Original Paper

Propofol Alters Long Non-Coding RNA Profiles in the Neonatal Mouse Hippocampus: Implication of Novel Mechanisms in Anesthetic-Induced Developmental Neurotoxicity

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Key Words

Apoptosis • Developmental neurotoxicity • Long non-coding RNAs • Microarray • Bioinformatics analysis

Abstract

Background: Propofol induces acute neurotoxicity (e.g., neuroapoptosis) followed by impairment of long-term memory and learning in animals. However, underlying mechanisms remain largely unknown. Long non-coding RNAs (lncRNAs) are found to participate in various pathological processes. We hypothesized that lncRNA profile and the associated signaling pathways were altered, and these changes might be related to the neurotoxicity observed in the neonatal mouse hippocampus following propofol exposure. **Methods:** In this laboratory experiment, 7-day-old mice were exposed to a subanesthetic dose of propofol for 3 hours, with 4 animals per group. Hippocampal tissues were harvested 3 hours after propofol administration. Neuroapoptosis was analyzed based on caspase 3 activity using a colorimetric assay. A microarray was performed to investigate the profiles of 35,923 lncRNAs and 24,881 messenger RNAs (mRNAs). Representative differentially expressed lncRNAs and mRNAs were validated using reverse transcription quantitative polymerase chain reaction. All mRNAs dysregulated by propofol and the 50 top-ranked, significantly dysregulated lncRNAs were subject to bioinformatics analysis for exploring the potential mechanisms and signaling network of propofol-induced neurotoxicity. **Results:** Propofol induced neuroapoptosis in the hippocampus, with differential expression of 159 lncRNAs and 100 mRNAs (fold change $\pm$

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2.0, $P < 0.05$). Bioinformatics analysis demonstrated that these lncRNAs and their associated mRNAs might participate in neurodegenerative pathways (e.g., calcium handling, apoptosis, autophagy, and synaptogenesis). **Conclusion:** This novel report emphasizes that propofol alters profiles of lncRNAs, mRNAs, and their cooperative signaling network, which provides novel insights into molecular mechanisms of anesthetic-induced developmental neurodegeneration and preventive targets against the neurotoxicity.

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Introduction

Propofol is one of the most commonly used pediatric anesthetics. Mounting evidence from animal studies has demonstrated that propofol induces neuronal death followed by cognitive dysfunction (e.g., impaired learning and memory ability) that are persistent into adulthood in animal models [1-7]. The observed neurotoxicity was specific to the brain growth spurt during the developmental period [8-12]. Since many developmental events such as neurogenesis and synaptogenesis occur during this period, neuronal death may not be the only consequence of propofol exposure. Several abnormal changes, such as decreased neural stem cell proliferation, altered neurogenesis, neurite retraction, and dendrite growth reduction, have already been demonstrated [13-16]. So far, cellular and molecular events altered by propofol, such as intracellular calcium levels, neurotrophin expression, mitochondrial signaling (e.g., mitochondrial fission) have been identified as potential contributors to this neurodevelopmental dysfunction [3, 12, 17-19]. However, the detailed mechanisms by which this toxicity occurs in developmental brains remain largely unknown. Additionally, the findings that propofol can attenuate cellular injury in other types of cells (e.g. cardiomyocytes and liver cells) [20, 21] call for greater understanding as to why propofol is specifically harmful to the developing brain.

Long non-coding RNAs (lncRNAs) are composed of at least 200 nucleotides. So far, over 30,000 lncRNAs have been identified; however, very few of these lncRNAs have been characterized for their functions and underlying mechanisms [22]. lncRNAs are rapidly emerging as major regulators of cellular and molecular events. lncRNA profiles and lncRNA/messenger RNA (mRNA) interactions have been implicated as spatially and temporally dynamic during neurogenesis [23], making them interesting candidates with underlying neurotoxic phenotypes resulting from developmental anesthetic exposure. One recent study indicates the important roles of lncRNAs in anesthetic neurotoxicity. The authors found that brain-derived neurotrophic factor antisense (BDNF-AS) lncRNA was upregulated by the intravenous anesthetic agent ketamine in cultured mouse stem cell-derived neurons. BDNF-AS-induced downregulation of BDNF decreased neurite growth and increased apoptosis [24]. The effort of this study focused on a single lncRNA dysregulated by ketamine; however, the role of lncRNAs in propofol-induced development has not been explored yet. We hypothesized that lncRNA profile and the associated signaling pathways were altered, and these changes might be related to the neurotoxicity observed in the neonatal mouse hippocampus following propofol exposure.

