Calpain-TRPC6 Signaling Pathway Contributes to Propofol-Induced Developmental Neurotoxicity in Rats

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Research Article

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Abstract

Background

Compelling experimental evidence suggests a risk of neuronal damage following early childhood exposure to anesthesia and sedation drugs, including propofol. We investigated whether the transient receptor potential canonical 6 (TRPC6) channel could protect neonatal rats against developmental neurotoxicity following prolonged exposure to propofol. The potential role of calpain, a neuronal TRPC6 protease, was also investigated.

Methods

Postnatal day 7 rats were exposed to five bolus injections of 25 mg/kg propofol or 10% intralipid at hourly intervals. Acute neuronal injury was assessed by the expression pattern of terminal deoxynucleotidyl transferase nick-end labeling staining and cleaved-caspase-3 in the prefrontal cortex. The Morris water maze test was used to evaluate learning and memory functions in later life. Pretreatments consisting of intracerebroventricular injections of a TRPC6 agonist, TRPC6 inhibitor, or calpain inhibitor were used to confirm the potential role of a calpain-TRPC6 pathway.

Results

Prolonged exposure to propofol induced acute neuronal injury, downregulation of TRPC6, and enhancement of calpain activity in the prefrontal cortex up to 24 h after anesthesia. It also induced later behavioral disorders, manifesting as longer escape latency and as fewer platform-crossing times and less time spent in the target quadrant during postnatal days 35–42. These propofol-induced effects were attenuated by TRPC6 agonist and calpain inhibitor while exaggerated by TRPC6 inhibitor. Treatment with calpain inhibitor also attenuated the propofol-induced TRPC6 downregulation in the prefrontal cortex.

Conclusions

A calpain-TRPC6 signaling pathway contributes to propofol-induced acute neuronal injury and long-term behavioral disorders in neonatal rats.

Introduction

Compelling evidence from animal studies have revealed that most general anesthetics link to acute widespread neuronal cell death in the developing brain followed by learning and memory abnormalities later in life. The very rapid developmental changes of young brains make them particularly vulnerable to neurotoxicity from anesthesia [1, 2]. In human, the period of brain rapid developmental changes ranging from about the third trimester of pregnancy to the third year after birth, which corresponds to postnatal

days (PND) 1–10 in rats [3, 4]. Propofol anesthesia administered to this period of neonatal rats, either in a single large dose, or in multiple small doses, could induce significant nerve cell death in the brain and subsequent cognitive dysfunction [5–12]. Although propofol is one of the most common used anesthetic drugs in current pediatric anesthesia and intensive care practice [13], the cellular mechanisms and signaling pathways that underlie propofol-induced developmental neurotoxicity remain elusive.

Transient receptor potential canonical 6 (TRPC6) channel, a member of the transient receptor potential superfamily of non-selective cation channels abundantly expressed in most regions of mammalian brain, such as cerebellum, hippocampus, and cortex [14, 15], is emerging as a putative target for the prevention of neuronal damage in a wide range of brain diseases, such as Alzheimer's disease, cerebral ischemia, and traumatic brain injuries [16–20]. TRPC6 is known to be proteolyzed by activation of calpain and a calpain-TRPC6 signaling pathway has been deeply implicated in promoting neuronal survival against ischemic stroke [15, 16, 18, 21]. There is also evidence that the enhanced calpain-dependent signaling pathways after prolonged propofol administration is considered to be a potential mechanism underlying neuronal cell death in PND 7 rats [22]; however, whether these observations can translate to lasting effects on behavioral and cognitive development is not known. The hypothesis tested in the present study is that a calpain-TRPC6 signaling pathway participates in neuro-developmental deficits induced by prolonged exposure of propofol and may serve as a pharmacological target for the intervention of developmental neurotoxicity from anesthesia.

Materials And Methods

Animals

All experiments were performed in accordance with approved institutional animal care guidelines. PND7 Sprague Dawley rats (12–16g), half male and half female, were used in all experiments. The pups were housed in polypropylene cages, and the room temperature was maintained at 22°C with 12h light–dark cycle. Animals not intended to be killed immediately after anesthesia were allowed to recover in the incubator for 1h and were returned to their mothers to feed.

