WNK1 Antagonist Decreased Sevoflurane-induced Neurotoxicity in HT22 Hippocampal Neurons via the WNK1/NKCC1 Signalling Pathway

Yafan Bai  
Capital Medical University Affiliated Beijing Friendship Hospital Department of Anesthesiology  
https://orcid.org/0000-0003-2449-4909

WenJing Li  
Capital Medical University Affiliated Beijing Friendship Hospital Department of Anesthesiology

JunFa Li  
Capital Medical University Department of Neurobiology and Beijing Institute for Brain Disorders

LiXin An  (anlixin8120@163.com)  
https://orcid.org/0000-0001-6344-4010

Research article

Keywords: WNK-463, sevoflurane, neurotoxicity, WNK1/NKCC1 pathway

DOI: https://doi.org/10.21203/rs.3.rs-29017/v1

License: ☒ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

**Background:** Children repeatedly exposed to anaesthesia have a high risk of cognitive impairment. The GABA$_A$R plays an important role in the neurotoxicity caused by sevoflurane, but the mechanism of its regulation in this context is unknown. The present study aimed to reveal the WNK1/NKCC1 pathway as a regulator of the neurotoxicity caused by sevoflurane on the HT22 hippocampal neuron cell line.

**Methods:** In this study, HT22 hippocampal neurons were used as the research object. A model of sevoflurane treatment was established. HT22 cultured hippocampal neurons were divided into three groups: control group, 4.1% sevoflurane treatment group for 6h, WNK-463 (specific antagonist of WNK1, 1µmol) + sevoflurane treatment group. Cell viability and the optimum concentration of WNK-463 were measured by MTS method. Indicators of neuron injury: cell viability was detected by MTS method, cell apoptosis was detected by Tunel method, and the content of cleaved caspase-3 protein apoptosis factor was detected by Western blot. Pathway protein detection: the expression of WNK1, NKCC1 was detected. Calcium imaging measures intracellular calcium ion concentration and verifies downstream targets.

**Results:** The neurotoxic effects of sevoflurane on hippocampal neurons were observed. Cell viability was reduced, the apoptotic cell rate was increased, and cleaved caspase-3 was upregulated after 4% sevoflurane exposure for 6 h. WNK-463 downregulated the protein expression of cleaved caspase-3, increased cell viability and decreased apoptosis in sevoflurane-injured neurons. Compared with the control HT22 cells, sevoflurane increased the expression of WNK1 kinase and NKCC1 protein, whereas WNK-463 reversed this increase without affecting the control HT22 cells. Sevoflurane exposure in HT22 cells caused intracellular Ca$^{2+}$ concentrations to increase, while WNK-463 reversed this change.

**Conclusion:** This study demonstrated a neuroprotective role of the WNK1 antagonist WNK-463 in sevoflurane-induced neurotoxicity. WNK-463 promoted hippocampal neuron viability and reduced the apoptosis and intracellular calcium overload caused by sevoflurane on HT22 hippocampal neurons, possibly via the modulation of the WNK1/NKCC1 pathway.

1. **Background**

The neurotoxic effect caused by general anaesthesia in the developing brain has caused continual concern. Numerous animal studies have found that an exposure to most general anaesthetics in mammals at their peak of development leads to a range of morphological changes$^{[1-3]}$. These changes include neurodegenerative changes$^{[3, 4]}$, neural apoptosis or cell death, and impaired neurogenesis$^{[5]}$. In addition, anaesthesia exposure in infancy has also been associated with memory impairment, behavioural disorders, and poor intellectual development persisting to early adulthood$^{[6-10]}$. Repeated and prolonged exposure to general anaesthetics in the developing brain is still a high-risk factor for neuronal injury$^{[6, 11, 12]}$. Therefore, it is of great significance to explore the mechanism of neurotoxicity caused by general anaesthetics in developing neurons.
The γ-aminobutyric acid type A receptor (GABA\textsubscript{A}R) plays an important role in the mechanism of general anaesthesia\textsuperscript{[13, 14]}. Sevoflurane, a representative inhaled general anaesthetic, stimulates GABA\textsubscript{A}R to induce the flow of chloride ions (Cl\textsuperscript{−})\textsuperscript{[14]}. In the adult brain, GABA\textsubscript{A}R activation triggers Cl\textsuperscript{−} flowing into cells, membrane hyperpolarization and synaptic inhibition. Conversely, in the developing brain, GABA\textsubscript{A}R activation triggers Cl\textsuperscript{−} flowing out of cells, membrane depolarization and synaptic excitation\textsuperscript{[15, 16]}. The difference in neuronal excitability is due to different chlorine ion concentrations in cells ([Cl\textsuperscript{−}]i) at different stages\textsuperscript{[16, 17]}. This developmental "switch" in GABA\textsubscript{A}R function from excitability to inhibition has been attributed to the difference in the [Cl\textsuperscript{−}]i of immature versus mature neurons\textsuperscript{[17]}. This difference in [Cl\textsuperscript{−}]i at different stages results from the conversion of receptors with a dominant function from Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter 1 (NKCC1) to K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter 2 (KCC2)\textsuperscript{[17]}.