Thus, the current study profiles dysregulated lncRNAs and mRNAs in the hippocampus (a brain region associated mainly with memory) from propofol-treated 7-day-old mice relative to control mice. Based on differentially expressed lncRNAs and mRNAs between propofol and control groups, we performed bioinformatics analysis of mRNA involvement, lncRNA/mRNA interaction, and proposed cellular and molecular signaling networks that might be related to initiation and progression of neurodegeneration following propofol exposure.

Materials and Methods

Animal studies

All studies were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The greatest vulnerability of the developing brain to anesthetics occurs within the first 2 postnatal weeks, peaking at approximately postnatal day 7 [25]. Thus, 7-day-old mice were used for the current study.

Anesthetic exposure

Mice were exposed to propofol (ZOETIS) or the vehicle control 10% intralipid (Fresenius Kabi AB) for 3 hours. The dose of propofol intraperitoneally injected was 50 mg/kg. This dose of propofol was selected based on previous reports showing that 1) the dosage required to induce a surgical plane of anesthesia in mice was 200 mg/kg, and 2) sub-anesthetic doses of 50 and 100 mg/kg propofol triggered neuroapoptosis. It was also reported that single intraperitoneal injection of propofol maintained anesthesia in mice for approximately 90 minutes [26]. Therefore, we administered propofol every 90 minutes throughout the experiments for a total of 2 times. Following injection, animals were placed on a heated pad (37°C). Mice were euthanized 3 hours following 3-hour propofol exposure for further tissue harvest used for Western blot analysis and lncRNA and mRNA profile assay of the hippocampus.

Caspase 3 activity assay

Caspase 3 enzyme activity assay was analyzed using a Caspase 3 Colorimetric Assay Kit (Sigma Aldrich, MO, USA). Briefly, the caspase 3 activities in the cell lysate were analyzed by administration of the caspase 3 substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DECD-pNA) and incubated for 90 minutes. The concentration of the reaction product pNA was detected at 405 nm and caspase 3 enzyme activity was calculated using the following formula:

$$\frac{\mu\text{mol caspase 3 reaction product} \times \text{dilution factor}}{\text{nl (sample lysate)} \times \text{min (reaction time)} \times \text{mg (protein)}} \times 100\%$$

RNA extraction

RNA was extracted using a phenol-chloroform method as described in our previous publication [19]. The extracted RNA was used for microarray and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays as described below.

Microarray assay

Mouse lncRNA Expression Microarray V3.0 assay and data analysis were performed by Arraystar Inc. (Rockville, MD) to evaluate global expression levels of 35,923 lncRNAs and 24,881 mRNAs in the hippocampus. Prior to the microarray assay, RNA was subject to RNA quality control analysis of RNA quantity, purity, integrity, and genomic DNA contamination and probe label efficiency. The RNA was converted to complementary DNA (cDNA) and then hybridized to Arraystar's custom lncRNA or mRNA probes. The significantly differentially expressed lncRNAs and mRNAs were defined by expressing above ± 2.0 fold change and $p < 0.05$ between propofol and control samples, and are shown in volcano plots or heatmaps. Volcano plot depicts the p -value against the fold change ratio, with genes with the largest magnitude fold change in abundance being most displaced from the center of the plot (0 on the X-axis). The heatmap shows the gene profile of all samples analyzed. Data were normalized by a $\log_2$ transformation of the fold change and a $-\log_{10}$ transformation of the p -value.