Experimental protocol

Three experiments were performed (Fig. 1). In experiment one, pups received five consecutive intraperitoneal injections of propofol (AstraZeneca, London, UK) or the vehicle control 10% intralipid (Fresenius Kabi AB, Uppsala, Sweden), at a rate of one injection per hour with a 0.5-mL tuberculin syringe and a 30-gauge needle. Our pilot study found that the sedation time (loss of the righting reflex) produced by a single injection of 25 mg/kg propofol was 51 ± 7 min. We thus chose a total of five consecutive intraperitoneal injections of propofol at 60 min intervals for a 5-h anesthesia. Control animals received five intraperitoneal injections of intralipid with the same volume, speed, and intervals as the injection of propofol. During the anesthesia, the animals were kept in a neonatal incubator using a heating pad (Harvard Apparatus, Holliston, Massachusetts, USA) to maintain stable body temperatures at 37.0–38.0 °C and were provided with 2L/min oxygen to reduce potential low oxygen stress after the loss of righting

reflex. Arterial oxygen saturation, heart rate, and breath rate were continuously monitored by a Pulse Oximeter (Harvard Apparatus, Holliston, Massachusetts, USA) attached to the abdomen. Some animals were then decapitated and sampled either immediately after the infusion (designated as the 0 h time point), or after the recovery periods that lasted 4, 12, or 24 h following the termination of infusion (designated as the 4, 12, and 24 h time points, respectively). Another set of animals received open-field test at PND 25 and MWM tests during PND 35–42.

A separate group of pups ($n = 10$) was used to rule out the possibility that physiological stress may have induced neuronal injury. During propofol infusion, the arterial oxygen saturation, heart rate, and breath rate were monitored by using the Pulse Oximeter (Harvard Apparatus, Holliston, Massachusetts, USA). However, it was not feasible to apply the Pulse Oximeter to the control pups received the infusion of intralipid. Fortunately, we found that the control group pups were tolerant of the 5-h infusion with minimum abnormal behavior. At the immediate termination of the 5th injection of propofol or intralipid, the pups were sacrificed using decapitation and sampled arterial blood from left ventricle using a 32-gauge hypodermic needle, and then analyzed arterial blood gases immediately for partial pressure of O_2 (PaO_2), saturation of O_2 (SO_2), partial pressure of CO_2 ($PaCO_2$), HCO_3^- , Na^+ , K^+ , Ca^{2+} , blood glucose, and pH values (iStat analyzer, Abaxis, Union City, CA, USA).

In experiment two, pups were pretreated with agonist and antagonist of TRPC6 using right intracerebroventricular (ICV) drugs injection 30 min before the 5-h infusion. The animals were randomly divided into 5 groups: intralipid + ICV injection of vehicle (Veh + veh), propofol + ICV injection of vehicle (P + veh), propofol + ICV injection of hyperforin 5 μ M (P + H5), propofol + ICV injection of hyperforin 10 μ M (P + H10), and propofol + ICV injection of SKF96365 20 μ M (P + SKF). The ICV injection of vehicle was 0.01% dimethyl sulfoxide. Right ICV pretreatments were performed under sevoflurane anesthesia 30 min before the first intraperitoneal injection of propofol or intralipid. Before surgery, the pups were briefly put into a transparent anesthesia box with 8% sevoflurane for 2 min. Depth of anesthesia the tail was confirmed by gently pinching. The anesthetized pups were then placed into a stereotaxic frame where the head was secured in a flexed-forward position. The injection was performed in right ventricle and was 2 mm rostral to bregma, 1.5 mm lateral, and 2 mm deep to the skull surface [23]. The accuracy of ICV injection was verified by methylene blue in our preliminary experiments. A small volume of fluid (2.5 μ L) was injected at a constant rate of 2.5 μ L/min using a Hamilton syringe with a 27-gauge needle. Some animals were sacrificed 4 hours after the end of the 5-h infusion. The remaining animals received behavior tests as above-mentioned in experiment one.

In experiment three, pups were pretreated with agonist and antagonist of TRPC6 using right intracerebroventricular (ICV) injection of calpain specific inhibitor calpeptin or vehicle 30 min before the 5-h infusion. The animals were randomly divided into 3 groups: intralipid + ICV injection of vehicle (Veh + veh), propofol + ICV injection of vehicle (P + veh), and propofol + ICV injection of calpeptin 20 μ M (P + CA). Then some animals were sacrificed and sampled 4 hours after the end of the 5-h infusion and the others received behavior tests as above-mentioned.