WNK (with no lysine) kinases are Cl\textsuperscript{−}-sensing kinases, including WNK1, that regulate the activities of NKCC1 and KCC2\textsuperscript{[17]}. In our previous study, we found that sevoflurane's neurotoxicity on the developing brain was related to the changes in the transition of GABA\textsubscript{A}R a1 and a2\textsuperscript{[18]}. This neuronal damage was decreased by the NKCC1 inhibitor bumetanide\textsuperscript{[18]}. Stevens RA\textsuperscript{[19]} also found that an NKCC1 inhibitor prevented ketamine neurotoxicity. Therefore, we hypothesized that when a WNK1 inhibitor (WNK-463) is administered, NKCC1 activity can be downregulated, the concentration of chloride ions in cells can be reduced, and the excitatory neurotoxic effects caused by sevoflurane can be decreased in the developing brain.

As the research object, hippocampal neuronal HT22 cells from mice\textsuperscript{[20]} were used to verify the above hypothesis and explore the neurotoxicity mechanisms of sevoflurane on neurons, with the aim to provide new ideas for ameliorating the neurotoxicity of inhalational anaesthetics.

2. Materials And Methods

2.1 Cell culture and treatment

The hippocampal neuronal HT22 cells of mice were purchased from the American Type Culture Collection (ATCC). HT22 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) containing 10% foetal bovine serum (Gibco, Grand Island, USA) and 1% penicillin-streptomycin solution (Life Technologies, Carlsbad, ON, Canada) at 37 °C in a humidified incubator with 5% CO\textsubscript{2}. The medium was completely changed every 72 h.

2.2 Exposure to sevoflurane and WNK-463 administration

The cell culture was exposed in an airtight plastic chamber with inlet and outlet connectors. The inlet port of the chamber was used to adjust the concentration of sevoflurane (AbbVie Inc., North Chicago, IL, USA), which was connected to a sevoflurane vaporizer. Subsequently, the chamber was gassed with a concentration of sevoflurane (4.1%) in the carrier gas (95% air/5% CO\textsubscript{2}) for 30 min as described
previously\textsuperscript{21}. The outlet of the chamber was used to monitor sevoflurane concentration through a gas monitor (PM 8060, Drager, Lübeck, Germany) until the target concentration was reached. The chamber was then kept tightly sealed for 6 h at 37 °C.

The WNK1 inhibitor WNK-463 was dissolved in dimethyl sulfoxide (DMSO) and diluted with sevoflurane-treated medium. The intervention group was treated with WNK-463 in the medium before sevoflurane anaesthesia and then treated with sevoflurane for 6 h together with the treatment group. The control cells were grown in a humidified atmosphere with 5% CO\textsubscript{2} at 37 °C.

2.3 MTS assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS) assay was used for cell proliferation analysis. The CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI, USA) is a colourimetric method for determining the number of viable cells in proliferation in cytotoxicity assays according to a previous study\textsuperscript{22}. The assays are performed by adding a small amount of the CellTiter 96® AQueous One Solution reagent directly into the culture wells, incubating for 2 h and then recording the absorbance at 490 nm with a 96-well plate reader.