RT-qPCR

In order to confirm the gene expression results obtained from the microarray, the expression level of 4 randomly selected differentially expressed lncRNA (Gm11985 and Ak156531) and mRNAs (Igfbp1 and Npas4) were further analyzed using RT-qPCR assay as shown in our previous publication [19]. Briefly, cDNA was synthesized using an RT2 First Strand Kit (Qiagen). For qPCR assay, cDNA was mixed with [™] SYBR Green Master Mix (Thermo Fisher Scientific), primers, and pure water (Qiagen). Triplicate samples were loaded

into 96-well plates (25 μ l/well) and RT-qPCR was performed using QuantStudio™ 6 Real-Time PCR detection system (Thermo Fisher Scientific) for 10 minutes at 95°C followed by 40 cycles of a denaturation step (15 seconds at 95°C) and a combined annealing/extension step (30 seconds at 60°C). The mean cycle threshold (Ct) values of triplicate wells for each sample were collected and the expression data was normalized to the endogenous control (beta-actin). Melting curves were monitored to validate the purity of the PCR product in each well.

Ingenuity Pathway Analysis of differentially expressed mRNAs and their associated signalling pathways

In order to study the possible mechanisms and signaling pathways of differentially expressed mRNAs in propofol-induced developmental neurotoxicity, we performed bioinformatics analysis of mRNAs using Ingenuity Pathway Analysis (IPA) software (Qiagen Bioinformatics). IPA performs a causal network analysis of gene involvement based on its known upstream regulators, downstream effects, and the gene's role in previously established pathways. The analysis reveals hypothetical disease mechanisms, toxicity pathways, and canonical physiological signaling pathways [27]. Based on IPA prediction of pathway clusters and involved genes from the Arraystar dataset, the selected pathways analyzed were associated with developmental anesthetic exposure and neurodegenerative phenotypes.

Analysis of involved signaling pathways of differentially expressed lncRNAs

Our data showed that there were 159 lncRNAs and 100 mRNAs dysregulated following propofol exposure. In this current study, we analyzed top-ranked 50 lncRNAs and all dysregulated mRNAs to predict lncRNA pathway using the following two bioinformatics analysis approaches as described in previous publications [28, 29]. The top 50 lncRNAs were selected based on the criteria with $p < 0.01$ and the highest absolute fold change value (propofol vs control). 1) lncRNA Gene Set Enrichment Analysis (lncRNA-GSEA) was used for analyzing the signaling pathways of lncRNAs and the roles of lncRNAs in developmental neurotoxicity. The correlation between the expression level of each top 50 lncRNA and all dysregulated mRNAs was analyzed. For each lncRNA, a list of correlation-based ranked mRNAs was constructed and subjected to GSEA. An association matrix between lncRNA and KEGG pathway was constructed using the value of [Normalized enrichment score] greater than 1 and a false-discovery rate (FDR) q-value threshold of 0.2. 2) lncRNA-mRNA co-expression interaction assay was used for analyzing the networks among lncRNAs and mRNAs. Correlation coefficients between the normalized data of the top 50 non-coding genes (lncRNA) and all dysregulated coding gene (mRNA) expression were calculated. mRNAs with the absolute value of Pearson correlation coefficients greater than 0.9 and FDR less than 0.05 were selected for generation of the coding and non-coding gene co-expression network map using the software Cytoscape V2.8.3. Literature searches for lncRNAs and mRNAs involved in neurodevelopmental and/or neurodegenerative conditions were performed by utilizing the National Center for Biotechnology Information Pubmed search engine, and searching for both gene name and gene abbreviation.

Statistics

The data were presented as mean $\pm$ standard deviation (SD). All data from individual experiments were obtained from 4 intralipid- and 4 propofol-treated mice ($n=4$), based on the previous data from our lab and similar studies in the field [30, 31]. For differentially expressed lncRNAs and mRNAs by microarray, Western blot analysis of cleaved caspase 3, and RT-qPCR analysis of gene expression were done. An independent two-tailed t-test was performed using SPSS (IBM SPSS Statistics 24, USA) to compare the values between propofol and control groups; $p < 0.05$ was considered statistically significant. Statistical analysis of the signaling pathway was described in the sections above.

