the change in the mRNA expression profile in aged mice after sevoflurane exposure is different from that in juvenile mice, especially in the hippocampus, which integrates memory and cognitive function [14]. Recent progress in genomics has enable us to comprehensively analyze cellular modifications at the gene expression level using transcriptome analysis. The DNA microarray technique has uncovered various mechanisms of diseases; however, there has been no

investigation of the association between POCD and the hippocampus by a transcriptome-wide association study.

In this study, the mRNA expression profiles of hippocampal cells in juvenile mice and in aged mice under sevoflurane anesthesia were investigated by using transcriptome analysis.

Materials and Methods

With approval from the Sapporo Medical University School of Medicine animal ethics committee (project number: 12-033) for this study, male C57/BL6 mice (8 weeks old, body weight of 20-25 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed at 22°C under controlled lighting (12:12-hour light/dark cycle) with food and water provided ad libitum. Eight male mice were assigned to two groups: a young group (8 weeks of age, n=4) and an aged group (35 weeks of age, n=4). In both groups, 3.5% sevoflurane (Maruishi Co., Ltd. Shizuoka, Japan) in 100% oxygen was provided to mice in a plastic chamber for 1 hour.

Then the mice were decapitated after being anesthetized with 3.5% sevoflurane. The brain of each mouse was immediately removed from the skull, frozen at -70°C with 2-methylbutane, and placed in a Petri dish containing ice-cold phosphate-buffered saline. The brain was cut along the longitudinal fissure of the cerebrum, and the regions posterior to the lambda were cut off using tissue matrices (Brain Matrices, EM Japan, Tokyo, Japan). Thereafter, the brain was placed with the cortex of the left hemisphere facing down and any non-cortical forebrain tissue was removed. Tissue blocks containing hippocampal cells were obtained using Brain Matrices (EM Japan). Meningeal tissue was removed from the hemisphere according to a previously described method [15]. Finally, dissected hippocampal cells were homogenized and lysed into six samples for each mouse using the RNeasy® Plus Micro Kit (Qiagen, Hilden, Germany) and QIAcube (Qiagen). Quality control for isolated RNA was performed using the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). For samples to pass the initial quality control step, it was necessary to quantify >1 μg of sample and to have an equivalent RNA integrity number (eRIN) of ≥ 8. The eRIN determined by a 2500 Bioanalyzer Instruments (Agilent Technologies) has been reported to provide accurate information [16]. Isolated RNA was then pooled into two samples per group and labeled. A cDNA library was prepared using TruSeq® RNA Library Prep Kits (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. RNA-seq was performed in the paired-end (101 cycles × 2) mode on an Illumina HiSeq 2500 platform (Illumina, Inc.).

Base call (.bcl) files for each cycle of sequencing were generated by Illumina Real Time Analysis software (Illumina, Inc.) and were analyzed primarily and de-multiplexed into a FASTQ (.fastq) file using Illumina's BCL2FASTQ conversion software (ver. 1.8.4, Illumina, Inc.). Raw paired-end RNA-seq reads in FASTQ formats were assessed for base call quality, cycle uniformity, and contamination using FastQC (http://www.bioinformatics.bbsrc.ad.uk/projects/fastqc/). Mapping of the quality control-filtered paired-end reads to mouse genomes and quantification of the expression level of each gene were performed using R software (ver. 3.1.1 with TCC package) [17,18]. The quality control-filtered paired-end reads were mapped to public mouse genome data published by UCSC (NCBI37/mm9, http://genomes.UCSC.edu/). Differential gene sets were filtered to remove those with fold changes <1.5 (up- or down-regulated) and with a false discovery rate-corrected P value of 0.05. Sample size was calculated with the following parameters: power ≥ 0.8, probability level <0.05, and anticipated effect size=14.

Results

All total RNA samples had a quality ≥ 1 μg and eRIN value ≥ 8. The average base calls after primary filtration were 41,778,221 base pairs, and the average mean quality score (Phred quality score) was 37.1. We investigated changes in expression levels of a total of 37,681 genes (Supplementary Table 1). A total of 7,716 genes were filtered because they showed little change in mRNA expression levels. Microarray plotting showed a total of 7,027 genes that were expressed differentially between the maturing stages. The *Lhx9* gene was the most highly up-regulated in aged mice (Table 1). The *Htr5b* gene, which encodes the serotonin receptor, the *Cbln3* gene, which encodes cerebellin 3 precursor protein, and the *Gabra6* gene, which encodes the gamma amino butyric acid type A (GABAA) receptor alpha 6 subunits, were highly up-regulated in aged mice (log2 ratios being 7.48, 7.33, and 6.27, respectively). The *Epyc* gene was the most down-regulated gene in aged mice (Table 2). The *Oprd1* gene, which encodes the delta opioid receptor, the *Drd1a* gene, which encodes dopamine receptor D1A, and the *Adora2a* gene, which encodes adenosine A2a receptor were highly down-regulated in aged mice (log2 ratios being 7.64, 5.54, and 5.52, respectively).