Behavior Tests

Open-field test

An open field was used to detect the general locomotor activity. PND25 rats were first placed in the center of a 100×100 cm box. After a 5 min habituation to the box environment, the locomotor activity was recorded by a video camera for a total of 5 min. DigBehv software was used to analyze the total distance traveled while the rat was in the box. Activity was defined as the total traveling distance.

MWM test

The MWM test was used to measure the spatial learning and memory of the rats, and includes a place navigation test and space exploration test. Briefly, PND 35 rats of each group were trained in a 100 cm diameter pool (60 cm high) for 7 consecutive days. Stationary extra-maze cues are visible around the 2.5 × 2.5 diameter room. Swim path was recorded via a video tracking system (Ethovision XT, Noldus, Netherlands). Each training trial to find the hidden platform (10 cm diameter/clear plastic) consisted of four randomized placements in the pool. Rats were allowed a maximum of 60 sec to find the submerged platform and given a 1 min rest between trials. In the spatial probe test, the platform was removed, and the rats were allowed to swim for 60 sec before they were removed. The animals were released in the water in a location that was exactly opposite from where the platform was placed. Behavior was recorded with a video tracking system. The escape latency and swimming speed were used to assess the acquisition of the water maze. Duration in the target quadrant was used to assess whether the rats remembered the location of the platform. The escape latencies, the swimming speed, times across the platform and percentage time spent in the target quadrant were recorded for subsequent analysis. All experiments were conducted between 8:00 and 19:00 in 22°C.

All behavioral tests were conducted by a researcher who was blinded to the treatment conditions.

Immunohistochemistry

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining analysis was used to detect apoptotic cell death right after the cessation of propofol exposure. The pups were anesthetized with sevoflurane, sacrificed 4 hours after the end of the 5-h infusion, and then perfused transcardially with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The prefrontal cortex was post-fixed overnight at 4°C until it was paraffin embedded, sectioned, and stained immunohistochemically for TUNEL. The TUNEL assay was performed with a commercial kit (Life Technology, Carlsbad, CA, USA). The number of cells stained positively for TUNEL and DAPI in the prefrontal cortex was analyzed using Image-pro Plus software by two persons blinded to the treatments. The densities of the cells positively stained with TUNEL and DAPI in the prefrontal cortex were calculated by dividing the number of positive cells by the area size of that region. At least six brain tissue sections were chosen for analysis from each animal and the average value from these sections was taken as final value of this animal.

Immunoblotting

Brain tissues from the prefrontal cortex were dissected and frozen in dry ice. Equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween20 for 2 h. Membranes were then incubated overnight at 4°C with α -spectrin/ α -fodrin (Abcam, Cambridge, MA, USA), TRPC6 and cleaved-caspase-3 (Cell signaling technology, Danvers, MA, USA), followed by horse radish peroxidase-conjugated IgG. Labeled proteins were detected with the chemiDocXRS+ chemiluminescence imaging system. Protein bands were quantified by persons blinded to the treatments using image lab image acquisition and analysis software.

Statistical analysis

Data were expressed as means $\pm$ standard deviation (SD) unless otherwise noted. Shapiro-Wilks test was used to determine normality. Alterations of expression of the mRNA and proteins detected, physiologic parameters and the behavioral responses to mechanical and thermal stimuli over time among groups were tested with one-way or two-way analysis of variance with repeated measures followed by multiple comparison tests using the Bonferroni method when statistical significance reached. Non-normally distributed data including time spent in the target quadrant and platform crossing times were analyzed by Mann-Whitney test or Kruskal-Wallis test. P value < 0.05 was considered to indicate statistical significance.

Results

Physiological homeostasis during a prolonged exposure of propofol

To investigate whether the prolonged exposure of anesthesia cause disturbance of physical homeostasis in the PND 7 rats, arterial oxygen, respiratory rate, body temperature, and heart rate were continuously monitored and recorded. No significant differences in these physiological parameters had been discovered between the groups during a 5-h exposure of propofol (data not shown). Artery blood was collected for blood gas analysis at the immediate end of the 5-h exposure and there is no statistically significant difference between the groups (Table 1). These findings suggested that the administered doses of propofol have no side effect on the physiological parameters in neonatal rats.