2.4 TUNEL assay

The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) method was performed to label the 3'-terminus of the fragmented DNA of apoptotic cells according to the manufacturer's protocol (One Step TUNEL Apoptosis Assay Kit, Beyotime, China). Cell slices were fixed with 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) for 1 h and washed with 1x PBS 3 times for 5 min. After permeabilization with 1x PBS containing 0.1% Triton X-100 for 2 min on ice, the cell slices were incubated with a TUNEL working solution for 1 h at 37 °C in darkness. The Cy3-labelled TUNEL-positive cells were examined with fluorescence microscopy (Olympus IX710 Camera, Japan). The cells with green fluorescence were considered apoptotic cells. To count the number of apoptotic cells, we selected the same-sized areas of each cell slice for analysis with Image Pro Plus software.

2.5 Measurement of intracellular calcium levels ([Ca\textsubscript{2+}]i)

Cell density measurement and experimental grouping were similar to those in the section on intracellular reactive oxygen species (ROS) assay. According to before the treatment method of cells after collecting, learned that, the supernatant fluid to join without phenol red culture medium, HBBS wash three times, to join the Fluo – 4, AM concentration of 5 umol/L working liquid Beyotime biological co., LTD. (Shanghai), incubation in 37 °C for 30 min, using Hank's balanced salt solution (HBBS) flushing cells 3 times, and continue in HBSS incubation for 30 min, under laser confocal microscope test, excitation wavelength of 494 nm, emission wavelength of 516 nm.

2.6 Western blot analysis

The protein used for western blot analysis was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). The protein concentrations were quantified using the BCA™ Protein Assay Kit (Pierce,
Equal amounts of protein were resolved over 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The primary antibodies were caspase-3 antibody (#9662, 1:1000 dilution), WNK1 antibody (NB600-225, 1:500 dilution) and NKCC1 antibody (#14581, 1:500 dilution). The membranes were then incubated with the secondary antibodies (Abcam) for 1 h at room temperature. Positive signals were visualized by enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, UK). The intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, CA, USA).

2.7 Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the means ± standard deviations (SD). Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). The P-values were calculated using t analysis. A P-value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1 WNK-463 alleviated the sevoflurane-induced reduction in cell activity

The MTS method was used to detect hippocampal neuronal HT22 cell activity. The mean value of the normal control group was 100% and that of the sevoflurane group was 75.40%; the value was significantly decreased (P < 0.0001, Fig. 1A). We chose WNK1 antagonists (WNK-463) as interventions to determine the best concentration of WNK-463. We used different concentrations of WNK-463 on HT22 cells after sevoflurane treatment. From the MTS results (absorption was detected at 490 nm using a microplate reader), we found that with the increase in WNK-463 concentration, the protective effect was gradually enhanced and reached a peak at 1 µmol before it decreased. The maximum protective effect occurred at a concentration of 1 µmol, which was used in our subsequent experiments (P < 0.01, Fig. 1B).

WNK-463 (1 µmol) was used in normal HT22 cells (control cells) and sevoflurane-treated HT22 cells (Fig. 1C). We found that compared to normal HT22 cells, WNK-463 did not change the MTS in HT22 cells but alleviated the sevoflurane-induced reduction in cell activity. Sevoflurane obviously reduced HT22 cell activity, and 1 µmol WNK1 receptor antagonist showed a significant protective effect (P < 0.001, Fig. 1A).

3.2 WNK-463 decreased the HT22 cell apoptosis caused by sevoflurane

To investigate whether sevoflurane can induce apoptosis, the cells were exposed to sevoflurane (0% or 4.1%) for 6 h, and a TUNEL assay was used to measure cell apoptosis. In the field of the fluorescence microscope, compared with those of the control group (0% sevoflurane), the fluorescence intensity and
quantity of labelled cells treated with sevoflurane were obviously increased, and the number of apoptotic cells in the WNK-463 group was significantly reduced (Fig. 2A, B, C). The results showed that sevoflurane obviously increased the number of apoptotic cells, while WNK-463 significantly reduced the apoptosis caused by sevoflurane ($P < 0.0001$, Fig. 2D).

To further verify apoptosis, the level of cleaved caspase-3 was investigated in HT22 cells after treatment with sevoflurane using western blot analysis. The results indicated that cleaved caspase-3 protein was significantly upregulated in cells after sevoflurane treatment compared to the control cells. Moreover, caspase-3 activity was significantly reduced after WNK-463 treatment compared to the sevoflurane group ($P < 0.05$, Fig. 2F). These results suggested that sevoflurane induces apoptosis in HT22 cells, which was inhibited by WNK-463.