Gene name	Gene description	Log2 ratio
Lhx9	LIM homeobox protein 9	9.21
Pou4f1	POU domain, class 4, transcription factor 1	8.72
Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	7.48
4631426E05Rik	RIKEN cDNA 4631426E05 gene	7.44
Cbln3	Cerebellin 3 precursor protein	7.33
Gpr151	G protein-coupled receptor 151	7.08
Irx3	Iroquois related homeobox 3 (Drosophila)	6.84
Umodl1	Uromodulin-like 1	6.72
Slc5a1	Solute carrier family 5 (sodium/glucose cotransporter), member 1	6.71

Irx2	Iroquois related homeobox 2 (Drosophila)	6.48
Iyd	Iodotyrosine deiodinase	6.41
Nrk	Nik related kinase	6.36
Hes3	Hairy and enhancer of split 3 (Drosophila)	6.35
Gabra6	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6	6.27
Bcl2l15	Bcl2-like 15	6.2
Gsbs	G substrate	6.13
Ntng1	Netrin G1	6.1
OTTMUSG00000003311	Predicted gene, OTTMUSG00000003311	5.97
Irx1	Iroquois related homeobox 1 (Drosophila)	5.96
Tyrp1	Tyrosinase-related protein 1	5.94
Lhfpl1	Lipoma HMGIC fusion partner-like 1	5.94
Gm941	Gene model 941, (NCBI)	5.89
Gtf2a1l	General transcription factor IIA, 1-like	5.88
Vsig8	V-set and immunoglobulin domain containing 8	5.69
Ldlrad2	Low density lipoprotein receptor A domain containing 2	5.64
Epb4.2	Erythrocyte protein band 4.2	5.61
Cnn1	Calponin 1	5.58
Epha1	Eph receptor A1	5.57
Tmem182	Transmembrane protein 182	5.56
4933436C20Rik	RIKEN cDNA 4933436C20 gene	5.53
Barhl2	BarH-like 2 (Drosophila)	5.52
EG667705	Predicted gene, EG667705	5.47
Avil	Advillin	5.4
Gpx2	Glutathione peroxidase 2	5.38
Aqp6	Aquaporin 6	5.38
Trim40	Tripartite motif-containing 40	5.33
Irx5	Iroquois related homeobox 5 (Drosophila)	5.31
Slc43a3	Solute carrier family 43, member 3	5.3
Wnt9b	Wingless-type MMTV integration site 9B	5.25
Ptprq	Protein tyrosine phosphatase, receptor type, Q	5.24
1700024G13Rik	RIKEN cDNA 1700024G13 gene	5.23
Slc17a6	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6	5.14
Gabrr1	Gamma-aminobutyric acid (GABA-C) receptor, subunit rho 1	5.12
Adamts19	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 19	5.12
Ramp3	Receptor (calcitonin) activity modifying protein 3	5.11

Neurog2	Neurogenin 2	5.09
Chrnb3	Cholinergic receptor, nicotinic, beta polypeptide 3	5.09
A530057A03Rik	RIKEN cDNA A530057A03 gene	5.08
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	5.06
1810019J16Rik	RIKEN cDNA 1810019J16 gene	5.02
Rspo4	R-spondin family, member 4	5.01
Gucy2c	Guanylate cyclase 2c	5

Table 1: Genes those are highly up-regulated in aged mice.