Table 1
Arterial blood physiological parameters between propofol exposure and intralipid control

Items	Control intralipid exposure rats	Propofol exposure rats
pH (mmol•L ⁻¹)	7.32±0.13	7.29±0.09
PO ₂ (mmHg)	97.40±1.84	96.10±1.91
SO ₂ (%)	98.16±2.72	96.22±3.53
PaCO ₂ (mmHg)	43.41±3.66	47.06±4.42
HCO ₃ (mmol•L ⁻¹)	27.30±3.30	29.45±3.58
Na ⁺ (mmol•L ⁻¹)	140.40±2.50	138.30±4.27
K ⁺ (mmol•L ⁻¹)	3.45±0.04	3.46±0.07
Ca ²⁺ (mmol•L ⁻¹)	1.38±0.03	1.36±0.06
Glucose (mg •dL ⁻¹)	9.24±0.09	9.31±0.13
Data are presented as mean ± SD (n=10). pH= arterial hydrogen ion concentration; SO ₂ = oxygen saturation; PaO ₂ = arterial oxygen partial pressure; PaCO ₂ = arterial carbon dioxide tension; HCO ₃ = bicarbonate radical. Propofol exposure did not affect arterial blood gas values and blood glucose levels significantly.		

Long-term behavioral disorder after a prolonged exposure of propofol

MWM was used for testing spatial learning and memory in the PND 35–42 rats. Propofol treated rats had significant longer escape latency when compared with intralipid treated rats during PND 39–41 (all $P < 0.01$, two-way repeated measure ANOVA with Bonferroni post-hoc test; Fig. 2A). A probe trial was then used 24 h after the last training of MWM to evaluate the reference memory. Propofol treatment decreased platform crossing times as well as time spent in the target quadrant when compared with placebo (both $P < 0.01$, Mann-Whitney test; Fig. 2C and D), indicating that propofol treated rats had long-term spatial learning and memory deficits. There is no statistically significant difference in the swimming speed between the groups (Fig. 2B). Overall locomotor activity functions were also evaluated using open field tests and there is no statistically significant difference between the groups as well (Fig. 2E).

Acute neuronal injury after a prolonged exposure of propofol

Several anesthetic drugs including propofol have been linked to significant cell death in neonatal rat cerebral cortex [5, 9, 22]. As shown in Fig. 3, significant increases in number of TUNEL–positive cells and cleaved-caspase-3 expression in the neonatal prefrontal cortex were induced at 0, 4, 12, and 24 h during the recovery from the propofol anesthesia (quantitative values of number of TUNEL–positive cells were 5.00, 7.56, 11.25, and 5.06–fold induction, and those on cleaved–caspase-3 expression were 1.49, 1.21,

1.78, and 2.03–fold induction, when compared with the vehicle, respectively; $P < 0.01-0.05$), indicating an acute neuronal injury induced by a prolonged exposure of propofol. We thus chose the timepoint of 4 h after propofol anesthesia in the subsequent experiments to manifest the high acute neuronal injury.

TRPC6 contributes to acute neuronal injury after a prolonged exposure of propofol

To explore the potential role of TRPC6 in propofol-induced neuronal injury, we first examined expression levels of this protein in the neonatal prefrontal cortex. Immunoblotting analysis and the following densitometry showed that the level of TRPC6 was down-regulated at 0, 4, 12, and 24 h after the propofol anesthesia (densitometric values were 0.74, 0.68, 0.5, and 0.86–fold decreases compared with the control treatment, respectively; all $P < 0.01$, Bonferroni post-hoc test; Fig. 4A). To understand the role of TRPC6 in propofol-induced neuronal injury, we pretreated the rat pups with ICV injection of either TRPC6 agonist (hyperforin) or inhibitor (SKF96365) before the prolonged exposure. As expected, pretreatment with ICV injection of 5 μ M and 10 μ M hyperforin attenuated the down-regulated expression of TRPC6, whereas pretreatment with ICV injection of 20 μ M SKF96365 exaggerated the down-regulation of this protein 4 h after the prolonged interventions (Fig. 4B). We then examined the pretreated effects on number of TUNEL-positive cells and cleaved-caspase-3 expression in the prefrontal cortex. With TRPC6 agonist, the number of TUNEL-positive cells and caspase-3 expression at the termination of propofol exposure were significantly reduced when compared with the propofol only treatment. In consistence, with TRPC6 antagonist, the number of TUNEL-positive cells and cleaved-caspase-3 expression were exaggerated as compared with the propofol only treatment (Fig. 4C-E). Taken together, these data indicate that propofol-induced acute neuronal damage involve a certain level of TRPC6 in the neonatal prefrontal cortex.