Results

Propofol induces neuroapoptosis in the hippocampus

Propofol exposure for 3 hours induced 30% increase of caspase 3 enzyme activity (an apoptosis indicator) in the hippocampus of P7 mice compared with mice exposed to the 10% intralipid vehicle control (Fig. 1).

Fig. 1. Propofol induces neuroapoptosis in the 7-day-old mouse hippocampus by analysis of caspase 3 activity. n=4 samples per group; *: p<0.05.

Fig. 2. Propofol alters messenger RNA (mRNA) profiles in the mouse hippocampus. (A) The images of denaturing agarose gel electrophoresis of total RNAs isolated from 4 intralipid- or 4 propofol-treated mouse hippocampal tissues. (B) The box plots display similar distributions of normalized mRNA intensity values across 4 control and 4 propofol samples, suggesting that the microarray data can be used for further analysis. Each box plot consists of a box with a central line and two tails. The central line represents the median of mRNA intensity values whereas the tails represent the upper and lower quartiles. (C) Heatmap showing profiles and the hierarchical clustering of differentially expressed mRNAs between intralipid- and propofol-treated mouse hippocampus (n=4; p<0.05). The red signal refers to high relative expression and the green signal referred to low relative expression.

Propofol induces the alteration of mRNA profiles

Each sample was confirmed to be suitable for microarray assay based on the RNA quantity, purity (data not shown), integrity, and appropriate probe label efficiency (Fig. 2A). The distribution of normalized intensity values between two groups indicated that these data were suitable for further analysis (Fig. 2B). Out of 24,881 mRNA transcripts, there were 67 upregulated mRNAs (propofol vs. control) and 33 downregulated (above ± 2.0 fold change, $p < 0.05$) as shown in the heatmap (Fig. 2C). The expression level of 2 representative altered mRNAs, *Igfbp1* and *Npas4*, was further validated using RT-qPCR ($p = 0.019$ for *Igfbp1* and $p = 0.021$ for *Npas4*; Fig. 3A) and the data were consistent with what obtained from microarray assay.

Fig. 3. (A) qRT-PCR validation of 2 representative altered mRNAs from array data (*Igfbp1* and *Npas4*). ($n = 4$; $*p < 0.05$). (B) qRT-PCR validation of two representative altered lncRNAs from array data. ($n = 4$; $*p < 0.05$).

Propofol induces the alteration of lncRNA profiles

The general distribution of normalized intensity values between control and propofol groups was similar (Fig. 4A). The volcano plot illustrated the differentially expressed lncRNAs between control and propofol groups (Fig. 4B). Out of total 35,923 lncRNA transcripts, there were 107 upregulated and 52 downregulated lncRNAs in the propofol versus control group as shown in the heatmap (above ± 2.0 fold change, $p < 0.05$) (Fig. 4C). Two representative altered lncRNAs (*Gm11985* and *Ak156531*) from the microarray data were further validated using RT-qPCR (Fig. 3B) and the results were consistent with the array data ($p = 0.034$ for *Gm11985* and $p = 0.021$ for *Ak156531*). Intergenic, exon sense-overlapping, natural antisense, intronic antisense, and bidirectional lncRNAs represent 50.6%, 22.7%, 12.2%, 11.7%, and 2.8% of all 159 dysregulated lncRNAs, respectively (Fig. 4D). There were seven altered lncRNAs whose nearby coding genes (mRNAs) were also dysregulated following propofol treatment ($p < 0.05$, fold change $> \pm 2$): *Gm11985/Igfbp1*, *AK153212/Ly6c1*, *AK153212/Ly6a*, *AK156531/Npas4*, *AK156997/Tmem174*, *A730032A03Rik/Slpi* and *Gm3294/Fam71f1* (Table 1).

