

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

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Research

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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 TRPC6 channel could protect neonate rats against neuroapoptosis following prolonged exposure to propofol. The potential role of calpain, a neuronal TRPC6 protease, was also investigated.

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

Results: Prolonged exposure to propofol induced neuronal injury, downregulation of TRPC6, and enhancement of calpain activity in the cerebral cortex up to 24 h after anesthesia. It also induced long-term behavioral disorders, manifesting as longer escape latency at PND40 and PND41 and as fewer platform-crossing times and less time spent in the target quadrant at PND42. These propofol-induced effects were attenuated by treatment with the TRPC6 agonist and exaggerated by the TRPC6 inhibitor. Pretreatment with the calpain inhibitor alleviated the propofol-induced TRPC6 downregulation and neuronal injury in the cerebral cortex.

Conclusions: A calpain-TRPC6 signaling pathway contributes to propofol-induced acute cortical neuron injury and long-term behavioral disorders in rats.

Introduction

Compelling evidence has emerged from animal studies that most general anesthetics link to acute widespread neuronal cell death in the developing brain that leads to behavior, learning and memory abnormalities later in life. It has been suggested that 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 up to third year after birth, which corresponds to postnatal days (PND) 1–10 in rats [3, 4]. Propofol anesthesia administered to this period of rats, either as a single large dose, or in multiple doses, can 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 pediatric anesthesia and intensive care practice, and attained its discoverer, John B. Glen, the 2018 Lasker ~ DeBakey clinical medical research award [13], the cellular mechanisms and signaling pathways that underlie propofol-induced developmental neurotoxicity remain elusive.

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]. It has also been reported in recent years that activation of calpain leads to proteolysis of TRPC6, 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 the developmental brain [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 be a pharmacological target for the prevention and treatment of developmental neurotoxicity after general 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 groups and drug administration

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 rats 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 (LORR). Arterial oxygen saturation, heart rate, and breath rate were continuously monitored by a Pulse Oximeter (Harvard Apparatus, Holliston, Massachusetts, USA) attached to the abdomen. The rats were then decapitated 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). This schedule was chosen in order to assess a range of post-exposure effects.

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 O₂ (PaO₂), saturation of O₂ (SO₂), partial pressure of CO₂ (PaCO₂), HCO₃⁻, Na⁺, K⁺, Ca²⁺, blood glucose, and pH values (iStat analyzer, Abaxis, Union City, CA, USA).

The further experiments were performed to use right intracerebroventricular (ICV) drugs injection to assure the relationship between calpain-TRPC6 pathway and the degree of cortical neurons injury following propofol anesthesia. The rats were randomly divided into six group: intralipid group (Vehicle group), propofol group (Prop group), propofol + ICV injection of hyperforin 5μM (P + H5 group), propofol + ICV injection of hyperforin 10μM (P + H10 group), propofol + ICV injection of SKF96365 20μM (P + S group), and propofol + ICV injection of calpeptin 20 μM (P + C group). Right ICV injection was performed under sevoflurane anesthesia 30 min before the first intraperitoneal injection of propofol or intralipid. Before surgery, the pup was briefly anesthetized in a transparent anesthesia box with 8% sevoflurane. Depth of anesthesia by gently pinching the tail was confirmed. The animal was then placed into a stereotaxic frame where the head was secured in a flexed-forward position. A gas anesthesia mask was placed over the snout to continue the anesthesia at 3.5% sevoflurane and 50% oxygen. Sevoflurane concentration and the levels of oxygen and carbon dioxide were monitored through a sampling line connected to the anesthesia mask using a gas analyzer. 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 μL) was injected at a constant rate of 2.5 μL/min using a Hamilton syringe with a 27-gauge needle.

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.

Immunohistochemistry

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) staining analysis was used to detect cortical apoptotic cell death after the cessation of propofol exposure. The pups were anesthetized with sevoflurane and perfused transcardially with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Their brains were post-fixed overnight at 4°C until they were paraffin embedded, sectioned, and stained immunohistochemically for TUNEL. The TUNEL assays were performed with a commercial kit (Life Technology, Carlsbad, CA, USA). The TUNEL-positive nuclei with chromatin condensation and fragmented nuclei were considered to represent apoptosis. At least six tissue sections in the frontoparietal region 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 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 SEM unless otherwise noted. 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. Time spent in the target quadrant and platform crossing times among groups 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 propofol exposure 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 prolonged exposure of propofol (data not shown). Artery blood was collected for blood gas analysis at the immediate end of infusion and no statistical differences were found between the groups (Table 1). These findings suggested that the administered dose of propofol has no side effect on the physiological parameters in these neonate 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. 1A). No significant changes in the swimming speed were noted between the groups (Fig. 1B). 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. 1C and D), indicating that propofol treated rats had long-term spatial learning and memory deficits. We also evaluated the overall locomotor activity functions using open field tests and found that a 5-h exposure of propofol did not produce any significant differences in locomotor distance when compared with intralipid control (Fig. 1E).