TRPC6 contributes to long-term behavioral disorder after a prolonged exposure of propofol

We next examined the effect of pharmacological manipulations of the TRPC6 on long-term spatial learning and memory deficits after the prolonged exposure of propofol. As expected, pretreatment with ICV injection of TRPC6 agonist rescued the propofol-induced increase in escape latency when compared with vehicle injection, and pretreatment with ICV injection of TRPC6 antagonist exaggerated the propofol-induced increase in escape latency (Fig. 5A). No significant changes in the swimming speed were observed among the ICV injected rats (Fig. 5B). The probe trial on PND 42 showed that pretreatment with hyperforin increased platform crossing times and time spent in the target quadrant when compared with vehicle treatment in the propofol-treated rats (Fig. 5C and D), indicating that TRPC6 contributes to the long-term spatial learning and memory deficits induced by prolonged propofol anesthesia. In open field tests, the rats given ICV injection of 5 μ M, 10 μ M hyperforin, or 20 μ M SKF96365 did not produce any significant differences in locomotor distance when compared with the rats pre-treated with vehicle (Fig. 5E), indicating that TRPC6 intervention may not affect overall locomotor activity level.

Calpain as upstream of TRPC6 to suppress acute neuronal injury after a prolonged exposure of propofol

To explore the potential involvement of calpain in propofol-induced cortical injury, we first examined calpain activity using expression levels of SBDP145, a calpain specific spectrin breakdown product [24].

As compared with the control group, propofol induced a significant increase in the expression of SBDP145 from 0 to 24 h after the prolonged infusion (1.68, 1.53, 1.35, and 1.2 -fold induction when compared with the control vehicle, respectively; Fig. 6A). With the pretreatment of ICV injection of calpeptin, a calpain specific inhibitor, the immediate induction of SBDP145 in the prefrontal cortex was markedly suppressed in comparison to propofol anesthesia only (Fig. 6B). Similarly, the propofol-induced down-regulation of TRPC6 expression was reversed in the presence of calpeptin pretreatment (Fig. 6C). Western blot analysis showed that calpeptin pretreatment also alleviated the propofol-induced increase in levels of cleaved-caspase-3 in the prefrontal cortex (Fig. 6D). The TUNEL assay showed that the propofol-induced increase in number of TUNEL-positive cells, as seen at the termination of propofol exposure, was also significantly attenuated in the presence of calpeptin pretreatment (Fig. 6E and F). Collectively, our data indicate that calpain acts on the upstream of TRPC6 to suppress acute neuronal injury after propofol anesthesia.

Discussion

Various studies done in developing rodents and primate models reported that neuroapoptosis plays an important role in the pathogenesis of neurodevelopmental deficits induced by prolonged exposure or multiple exposures of propofol [6–12, 22, 25]. The US Food and Drug Administration (FDA) has released a warning label about possible negative effects of general anesthetics including propofol on brain development in fetuses or children younger than 3 years [26]. Here we demonstrated that a 5-h prolonged exposure to propofol anesthesia is sufficient to induce neurodevelopmental deficits in neonatal rats. This is in accordance with the warning label mandated by FDA and confirms the developing brain is vulnerable to repeated or prolonged exposure of general anesthetics. In our study, propofol was administered in repeated injection of 25 mg/kg propofol in five boluses at 1-hour intervals, which mainly mimic prolonged exposure to the anesthetics in clinical practice, such as children who need prolonged general anesthesia or operations of long duration.

More importantly, we demonstrated for the first time that propofol-induced neurodevelopmental deficits involve a calpine-TRPC6 signaling pathway in the neonatal prefrontal cortex. The neonatal cortical neurons where we noted a potentiating effect of TRPC6 channel interventions on the neurodevelopmental deficits are known for functional expression of TRPC6 [24, 27]. TRPC6 has been demonstrated to be important for neuronal survival [16, 24], synapse formation [28], and neurite outgrowth [29] during neural development. Several studies found that an abnormal TRPC6 expression is related to neuronal injury induced by stroke and epilepsy [15–18]. In view of these findings, we focused on the role of TRPC6 in the prefrontal cortex injury after a prolonged exposure of propofol. Our results showed that pretreatment with TRPC6 agonist decreased the number of TUNEL-positive cells and cleaved-caspase-3 expression in the prefrontal cortical neurons up to 24 h after the exposure of propofol, and improved neurodevelopmental ability of the young rats during PND 35-42, whereas pretreatment with TRPC6 inhibitor exaggerated these propofol-induced effects. Overall locomotor activity was not impaired after the pharmacological manipulations of TRPC6. Combination of the previous findings with our results, it is reasonable to

conclude that maintaining a stable TRPC6 expression is critical for normal functions of the developing brain.