Gene name	Gene description	Log2 ratio
Epyc	Epiphycan	-8.38
Oprd1	Opioid receptor, delta 1	-7.64
Sh3rf2	SH3 domain containing ring finger 2	-7.25
Clspn	Claspin homolog (Xenopus laevis)	-7.23
Dlx5	Distal-less homeobox 5	-6.89
Ovol2	Ovo-like 2 (Drosophila)	-6.86
Actn2	Actinin alpha 2	-6.43
Gm1337	Gene model 1337, (NCBI)	-6.3
3110039M20Rik	RIKEN cDNA 3110039M20 gene	-6.28
Cd4	CD4 antigen	-6.08
Krt9	Keratin 9	-5.99
Ankk1	Ankyrin repeat and kinase domain containing 1	-5.94
Nkx2-1	NK2 homeobox 1	-5.92
Bcl11b	B-cell leukemia/lymphoma 11B	-5.89
Nxph2	Neurexophilin 2	-5.86
Fgf3	Fibroblast growth factor 3	-5.64
Ucn3	Urocortin 3	-5.62
Drd1a	Dopamine receptor D1A	-5.54
Kcnv1	Potassium channel, subfamily V, member 1	-5.53
Tgm3	Transglutaminase 3, E polypeptide	-5.52
Adora2a	Adenosine A2a receptor	-5.52
Gucy2g	Guanylate cyclase 2g	-5.42
Hs3st2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	-5.28
Gpr88	G-protein coupled receptor 88	-5.28
Rspo2	R-spondin 2 homolog (Xenopus laevis)	-5.27
Brs3	Bombesin-like receptor 3	-5.26
Indo	Indoleamine-pyrrole 2,3 dioxygenase	-5.25
Kcnj4	Potassium inwardly-rectifying channel, subfamily J, member 4	-5.22
Kcnh4	Potassium voltage-gated channel, subfamily H (eag-related), member 4	-5.21
Dlx6	Distal-less homeobox 6	-5.13
Tpsg1	Tryptase gamma 1	-5.11
Tbr1	T-box brain gene 1	-5.09
Arx	Aristaless related homeobox gene (Drosophila)	-5.09
Lhx6	LIM homeobox protein 6	-5.09
Ccdc88c	Coiled-coil domain containing 88C	-5.05
Serpina9	Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 9	-5.03

Table 2: Genes that are highly down-regulated in aged mice.

Discussion

We first confirmed the quality of RNA samples for transcriptome analysis. The quality and amount of RNA samples are likely to vary depending on the type, state, and part of tissue, and it confirmation of the quality is an important requirement for transcriptome analysis [19]. Using a previously described method, we homogenized some of the hippocampal cells without any tissue fixation and freezing technique [15]. Consequently, we were able to obtain quality-controlled RNA samples in this study [20]. We investigated a total 37,681 genes using data published data by UCSC. A total of 18,814 genes showed very small average expression levels of mRNA, namely less than 1 count per sample, in the hippocampus of both juvenile and aged mice. In the remaining 18,867 genes, we found that a total of 7,027 genes were differentially expressed between the groups in this study. These data might support that the mRNA expression levels in hippocampus cells are different depending on the maturing stage and suggest mechanisms underlying the differences in efficacy of sevoflurane among maturing stages. Understandably, since a very large number of genes were expressed differently in the two groups, we could not identify the factor that critically alters the effect of sevoflurane in this study. Further study is needed to identify the factor that alters the effect of sevoflurane.

Next, we demonstrated that the *Lhx9* gene was the most up-regulated gene in aged mice. In our previous study, the *Lhx9* gene was found to be the most down-regulated gene in anesthetized juvenile mice, and we therefore could not determine whether the *Lhx9* gene was up-regulated in aged mice by sevoflurane *per se* [10]. However, the *Lhx9* gene showed divergent mRNA expression between juvenile and aged mice in the hippocampus. The *Lhx9* gene encodes a LIM-homeodomain factor that is essential for the development of gonads, spinal cord interneurons, and thalamic neurons [11,21,22]. In juvenile mice, sevoflurane might suppress brain development *via* LIM-homeodomain factors or compensate for the hyperexcitability of the thalamocortical network by suppressing LIM-homeodomain factors [23], while sevoflurane exposure might increase *Lhx9* gene expression or not change its expression. If it is assumed that expression of the *Lhx9* gene enhances neuroinflammation in the mouse hippocampus, sevoflurane might not induce neuroinflammation in aged mice or the neuroprotective mechanism might be vulnerable in aged mice. Expression of the *Lhx9* gene might contribute to the development of POCD, and this could be the focus of future research.

The *Htr5b* gene and the *Cbln3* gene were also highly up-regulated in aged mice in this study. Serotonin receptors encoded by the *Htr5b* gene are widely distributed in the central or peripheral nervous system and play a role in neurotransmission [24]. Serotonin antagonists are used as anti-emetic agents in chemotherapy induced emesis and postoperative nausea and vomiting. Our previous results also showed that serotonin receptor genes were not up-regulated by sevoflurane exposure in juvenile mice. These results might suggest that serotonin antagonists are more effective for postoperative nausea and vomiting in aged patients. The *Cbln3* gene is known as a protein-coding gene that accumulates at parallel fiber-Purkinje cell synapses, and the proteins provide an anatomical basis for a common signaling pathway regulating circuit development and synaptic plasticity in the cerebellum [25]. Assuming that the expression level of the *Cbln3* gene is increased because it acts protectively against neuroinflammation caused by sevoflurane, the juvenile brain might be more prone to neuroinflammation caused by sevoflurane. Therefore, further investigation is needed to determine whether the *Cbln3* gene has a protective effect in the hippocampus.