Propofol-induced altered mRNAs are involved in apoptosis and neurodegeneration signaling pathways

IPA software was used to analyze signaling pathways of the differentially expressed mRNA (± 2.0 fold cut-off). Specifically, we selected the signalling pathways that were associated with 1) brain developmental events (e.g., synaptic plasticity), and 2) previously-documented anesthesia neurotoxicity phenotypes and cellular events (e.g., apoptosis and

Fig. 4. Propofol alters profiles of long non-coding RNAs (lncRNAs) in the mouse hippocampus. (A) The box plots showing the similar distributions of normalized lncRNA intensity values from 4 control and 4 propofol samples. Each box plot consists of a box with a central line and two tails. The central line represents the median of lncRNA intensity values, whereas the tails represent the upper and lower quartiles. (B) The volcano plot illustrating the differentially expressed lncRNAs (red dots) between control and propofol groups. These altered lncRNAs were either downregulated (left red dots) or upregulated (right red dots) following propofol exposure. (C) Heat map showing the profiles and hierarchical clustering of differentially expressed lncRNAs in intralipid- and propofol-treated mouse hippocampus ($n=4$; $p<0.05$). The red signals refer to high relative expression and the green signals indicate low relative expression. Among 24,881 lncRNAs analyzed, 159 lncRNAs were differentially expressed following propofol exposure. (D) Pie chart showing the ratio of various types of dysregulated lncRNAs. Specifically, over 50% total dysregulated lncRNAs were intergenic lncRNAs.

Table 1. Propofol-induced individual dysregulated lncRNAs and their neighbor protein-coding genes which are also altered following propofol exposure. Note: chr is the abbreviated chromosome. P value and fold change is shown as propofol vs. control. Expression changes are shown compared with control group

lncRNA symbol	Genomic location of lncRNAs	lncRNA p-value	lncRNA fold change	lncRNA change		FDR	lncRNA's neighbor protein-coding gene symbol	mRNA p-value	mRNA fold change	mRNA change		FDR
				Propofol vs. control						Propofol vs. control		
Gm11985	chr11	0.036	2.2	down	0.59	Igfbp1	0.005	3.7	up		0.52	
AK153212	chr15	0.004	2.3	up	0.42	Ly6c1	0.040	2.2	up		0.72	
AK153212	chr15	0.004	2.3	up	0.42	Ly6a	0.007	2.0	up		0.54	
AK156531	chr19	0.008	2.6	down	0.45	Npas4	0.001	4.2	down		0.40	
AK156997	chr13	0.039	2.1	up	0.59	Tmem174	0.022	3.0	up		0.66	
A730032A03Rik	chr2	0.042	2.7	up	0.61	Slpi	0.034	2.1	up		0.71	
Gm3294	chr6	0.031	2.9	down	0.58	Fam71f1	0.015	3.4	up		0.62	

Fig. 5. Bioinformatics analysis of signaling pathways of propofol-induced altered mRNAs in the neonatal mouse hippocampus using IPA software. The selected pathways shown in the left column were associated with developmental anesthetic exposure and neurodegenerative phenotypes based on previous publications included in the IPA database. The right column lists altered mRNAs that were associated with the signaling pathway shown in the left column. The green and red arrows, respectively, represent the mRNA downregulation or upregulation following propofol exposure.

dysregulated calcium signaling). The respective dysregulated mRNA clusters influencing these physiological and pathological processes were listed in Fig. 5. For example, among the 25-dysregulated mRNAs that were reported to participate in the apoptosis process, 11 mRNAs were downregulated while another 14 mRNAs were upregulated in the propofol-treated hippocampus compared with the control group.

Fig. 6. Bioinformatics analysis of neuro-degenerative signaling pathways of top 50-ranked altered lncRNAs. The green and red arrows, respectively, representative lncRNA downregulation and upregulation following propofol exposure.