**3.3 WNK-463 inhibited the WNK1 kinase concentration increase caused by sevoflurane**

To explore the mechanism of apoptosis and the protective mechanism of the WNK1 inhibitor, the expression levels of WNK1 kinase proteins were investigated in HT22 cells after treatment with sevoflurane. The results indicated, compared with those in the control group, that the WNK1 kinase proteins in cells in the sevoflurane group were significantly upregulated ($P < 0.001$; Fig. 3). There was no significant difference between the WNK-463 group and the control group, which may be due to the low expression of WNK1 kinase under normal conditions. However, after exposure to sevoflurane, the WNK1 concentration was significantly increased, and this high expression could be inhibited by WNK-463. These results suggested that WNK1 kinase may play an important role in the neurotoxicity of sevoflurane.

**3.4 WNK-463 inhibited the NKCC1 concentration increase caused by sevoflurane**

To verify the role of WNK1/NKCC1 signaling pathway, the expression levels of NKCC1 proteins were investigated in HT22 cells after treatment with sevoflurane. The results indicated, compared with those in the control group, that the NKCC1 proteins in cells in the sevoflurane group were significantly upregulated after exposure to sevoflurane, and this high expression could be inhibited by WNK-463. ($P < 0.05$; Fig. 4). These results suggested that WNK1/NKCC1 signaling pathway may play an important role in the neurotoxicity of sevoflurane.

**3.5 WNK-463 reduced the calcium overload in HT22 cells after exposure to sevoflurane**

Calcium ion imaging technology (Fluo-4) was used to detect the basic values of calcium in the cells. The Fluo-4 images showed that the fluorescence intensity of the sevoflurane treatment group was significantly enhanced compared to that of the control group. However, compared with the sevoflurane group, the WNK-463 treatment group showed a weak fluorescence intensity differ from the control group.
(Fig. 5A, B, C), indicating that the WNK1 inhibitor reduced the calcium overload caused by sevoflurane. The results indicated, compared with those in the control group, that the calcium ion in cells in the sevoflurane group were significantly upregulated (P < 0.01; Fig. 3)

4. Discussion

Our study showed that the hippocampal neuronal HT22 cell had lower cell activity and higher apoptosis rate after exposure to sevoflurane. These changes are related to the overload of intracellular calcium and the high expression of WNK1 kinase. However, the WNK1 inhibitor WNK-463 could improve cell activity, decrease the apoptosis caused by sevoflurane, downregulate the expression of WNK1 and alleviate calcium overload. Therefore, we propose that the WNK1/NKCC1 signalling pathway may play a major role through intracellular calcium overload in sevoflurane-induced neurotoxicity, which may be a potential protective mechanism against sevoflurane-related neurotoxicity.

Most general anaesthetics are excitants of GABA$_A$R and inhibitors of the N-methyl-D-aspartate (NMDA) receptor. Currently, mechanisms have been shown, such as the hypothesis of neurotrophic apoptosis$^{[2, 3]}$, mitochondrial damage and accumulation of ROS$^{[23]}$, the effect of inflammatory factors$^{[16, 24]}$, the excitatory neurotoxicity hypothesis$^{[3]}$, the effects on the regeneration of neurons, changes in receptor subtypes$^{[18]}$, and changes in ion concentration$^{[19–25]}$. In our previous study, we found that sevoflurane can induce neuroapoptosis in neonatal rats and cause behavioural changes during development, and that GABA$_A$R played a role in sevoflurane's neurotoxicity in the developing brain$^{[18]}$. The toxicity of sevoflurane was demonstrated more intuitively at the cellular level. These results were similar to the findings of many studies$^{[19]}$.