Acute cortex injury after a prolonged exposure of propofol

Several anesthetic drugs including propofol have been linked to significant cell death in neonate rat cerebral cortex [5, 9, 22]. As shown in Fig. 2, significant increases in number of TUNEL-positive cells and cleaved-caspase-3 expression in the neonate cortex were induced at 0, 4, 12, and 24h during the recovery from the propofol anesthesia (quantitative values of number of TUNEL-positive cells were 7.00, 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; all $P < 0.01$), indicating an acute cortex injury induced by a prolonged exposure of propofol. We thus chose the timepoint of 4 h after anesthesia in the subsequent experiments to manifest the high acute cortex injury.

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

To explore the potential role of TRPC6 in propofol-induced cortical injury, we first examined expression levels of this protein in the neonate cerebral 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. 3A). To understand the role of TRPC6 in propofol-induced neurodegeneration, we pretreated the rat pups with ICV injection of either TRPC6 agonist (hyperforin) or inhibitor (SKF96365) before the prolonged interventions. As expected, pretreatment with ICV injection of 5 μM and 10 μM hyperforin attenuated the down-regulated expression of TRPC6, whereas pretreatment with ICV injection of 20 μM SKF96365 exaggerated the down-regulation of this protein 4 h after the prolonged interventions (Fig. 3B). We then examined the pretreated effects on number of TUNEL-positive cells and cleaved-caspase-3 expression in the cortex. With 5 μM and 10 μM hyperforin, 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 20 μM SKF96365, the number of TUNEL-positive cells and cleaved-caspase-3 expression were exaggerated as compared with the propofol only treatment (Fig. 3C-E). Taken together, these data indicate that propofol-induced acute cortex damage involve a certain level of TRPC6 in the neonate cerebral 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 prolonged propofol anesthesia. As expected, pretreatment with ICV injection of 5 μM and 10 μM hyperforin rescued the propofol-induced increase in escape latency when compared with vehicle injection, and pretreatment with ICV injection of 20 μM SKF96365 exaggerated the propofol-induced increase in escape latency (Fig. 4A). No significant changes in the swimming speed were observed among the ICV injected rats (Fig. 4B). 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. 4D and E), 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 μM , 10 μM hyperforin, or 20 μM SKF96365 did not produce any

significant differences in locomotor distance when compared with the rats pre-treated with vehicle (Fig. 4F), indicating that TRPC6 intervention may not affect overall locomotor activity level.

Calpain as upstream of TRPC6 to suppress acute cortical 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 h 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. 5A). With the pretreatment of ICV injection of calpeptin, a calpain specific inhibitor, the immediate induction of SBDP145 in the cortex was markedly suppressed in comparison to propofol anesthesia only (Fig. 5B). Similarly, the propofol-induced down-regulation of TRPC6 expression was reversed in the presence of calpeptin pretreatment (Fig. 5C). Western blot analysis showed that calpeptin pretreatment also alleviated the propofol-induced increase in levels of cleaved-caspase-3 in the cortex (Fig. 5D). 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. 5E and F). Collectively, our data indicate that calpain acts on the upstream of TRPC6 to suppress acute cortical 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 is sufficient to induce neurodevelopmental deficits in young 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 calpain-TRPC6 signaling pathway in the neonate rat cerebral cortex. The cortical neurons where we noted a potentiating effect of TRPC6 channel interventions on propofol-induced neurodegeneration are known for functional expression of TRPC6 in the early postnatal rat brain [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 cerebral cortex neuroapoptosis 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 neonate rat cerebral cortex up to 24 h after the exposure of propofol, and improved neurodevelopmental ability of the young rats in long-term, 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, it was demonstrated that TRPC6 knockdown inhibited isoflurane-induced toxic damage in SHSY5Y cells cultured with high glucose [30]. Please note that it was performed in an 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 TRPC6 downregulation, acute neuronal injury, and improved neurobehavior outcome, demonstrating a calpain-TRPC6 signaling mechanism for neurodevelopmental deficits induced by general anesthetics. Activation of calpain can be reflected by the protein levels of all-spectrin breakdown products of 145kDa (SBDP145). SBDP145 has been demonstrated to result from specific calpain proteolysis of all-spectrin, and can contribute to apoptotic or necrotic cell death of neurons [34, 35]. The results of the present study showed the appearance of breakdown products, calpain-specific SBDP145, was induced in the 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 rat 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 6 h after 3 exposures of propofol (20 mg/kg). We could not exclude the possibility that smaller dosage and less repeated exposures of propofol mostly contributed to discrepancy in activation of calpain over time. Nevertheless, the increased calpain cleavage products detected and the rescue effects of calpain inhibitor herein pointed to the proteolysis of TRPC6 and a potential role in neurotoxic responses. It has been reported that the activation of calpain causes proteolysis of TRPC channels in many cell types including neuron [15, 36], and calpain-mediated proteolysis of TRPC6 contributes to neuronal cell death, and inhibition of calpain activity prevents TRPC6 degradation and could promote neuronal survival in stroke [15, 16]. The present study 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 to activate or maintain stable expression level of both calpain and TRPC6 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 read and approved the final manuscript.