Interestingly, a recent study reported that TRPC6 knockdown inhibited isoflurane-induced toxic damage in SHSY5Y cells cultured with high glucose [30]. Please note that the study was performed in immortal neuroblastoma cells, and the relevance of the in vitro finding has not yet been reported. We inferred that potentially TRPC6 has both positive and negative roles in regulating neurotoxic damage induced by general anesthetics, depending on the diversity of stimuli and experimental models. That TRPC6 channel undergo remarkable pattern of changes during prenatal and postnatal stages of brain development [24, 31, 32], may explain, at least partially, the neurotoxic effects of general anesthetics predominant seen in young animals, but not in adult animals [25, 33].

We also found that pretreatment with calpeptin, a calpain specific inhibitor, counteracted the propofol-induced neuronal injury, neurobehavior outcome, and TRPC6 downregulation, demonstrating a calpain-TRPC6 signaling mechanism for the propofol-induced neurotoxicity. Activation of calpain can be reflected by the protein levels of all-spectrin breakdown products of 145kDa (SBDP145). SBDP145 is a specific calpain breakdown product of all-spectrin, and can contribute to apoptotic or necrotic cell death of neonatal neurons [34, 35]. The results of the present study showed the appearance of this breakdown product, calpain-specific SBDP145, was induced in prefrontal cortex up to 24 h after the exposure of propofol. Consistently, previous studies which used other models [34] also showed an accumulation of the SBDP145 in the neonatal cortex up to 24 h after cessation of anesthesia. In contrast, Milanovic et al. [22] reported that a transient increase in activation of calpain in the cortex of PND7 Wistar rats only at 4 h after 3 exposures of propofol (20 mg/kg). We could not exclude the possibility that smaller dosage and less repeated exposures of propofol contributed to discrepancy in activation of calpain over time. Nevertheless, the calpain cleavage products detected and the rescue effects of calpain inhibitor herein pointed to the proteolytic activity of calpain in neurotoxic responses. It has been reported that calpain cleaves of TRPC channels in many cell types including neuron [15, 36]. Inhibition of calpain activity has been shown to prevent TRPC6 degradation and promote neuronal survival in stroke [15, 16]. The present study thus confirmed that calpain-mediated TRPC6 channel proteolysis plays a critical role in neuronal survival in the developing brain.

Our study did not demonstrate the mechanisms by which propofol anesthesia exert neurotoxic effects on the activity of calpain. It has been reported that similar effects on calpain activation and apoptotic neuronal death could be induced by excitatory *N*-methyl-d-aspartate (NMDA) antagonists, γ -aminobutyric acid A receptor (GABAA) agonists, or ethanol, which combines mechanisms of both NMDA antagonist and GABAA agonist in the developing nervous system [37, 38]. Since ethanol and propofol both are known to activate GABAA receptors, can also act to block the NMDA receptors in the brain [38–40], a mechanism of combined both NMDA antagonist and GABAA agonist may thus be indicated. Our findings on the modulatory role of calpain-TRPC6 signaling pathway need to be further assessed in animals who survival longer.

In conclusion, to our knowledge, this is the first demonstration that a calpain-TRPC6 signaling pathway exists in propofol-induced neurotoxicity and cognitive deficits. This work suggests that new strategies acting through this signaling cascade may potentially ameliorate the developmental side effects of general anesthesia.

Declarations

Availability of data and materials: The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: All procedures were approved by Sun Yat-sen University Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: YJS, HPX, YG, and XR participated in and designed all of the experiment.; YJS, HPX, YG helped study conduct and data collection, YJS, QW, JZ, and XR analyzed the data and wrote the manuscript. YJS and XR supervised the experiments, analyzed the data, and wrote the manuscript. The authors approved the final manuscript and its post on a pre-print server at 02 Jul 2021, which can be accessed at <https://www.researchsquare.com/article/rs-658175/v1>.

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Figures

Figure 1

Protocol for experiments. P7 = Postnatal day 7, P35 = Postnatal day 35, P42 = Postnatal day 42.