Among all the neurotoxicity mechanisms of sevoflurane on the developing brain, GABA$_A$R excitatory toxicity is a very important aspect. GABA$_A$R is a ligand-gated chloride ion channel that mainly mediates inhibitory synaptic transmission. Recently, GABA$_A$R has been found to play an excitatory role in the immature brain and an inhibitory role in the adult brain$^{[15]}$. The conversion of GABA$_A$R depends on the concentration of chloride ions in cells at different stages of development ([Cl$^-$]). In the early stage of brain development in mammals and humans, chloride ions in immature neuron cells are in a high concentration state (15–20 mm). After the activation of GABA$_A$R, the channel opens and the Cl$^-$ outflow causes cell membrane depolarization and induces the action potential of neuron cells, showing the excitation effect$^{[15, 16]}$. This feature plays an important role in the process of neuron growth and synapse formation. Therefore, in the newborn brain, the depolarization of the GABA$_A$R current is a very important primary physiological and developmental neuroelectric loop that is activated. With the growth and maturation of neurons, the concentration of Cl$^-$ in neurons gradually decreases (~ 4 mM), and GABA$_A$ is transformed into an inhibitory neurotransmitter, which is used for the activation of GABA$_A$R. As a result, the internal flow of Cl$^-$ occurs, leading to the hyperpolarization of the cell membranes of neurons and inhibition of neurons.
In the present study, we found that WNK-463, an inhibitor of WNK1 kinase, may play a protective role in developing neurons. WNK1 kinase is a Cl\(^-\)-sensitive serine threonine protein kinase expressed in the central nervous system. Affected by intracellular chloride ions, this kinase can directly inhibit KCC2 activity and enhance NKCC1 activity\(^{[17]}\). Or postulated that SPS1-related proline/alanine-rich kinase (SPAK) or oxidative stress response protein (ORS-1) acts with KCC2 and NKCC1 and regulates nerve cell \([\text{Cl}^-]\)\(^{[25-27]}\). Friedel\(^{[28]}\) found that in the process of neuron development, GABA\(_A\)R is open, which transforms from the excitatory effect of immature neurons to the inhibitory effect of mature neurons, and an important guaranteed factor is that as KCC2 transports Cl\(^-\) synergistically out of cells, \([\text{Cl}^-]\) decreases. Our previous study demonstrated that the neurotoxic effect of sevoflurane may be related to the transformation of the GABA\(_A\) receptor \(\alpha\) from subtype 1 to subtype 2\(^{[18]}\). As an inhibitor of NKCC1, bumetanide can attenuate the increase in apoptosis induced by sevoflurane\(^{[18,29]}\). Stevens\(^{[19]}\) also found that the NKCC1 inhibitor alleviated the decline in the learning and memory function of newborn rats caused by ketamine. Therefore, we propose that as a cotransporter of NKCC1 and KCC2, WNK1 can affect the transformation of NKCC1-mediated Cl\(^-\) flow and affect the excitability of GABA\(_A\)R in the immature brain. In the present study, we found that sevoflurane caused a significant decrease in cell activity and an increase in apoptosis and cleaved caspase-3 expression in the hippocampal neuronal HT22 cells of neonatal rats. After the administration of the WNK1 inhibitor WNK-463, cell activity was improved, and apoptosis and the expression of cleaved caspase-3 were decreased. These results demonstrated that the WNK1 inhibitor was a protective factor against the neuroexcitatory toxicity of sevoflurane in developing neurons, which is consistent with our hypothesis.

We also found that sevoflurane exposure caused calcium overload in HT22 cells. In the early stage of neuronal development, the membrane receptors are stage-specific; the \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor is not yet fully functioning, and the activation of the NMDA receptor depends on the GABA\(_A\)R. The NMDA receptor has two characteristics: ligand-gate and voltage-gate. In the posterior synaptic membrane, the activation of GABA\(_A\)R leads to Cl\(^-\) outflow and excitatory post-synaptic potential (E\(_{\text{GABA}}\)). In addition, the calcium current generated by the NMDA receptor directly activates voltage-dependent Ca\(^{2+}\) channels (VDCCs), leading to increased intracellular Ca\(^{2+}\) and calcium overload. Calcium is an important regulator, and intracellular Ca\(^{2+}\) overload can lead to apoptosis through the activation of apoptosis-related enzymes, such as phospholipase, protease, and nucleic acid enzymes, as well as mitochondrial and cell membrane damage, and the generation of a large number of free radicals\(^{[30]}\). In the present study, the results of the Fluo-4 imaging of intracellular calcium showed a significant accumulation of Ca\(^{2+}\) in HT22 cells after treatment with sevoflurane. WNK1 inhibitor administration significantly decreased the calcium density, which demonstrated that the WNK1 inhibitor may influence NKCC1 and then GABA\(_A\)R excitability and intracellular Ca\(^{2+}\) overload in HT22 cells after sevoflurane exposure.