Involved Pathways	Propofol-induced altered lncRNAs
CALCIUM SIGNALING	AK156531↓, BG808371↓, ENSMUST00000121↓, ENSMUST00000124↓, ENSMUST00000134↓, ENSMUST00000135↓, ENSMUST00000181↓, TCONS_00020914↓, uc.98-↓, uc007cfx.1↑
AXON GUIDANCE	BG808371↓, ENSMUST00000124↓, ENSMUST00000134↓, TCONS_00020914↓
NEUROTROPHIN SIGNALING	BG808371↓, ENSMUST00000134↓, TCONS_00020914↓
REGULATION OF AUTOPHAGY	AK156531↓, uc.98-↓, uc007niz.1↓

Propofol-induced Dysregulated lncRNAs possibly form extensive signaling networks with dysregulated mRNAs and might be associated with anesthetic-induced adverse outcomes in the developing hippocampus

lncRNA-GSEA analysis showed the top 50 dysregulated lncRNAs are associated with many physiology- and pathology-related signaling pathways. These lncRNAs were either positively or negatively correlated with individual signaling pathways (Supplementary Fig. S1) (for all supplementary material see www.karger.com/doi/10.1159/000493875). We further focused on analyzing neurotoxicity-related pathways and found that 14 dysregulated lncRNAs were significantly involved in calcium signaling, axon guidance, regulation of autophagy, neurotrophin signaling disease, and long-term depression (Fig. 6). To understand the lncRNA-associated mRNA interaction, the coding/non-coding gene co-expression network study was performed using the dysregulated top 50 lncRNAs and all dysregulated gene-coding mRNA. The results showed that there were many lncRNA-mRNA interaction pairs in the propofol-treated mouse hippocampus (Supplementary Fig. S2). There was no previously published literature on any of the 159 propofol-altered lncRNAs, while 22 of 100 dysregulated mRNAs were previously implicated in neurodevelopmental and neurodegenerative conditions (Table 2).

Discussion

In this study, we utilized lncRNA and mRNA profiling to predict lncRNA-involved signaling pathways in propofol-induced developmental neurotoxicity in the mouse hippocampus by bioinformatics analysis. We found increased neuroapoptosis that was concurrent with an altered abundance of 159 lncRNAs and 100 mRNAs in mouse hippocampal tissues 3 h following propofol (3 h) exposure. These data suggest that a short-term propofol exposure induces significant changes of lncRNA and mRNA profiles. These dysregulated lncRNAs, mRNAs, and respective interactions between them may be related to several signaling pathways (e.g., calcium and synaptic plasticity) that were previously nominated for their involvement in anesthetic neurotoxicity. In addition to the neuroapoptosis observed in our study (Fig. 1), several reports suggest other propofol-induced side effects (e.g., dysregulated neurogenesis, abnormal dendritic development, and decreased neurotrophic factor expression) [12, 18, 19, 30, 32, 33]. Thus, the current study on the analysis of the signaling pathways based on altered lncRNA and mRNA involvement was not limited to focus on apoptosis alone but predicted pathways linked to multiple neurological outcomes. This opens more doors to study various

Table 2. Reported evidence of propofol-dysregulated mRNAs in neurodevelopmental and/or neurodegenerative conditions