Figure 2

Neonatal exposure to propofol causes long-term spatial memory and learning impairment. Rats were administered five bolus of 25 mg/kg propofol or 10% intralipid at postnatal day 7 and received Morris water maze test during postnatal days 35–42. A, Rats with the propofol anesthesia had longer escape latency compared with that in the rats with control intralipid. B, Average swimming speed. C, Times of the animal crossing the location of the removed platform. D, Time spent in target quadrant. E, Overall locomotor distance in open field test. Quantitative results are presented as means $\pm$ SD ($n = 10$), $**P < 0.01$. Veh = control intralipid; P = propofol.

Figure 3

Neonatal exposure to propofol increases number of TUNEL-positive cells and caspase-3 expression in the cerebral cortex. A and B, Representative photomicrographs and quantitation of TUNEL staining in the cortex at different timepoints after propofol exposure. C, Representative immunoblots and densitometric analysis of cleaved-caspase-3 in the cortex. β -actin was run as an internal standard for equal loading. Quantitative results are presented as means $\pm$ SD ($n = 6$), * $P < 0.05$ and ** $P < 0.01$. Vehicle = control intralipid; TUNEL = terminal deoxynucleotidyl transferase nick-end labeling.

Figure 4

TRPC6 contributes to caspase-3 expression and number of TUNEL-positive cells induced by neonatal exposure to propofol. A, Representative immunoblots and densitometric quantitation of TRPC6 in the right cortex after propofol exposure. B and C, Number of TUNEL-positive cells. D–F, Effects of pre-treatments with TRPC6 agonist and inhibitor on the levels of TRPC6 and cleaved-caspase-3 expression in the cortex 4 h after propofol anesthesia. β -actin was run as an internal standard for equal loading in immunoblots. Quantitative results are presented as means $\pm$ SD ($n = 6$), * $P < 0.05$ and ** $P < 0.01$. TUNEL = terminal deoxynucleotidyl transferase nick-end labeling; Veh + veh = intralipid + ICV injection of vehicle, P + veh = propofol + ICV injection of vehicle, P + H5 = propofol + ICV injection of hyperforin 5 μ M, P + H10 = propofol + ICV injection of hyperforin 10 μ M, and P + SKF = propofol + ICV injection of SKF96365 20 μ M.

Figure 5

TRPC6 contributes to long-term spatial memory and learning impairment induced by neonatal exposure to propofol. Neonatal rats were administrated with 5 h exposure of propofol or intralipid control after pretreatment with 5 μ M or 10 μ M hyperforin, or 20 μ M SKF96365, and then received Morris water maze (MWM) test and open field test. A, Pretreatment with 5 μ M and 10 μ M hyperforin attenuated the propofol-increased escape latency at PND 40 and 41, whereas rats pretreatment with 20 μ M SKF96365 enhanced the propofol-increased escape latency. B, Average swimming speed. C, Times of the animal crossing the location of the removed platform. D, The time spent in target quadrant. E, Over-all locomotor distance in open field test. Quantitative results are presented as means $\pm$ SD ($n = 10$), * $P < 0.05$ and ** $P < 0.01$. Veh + veh = intralipid + ICV injection of vehicle, P + veh = propofol + ICV injection of vehicle, P + H5 = propofol + ICV injection of hyperforin 5 μ M, P + H10 = propofol + ICV injection of hyperforin 10 μ M, and P + SKF = propofol + ICV injection of SKF96365 20 μ M.

Figure 6

Calpeptin counteracts calpain activity, TRPC6 expression, cleaved-caspase-3 expression, and number of TUNEL-positive cells induced by neonatal exposure to propofol. A, Representative immunoblots and densitometric quantitation of both α -II spectrin and its breakdown product SBDP145 for assessment of calpain activity in the cortex of PND7 rats at different timepoints after propofol exposure. B and C, Effect of calpeptin on calpain activity and TRPC6 expression right after propofol anesthesia. D, Effect of calpeptin on cleaved-caspase-3 expression and number of TUNEL-positive cells 4 h after propofol anesthesia. β -actin was run as an internal standard for equal loading in immunoblots. Results are presented as means $\pm$ SD ($n = 6$), * $P < 0.05$ and ** $P < 0.01$. Veh + veh = intralipid + ICV injection of vehicle, P + veh = propofol + ICV injection of vehicle, and P + CA = propofol + ICV injection of calpeptin 20 μ M.