Therefore, exposure to sevoflurane on the hippocampal neuronal HT22 cell line induced a decrease in cell activity and an increase in apoptosis, increasing the expression of cleaved caspase-3 and intracellular
Ca\(^{2+}\) overload. WNK1 inhibitor (WNK-463) administration may improve HT22 cell activity and decrease apoptosis and intracellular Ca\(^{2+}\) overload. Therefore, WNK1 plays an important role in neurotoxicity of the sevoflurane in the developing brain. This discovery demonstrates that the WNK1/NKCC1 pathway may be a signalling pathway in sevoflurane’s neurotoxicity, and WNK1 is a promising new target for reducing the toxicity of sevoflurane. There is a lack of research in this field, which needs further study.

5. Conclusion

The results of this study indicate that sevoflurane can inhibit the proliferation of HT22 cells and induce cell apoptosis, while WNK1 kinase antagonist can reverses these changes and produces protective effects. The WNK1/NKCC1 signaling pathway plays an important role. Reducing intracellular calcium influx may be a change in the downstream of this signaling pathway.

List Of Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| GABA\(_A\) | y-aminobutyric acid type A receptor |
| NMDA | N-methyl-D-aspartate |
| WNKs | with-no-lysine |
| NKCC1 | sodium-potassium-chloride cotransporter-1 |
| KCC2 | potassium-chloride cotransporter-2 |
| DMEM | Dulbecco’s Modified Eagle’s Medium Gibco |
| SDS | Sodium dodecyl sulfate |
| Tris | Trishydroxymethylaminomethane |
| PAGE | Polyacrylamine electrophoresis gel |
| PVDF | polyvinylidene fluoride |
| BCA | Bicinchoninic acid |
| PBS | Phosphate buffered saline |

Declarations

Ethics approval and consent to participate
Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This work was supported by the Research Foundation of Beijing Friendship Hospital, Capital Medical University [grant numbers yyqdkt2018-23].

**Authors' contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ya-Fan Bai, Wen-Jing Li. The first draft of the manuscript was written by Ya-Fan Bai. Jun-Fa Li and an Li-Xin An directed the completion of the experiment and manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

I would like to thank the department of Neurobiology and Beijing Institute for Brain Disorders of the Capital Medical University for its strong support and the hard work of all the experimenters.

**References**

1. Rappaport BA, Suresh S, Hertz S, et al. Anesthetic neurotoxicity—clinical implications of animal models. N Engl J Med. 2015; doi:10.1056/NEJMp1414786.

2. Ikonomidou C, Bosch F, Miksa M, et al. Blockade of nmda receptors and apoptotic neurodegeneration in the developing brain. Science.1999; 283:70–4.

3. Zheng SQ, An LX, Chen X, Wang Y. Sevoflurane Causes Neuronal Apoptosis and Adaptability Changes of Neonatal Rats. Acta Anaesth Scand. 2013; doi:10.1111/aas.12163.

4. Han XD, Li M, Zhang XG, et al. Single sevoflurane exposure increases methyl-CpG island binding protein2 phosphorylation in the hippocampus of developingmice. Mol Med Rep. 2015; doi:10.3892/mmr.2014.2751.
5. Briner A, De Roo M, Dayer A, et al. Volatile anesthetics rapidly increase dendritic spine density in the rat medial prefrontal cortex during synaptogenesis. Anesthesiology. 2010; doi:10.1097/ALN.0b013e3181cd7942.

6. Andropoulos DB, Greene MF. Anesthesia and Developing Brains - Implications of the FDA Warning. N Engl J Med. 2017; doi:10.1056/NEJMp170019.

7. Xia Y, Xu H, Jia C, et al. Tanshinone IIA Attenuates Sevoflurane Neurotoxicity in Neonatal Mice. Anesth Analg. 2017; doi:10.1213/ANE.0000000000001942.

8. Ju X, Jang Y, Heo JY, et al. Anesthesia affects excitatory/inhibitory synapses during the critical synaptogenic period in the hippocampus of young mice: Importance of sex as a biological variable. Neurotoxicology. 2019; doi:10.1016/j.neuro.2018.11.014.