Gene Symbol	Experimental model	Role in neurodevelopment and/or neurodegeneration	Reference
Alox15	Brain of Alzheimer's disease patients	15-lipoxygenase (Alox15) was decreased in the CA1 hippocampus of Alzheimer's disease patients.	[41]
Angptl4	Developing cerebral cortex in rats	Angptl4 was down-regulated in the developing cerebral cortex treated with antidepressant drugs for 21 days.	[42]
Arhgef6	Arhgef6 deficient mice	Arhgef6 deficient animals exhibited altered synaptic plasticity in the CA1 hippocampus, and changes in neural network morphology and function.	[43]
Cd163	Brain of rats on postnatal day 21	CD163-positive macrophages were increased in the hippocampus after MnCl ₂ -4H ₂ O exposure, likely disrupting neurogenesis.	[44]
Cdh7	Embryonic zebrafish Embryonic marmoset	Cdh7 expression in the central nervous system changes spatially and temporally during early development. In the transition between the pre- and postnatal period, cadherin abundance changes spatially and temporally over time.	[45] [46]
Dach2	Peripheral blood from two human brothers	Cdh7 influences migration of Purkinje cells to parasagittal domains during brain development.	[47]
Egr4	Planarians	Dach2 regulates brain and limb development, is observed in conjunction with congenital language disorder, growth retardation, intellectual disability, locomotion difficulties, and incontinence.	[48]
Gimap4	Bovine brain	Egr4 is essential for brain cell differentiation.	[49]
Has3	Has3 ^{-/-} mice	Gimap family is differentially expressed between maternal and paternal brain caudal lobe.	[50]
Hbp1	Mouse embryonic neural stem cells	Has3 knock-out reduces brain extracellular space, likely leading to seizures and altered neuron morphology.	[51]
Igfbp1	Mice with liver-specific transgenic expression of human Igfbp1	Hbp1 regulates neural proliferation during cortical development.	[52]
Lif	Igfbp1 transgenic mice mouse embryo NPAS4 ^{-/-} mice	Increased Igfbp1 resulted in decreased glial cell sensitivity to injury, decreased cell proliferation, and increased neurodegeneration in various brain regions. Impaired brain development was found in Igfbp1 overexpressing mice.	[53] [54]
Npas4	Sprague-Dawley rats Mouse hippocampal neurons Mouse embryonic stem cells	Lif signal-induced SOCS3 expression in the placenta leads to decreased cerebral neurogenesis. Short- and long-term memory formation is impaired in NPAS4 ^{-/-} mice. NPAS4 is required for memories of fear.	[55] [56] [57]
Npbwr1	Postnatal day 0-28 of the neuropeptide W (NPW) knock-in mouse	NPAS4 regulates abundance of activity-dependent genes and formation of synapses based on balance between excitatory and inhibitory activity.	[58]
Olig3	Mouse embryonic thalamus	Knockdown of Npas4 delayed timing of the differentiation process of embryonic stem cells.	[59]
Pde4b	Pde4b ^{-/-} mice	Npas4 regulates abundance of activity-dependent genes and formation of synapses based on balance between excitatory and inhibitory activity.	[60]
Pdk4	Motor cortex of monkeys	Olig3 is expressed in thalamic basal progenitor cells during development of the embryo.	[61]
Pirb	PirB ^{-/-} adult mice	Loss of Pde4b reduces locomotion, dopaminergic and serotonergic neuron activity, implicating a role in neuropsychiatric conditions.	[62]
Prx1l	Prx1l ^{-/-} mice	Pdk4 is positively correlated with physical activity.	[63]
Psmas5	HEK 293 cells	PirB regulates formation of hippocampal circuitry.	[64]
Trap2c	32-month-old female of Peruvian ethnicity Forebrain of developing mice	Prx1l is required for formation of subpopulations of glutamatergic dorsal horn neurons.	[65]
Txnip	BV2 mouse microglia cell line	Downregulation of PSMAS5 prevents cell proliferation, leading to impairment by amyloid precursor protein-induced inhibition of gene expression. A rare 20q13.2-q13.33 deletion was found in 1 female with intellectual deficits, no speech, hypotonia, growth retardation, unilateral cleft lip.	[66]
		Trap2c is required for the continuous expression of luteinizing hormone-releasing hormone.	[67]
		Txnip signaling is activated by thrombin, leading to inflammation and apoptosis in microglia.	[68]
			[69]

neurotoxic effects of anesthetics, novel mechanisms, and neuroprotective strategies. Propofol has recently been observed to decrease cervical carcinoma cell proliferation. Propofol also inhibited the migration of Hela cells possibly via the mechanisms of the reduced organization and decreased intensity of F-actin (the most important component of the cytoskeleton) [34]. These pathway analyses are helpful in contributing to greater mechanistic understanding the conserved across various physiological processes occurred in different types of cells following propofol treatment.