Notably, the *GABRA6* gene, which encodes GABAA receptor subunit alpha 6, was highly up-regulated in aged mice. The GABAA receptors increase tonic inhibition in somatostatin interneunons and alter circuit activity within the dentate gyrus [26]. GABAA receptors are also known to be a potential target of volatile anesthetics [27].

The *Epyc* gene was the most down-regulated gene in aged mice. The *Epyc* gene is located in the mapping interval of MYP3, which has been suggested to be a candidate gene for high myopia [28,29]. The EPYC protein is predominantly expressed in cartilage, and it is important for fibrillogenesis through the regulation of collagen fibrils [30,31]. It is unclear whether the *Epyc* gene is associated with the effect of sevoflurane. The *Oprd* gene, which encodes the delta-opioid receptor (OPRD), and the *Drd1a* gene, which encodes the dopamine receptor D1a, were also highly down-regulated in aged mice. The ghrelin, which is identified as the endogenous ligand for growth hormone secretagogue receptor 1 alpha, induces acute pain and increases OPRD-mRNA expression [32]. The serum growth hormone concentration in juvenile mice might be higher than that in aged mice and might cause the higher expression level of the *Oprd* gene in the brain. The methods used in this study might have been more harmful for juvenile mice than aged mice, or it is possible that juvenile mice are more likely to feel pain than aged mice. This result regarding the *Oprd* mRNA expressions suggest that juvenile mice should be treated without a painful sequence. Further investigation is needed to determine whether the treatment of mice affects the expression of the *Oprd* gene. The dopamine D1 receptor in the hippocampus is essential for the functional relationship between associative learning and synaptic strength at the CA3-CA1 synapse [33]. D1 receptor knock-out mice are known to have reduced spatial learning and fear learning. Sevoflurane *per se* might inhibit expression of the *Drd1a* gene in the hippocampus in aged mice and/or enhance expression of the *Drd1a* gene in juvenile mice. The juvenile mice showed more than 300 counts of *Drd1a*-mRNA per sample, while the aged mice showed less than 10 counts per sample in this study. Therefore, the difference between juvenile and aged mice in expression level of the *Drd1a* gene in the hippocampus suggests a difference in postoperative spatial cognitive function.

Interestingly, the *Adora2a* gene, which encodes adenosine A2a receptor, was also highly down-regulated in aged mice in this study. The adenosine modulation system mostly operates through inhibitory A1 receptors and facilitatory A2 receptors, and the adenosine receptors are mutually switching synaptic activities in the brain [34]. Brain insults up-regulate the adenosine A2a receptor through adaptive change of the brain, and adenosine A2a receptor bolsters neuronal plasticity. The *Adora2a* gene was reported to show an age-dependent decrease in the human hippocampus. In this study, the *Adora2a*-mRNA expression level was dramatically decreased in aged mice, whereas the published database showed that the mRNA expression level in the elderly human hippocampus was only half of that in the juvenile human [35]. This difference suggests that sevoflurane *per se* inhibits expression of the *Adora2a* gene in the hippocampus in aged mice, or the *Adora2a* gene expresses diversely among the animal species. The adenosine A2a receptor has been reported to be associated with caffeine-induced insomnia [36]. Down-regulation of the *Adora2a* gene might influence the excitation at emergence from general anesthesia and cause POCD in aged patients. Further study is needed to confirm the association between *Adora2a*-mRNA expression and POCD.

We could not determine whether the changes in mRNA expression levels of individual genes were caused by sevoflurane *per se* or other pathways. However, our results indicated that there was age-dependent variation in the mRNA expression profile. Although the molecular mechanisms of POCD after sevoflurane exposure were predicted in the present study, further experiments based on the regulation of individual genes are needed to confirm our speculations. Furthermore, we did not examine the behaviors of the animals that might suggest spatial learning, because the mRNA expression profile might change while recording their behavior. While our data cannot be directly extrapolated to humans, they might provide clues for the molecular mechanism of POCD. In addition, the sample size was small in this study, despite having been determined to obtain a power of ≥ 0.8, and we overlooked changes in the expression of genes that were expressed at low levels. Further studies with larger numbers of samples are needed to confirm the changes in genes that are expressed at low levels.

In conclusion, expression of the *Lhx9* gene, which is thought to be associated with neuronal inflammation, was the most highly up-regulated in aged mice. The *Epyc* gene, which encodes a protein related to the phospholipase-C pathway and ERK signaling, was the most down-regulated in aged mice. These findings may be useful for

exploring the mechanisms of POCD and neuronal inflammation after general anesthesia.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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