9. McCann ME, de Graaff JC, et al. Neurodevelopmental outcome at 5 years of age after general anaesthesia or awake- regional anaesthesia in infancy (GAS): an international, multicentre, randomised, controlled equivalence trial. Lancet. 2019; doi:10.1016/S0140-6736(18)32485-1.

10. Davidson AJ, Disma N, de Graaff JC, et al. Neurodevelopmental outcome at 2 years of age after general anaesthesia and awake-regional anaesthesia in infancy (GAS): an international multicentre, randomised controlled trial. Lancet. 2016; doi:1016/S0140-6736(15)00608-X.

11. Sun LS, Li G, Miller TL, et al. Association between a single general anesthesia exposure before age 36 months and neurocognitive outcomes in later childhood. JAMA. 2016; doi:10.1001/jama.2016.6967.

12. Gleich SJ, Flick R, Hu D, Zaccariello MJ. Neurodevelopment of children exposed to anesthesia: design of the Mayo Anesthesia Safety in Kids (MASK) study. Contemp Clin Trials. 2015; doi:1016/j.cct.2014.12.020.

13. Pontes A, Zhang Y, Hu W. Novel functions of GABA signaling in adult neurogenesis. Front Biol(Beijing). 2013; doi:10.1007/s11515-013-1270-2.

14. Kotani N, Akaike N. The effects of volatile anesthetics on synaptic and extrasynaptic GABA-induced neurotransmission. Brain Res Bull. 2013; doi:10.1016/j.brainresbull.2012.08.001.

15. Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist. 2012; doi:10.1177/1073858412438697.

16. Rivera C, Voipio J, Payne JA, et al. The K+/Cl− co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature. 1999; 397:251–255.

17. Friedel P, Kahle KT, Zhang J, et al. WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. Sci Signal. 2015; 30:ra65. doi:10.1126/scisignal.aaa0354.

18. Xie SN, Ye Hong, Li JF, An LX. Sevoflurane Neurotoxicity in Neonatal Rats is Related to an Increase in the GABAAR α1/ GABAAR α2 Ratio. J Neuro Res. 2017; doi:10.1002/jnr.24118.

19. Stevens RA, Butler BD, Kokane SS, et al. Neonatal inhibition of Na+/K+/2Cl− cotransporter prevents ketamine induced spatial learning and memory impairments. Neurotoxicol Teratol. 2017;
20. Li Y, Xia H, Chen L, Zhang X. Sevoflurane induces endoplasmic reticulum stress mediated apoptosis in mouse hippocampal neuronal HT22 cells via modulating miR-15b-5p/Rab1A signaling pathway. Int J Clin Exp Pathol. 2017; doi:1936-2625/IJCEP0057007.

21. Zhang L, Zhang J, Yang L, Dong Y. Isoflurane and sevoflurane increase interleukin-6 levels through the nuclear factor-kappa B pathway in neuroglioma cells. Br J Anaesth. 2013; 110Suppl 1:82-91. doi:1093/bja/aet115.

22. Liu Q, Wang G, Chen Y, et al. A miR-590/ACVR2a/Rad51B axis regulates DNA damage repair during mES cell proliferation. Stem Cell Reports. 2014; doi:1016/j.stemcr.2014.10.006.

23. Bai X, Yan Y, Canfield S, et al. Ketamine enhances human neural stem cell proliferation and induces neuronal apoptosis via reactive oxygen species-mediated mitochondrial pathway. Anesth Analg. 2013; doi:10.1213/ANE.0b013e3182860fc9.

24. Cui Y, Ling-Shan G, Yi L, et al. Repeated administration of propofol upregulated the expression of c-Fos and cleaved-caspase-3 proteins in the developing mouse brain. Indian J Pharmacol. 2011; doi:10.4103/0253-7613.89819.

25. Clayton G, Owens G, Wolff J, Smith R. Ontogeny of cation Cl− cotransporter expression in rat neocortex. Dev Brain Res. 1998; 109:281–92.

26. Chamma I, Chevy Q, Poncer JC, et al. Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. Front Cell Neurosci. 2012; doi:10.3389/fncel.2012.00005.