Our results showed that propofol elicited downregulation of 33 mRNAs and upregulation of 67 mRNAs (Fig. 2). IPA bioinformatics analysis indicates that some of these altered mRNAs are linked to apoptosis, autophagy, calcium handling, neurotrophin expression, neuronal morphology, and phenotypes (Fig. 5). For example, propofol-induced upregulation of interleukin-2-inducible T-cell kinase may be involved in acute calcium increase reported following anesthetic exposure, as previous literature proposed its involvement in the calcium-related and neuroprotective inflammatory response [35, 36]. Our mRNA array also found that oligodendrocyte factor 3 (oligo3) was downregulated by propofol. Since oligo3 influences neuron fate specification during the period of neurogenesis, propofol may alter neuron development and outgrowth, leading to altered synaptic plasticity and/or neural network formation. Existing information on the role of these genes in these non-anesthetic neurodegenerative conditions is extremely valuable in providing new insights on potential phenotypes related to anesthetic-induced neurotoxicity.

lncRNAs can be classified as antisense, intergenic, overlapping, intronic, and bidirectional with respect to their nearby protein-coding genes. The mechanisms of lncRNA action are complex and they can either regulate transcription machinery, chromatin modeling complexes, as well as mRNA and protein expression [37]. Our microarray data show upregulation of 107 lncRNA and downregulation of 52 lncRNA following propofol exposure. These altered lncRNAs belong to five categories of lncRNAs and over 50% of these lncRNAs were intergenic lncRNAs. Thus, these multiple types of dysregulated lncRNAs suggest possible various mechanisms of lncRNA action on propofol-induced neurotoxicity (Fig. 4).

Though unlikely due to the accompanying increased abundance of 107 lncRNAs and a decrease in 52 lncRNAs alone, the lncRNA and mRNA interactions likely play large roles in the downstream neuroapoptosis observed and other adverse effects reported. Bioinformatics analysis demonstrated the extensive signaling pathways and networks between dysregulated lncRNA and mRNAs that may be related to initiation and/or progression of propofol-induced neurotoxicity. Major lncRNA-involved pathways were apoptosis, neuronal morphology, calcium release, synaptic plasticity, and neurotrophin expression (Fig. 6 and Supplementary Figs. S1 and S2). For example, Gm11985-Igfbp1 (insulin growth factor binding protein 1), one of lncRNA and its nearby coding mRNA pair as shown in Table 1, was downregulated and upregulated, respectively in the propofol-treated hippocampus. Igfbp1 was reported to be elevated during various other kinds of brain injuries, such as hypoxic ischemia in rats [38] and multiple sclerosis in humans [39]. It is believed that insulin growth factor (Igf)/Igfbp1 axis is strongly implicated in brain injury, and Igfbp protein helps maintain Igf protein at the local brain injury site, thus playing a neuroprotective role [40]. However, the function of Gm11985 was barely studied before. Consistent with our data, it is possible that the propofol-induced downregulation of Gm11985 (chromosome 11: 7183156-7188569, negative strand) might lead to an increased level of its nearby downstream antisense Igfbp1 mRNA (Chromosome11: 7197786-7202546, positive strand) by complementary binding, in order to prevent neuronal cell damage conferred by propofol.

Conclusion

Collectively, this microarray analysis to investigate the abnormal profile of lncRNAs and mRNAs in the mouse hippocampus following propofol exposure is a new angle to explore in anesthetic-induced neurotoxicity. These dozens of changed lncRNAs and mRNAs

might interact in extensive signaling networks linked to multiple downstream disease phenotypes. These disease phenotypes not only include the widely recognized anesthetic-induced neuroapoptosis, but also other neurological outcomes (e.g., neuron morphology, myelination, and synaptic plasticity). In addition, these phenotypes might be related to complex mechanisms such as calcium release, neurotrophin expression, or autophagy. The roles of lncRNAs have just begun to be explored in the field of anesthetic-induced neurotoxicity. Our data provide insights into novel molecular mechanisms of anesthetic-induced developmental neurodegeneration and preventive strategies. In addition, this study provides a starting point for us and other researchers to study functional roles and novel molecular mechanisms of lncRNAs underlying developmental neurotoxicity.

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Disclosure Statement

The authors declare no competing interests.

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