27. Gagnon KB, England R, Delpire E. Volume sensitivity of cation-Cl− cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. Am J Physiol Cell Physio. 2006, l290:C134–C142. doi:10.1152/ajpcell.00037.2005

28. Friedel P, Kahle KT, Zhang J, et al. WNK1-regulated inhibitory phosphorylation of the KCC2 cotransporter maintains the depolarizing action of GABA in immature neurons. Sci Signal. 2015; 30:ra65. doi:10.1126/scisignal.aaa0354.

29. Heubl M, Zhang J, Pressey JC, et al. GABAA receptor dependent synaptic inhibition rapidly tunes KCC2 activity via the Cl−-sensitive WNK1 kinase. Nat Commun. 2017; doi:10.1038/s41467-017-01749-0.

30. Yang M, Wei H. Anesthetic neurotoxicity: Apoptosis and autophagic cell death mediated by calcium dysregulation. Neurotoxicol Teratol. 2017; doi:10.1016/j.ntt.2016.11.004.

Figures
WNK-463 alleviated sevoflurane-induced cell activity decreasing Figure 1. (A) The MTS used to measure cell viability in HT22 cells exposed to control or 4.1% Sev for 6 h (****P < 0.0001). (B) The apoptotic cells were detected using MTS after treatment with 4.1% Sev for 6 h at different concentrations of WNK-463; (C) The MTS was used to measure cell activity following treatment with 0% or 4.1% Sev for 6 h or added to 1µmol WNK-463 (The data represent the mean ± SD, n=6, ***P < 0.001).
Figure 2

WNK-463 decreased HT22 cell apoptosis caused by sevoflurane Figure 2. (A) TUNEL staining was performed on the Control HT22 cells; (B) TUNEL staining was performed on the HT22 cells exposed to 4.1% sevoflurane for 6 h; (C) Given WNK-463, TUNEL staining on the HT22 cells exposed to sevoflurane for 6 h; (D) Compared the apoptosis cell numbers among control, sevoflurane treatment and WNK-463+ sevoflurane treatment HT22 cells. Compared with the control group, sevoflurane increased the number of apoptosis cells. And WNK-463 decreased the apoptosis cells number compared with sevoflurane group (****P < 0.0001). (E) After exposure to sevoflurane, with or without WNK-463, the cleaved-caspase-3 and caspase-3 protein levels were measured using Western blot analysis. β-actin was used as an internal control; (F) Quantification of cleaved caspase-3 and caspase-3 expression at three replications. (The data represent the mean ± SD, n=6, *P < 0.05).
Figure 3

WNK-463 decreased HT22 cell apoptosis caused by sevoflurane Figure 2. (A) TUNEL staining was performed on the Control HT22 cells; (B) TUNEL staining was performed on the HT22 cells exposed to 4.1% sevoflurane for 6 h; (C) Given WNK-463, TUNEL staining on the HT22 cells exposed to sevoflurane for 6 h; (D) Compared the apoptosis cell numbers among control, sevoflurane treatment and WNK-463+ sevoflurane treatment HT22 cells. Compared with the control group, sevoflurane increased the number of apoptosis cells. And WNK-463 decreased the apoptosis cells number compared with sevoflurane group (****P < 0.0001). (E) After exposure to sevoflurane, with or without WNK-463, the cleaved-caspase-3 and caspase-3 protein levels were measured using Western blot analysis. β-actin was used as an internal control; (F) Quantification of cleaved caspase-3 and caspase-3 expression at three replications. (The data represent the mean ± SD, n=6, *P < 0.05).
Figure 4

WNK-463 inhibited NKCC1 increasing caused by sevoflurane Figure 4. (A) The NKCC1 protein levels measured using western blot analysis. β-actin was used as an internal control. (B) Quantification of NKCC1 expression at four groups. The data represent the mean ± SD based on the gray value of the control group, standardized analysis was performed. (*P < 0.05).
Figure 5

WNK-463 reduced the calcium overload in HT22 cells after exposure to sevoflurane Figure 5. (A) The fluo-4 Calcium ion imaging technology was performed on the Control HT22 cells; (B) Exposed to 4.1% sevoflurane for 6 h; (C) Given WNK-463, on the HT22 cells exposed to sevoflurane for 6 h; (D) Quantification of calcium ion imaging at three groups. The data represent the mean ± SD based on the gray value of the control group, standardized analysis was performed. (**P < 0.01)