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Figures

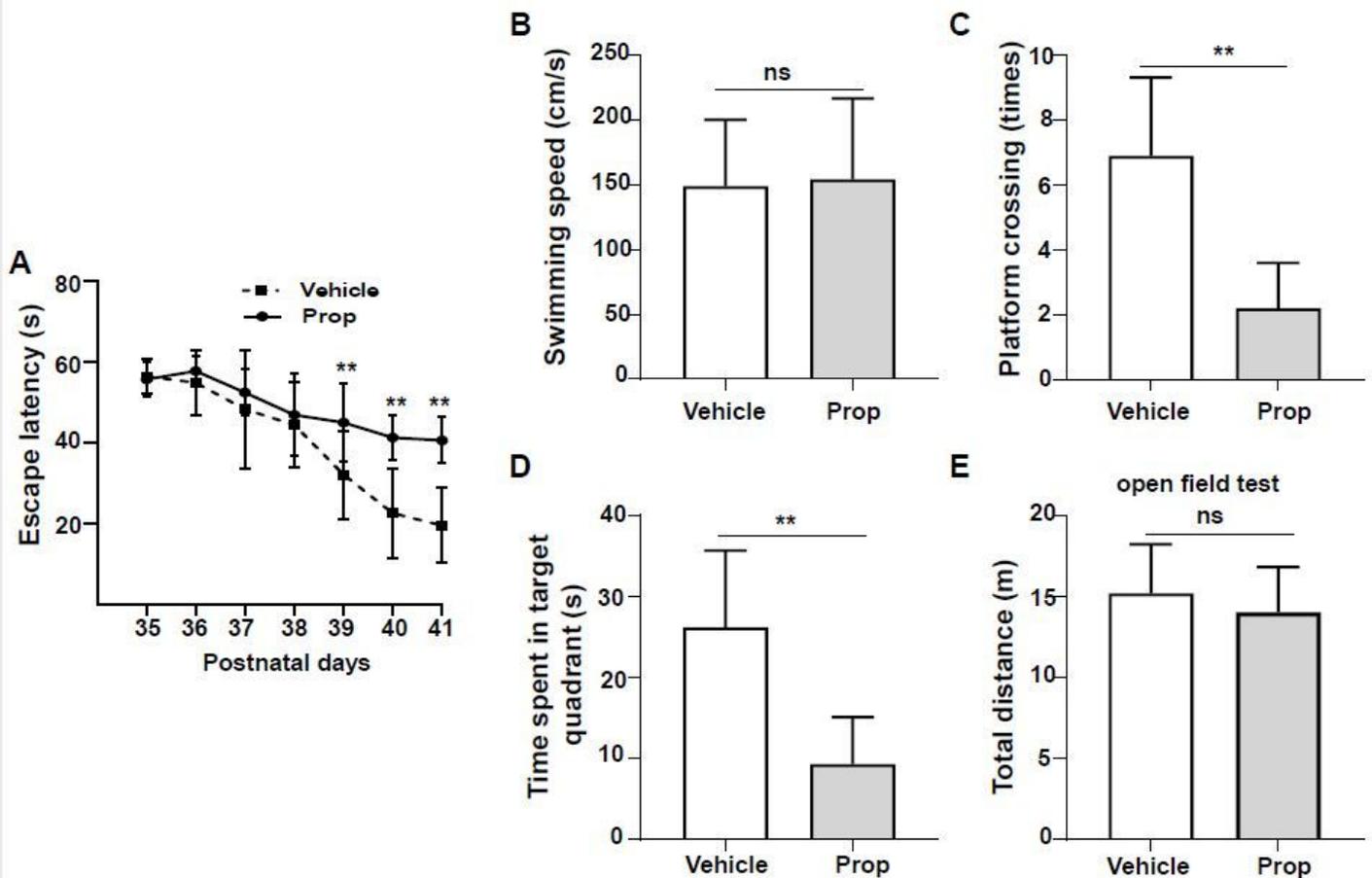


Figure 1

Neonatal exposure to propofol causes long-term spatial memory and learning impairment. Rats were administered five boluses 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 SEM (n = 10), **P < 0.01. Vehicle = control intralipid; Prop = propofol.

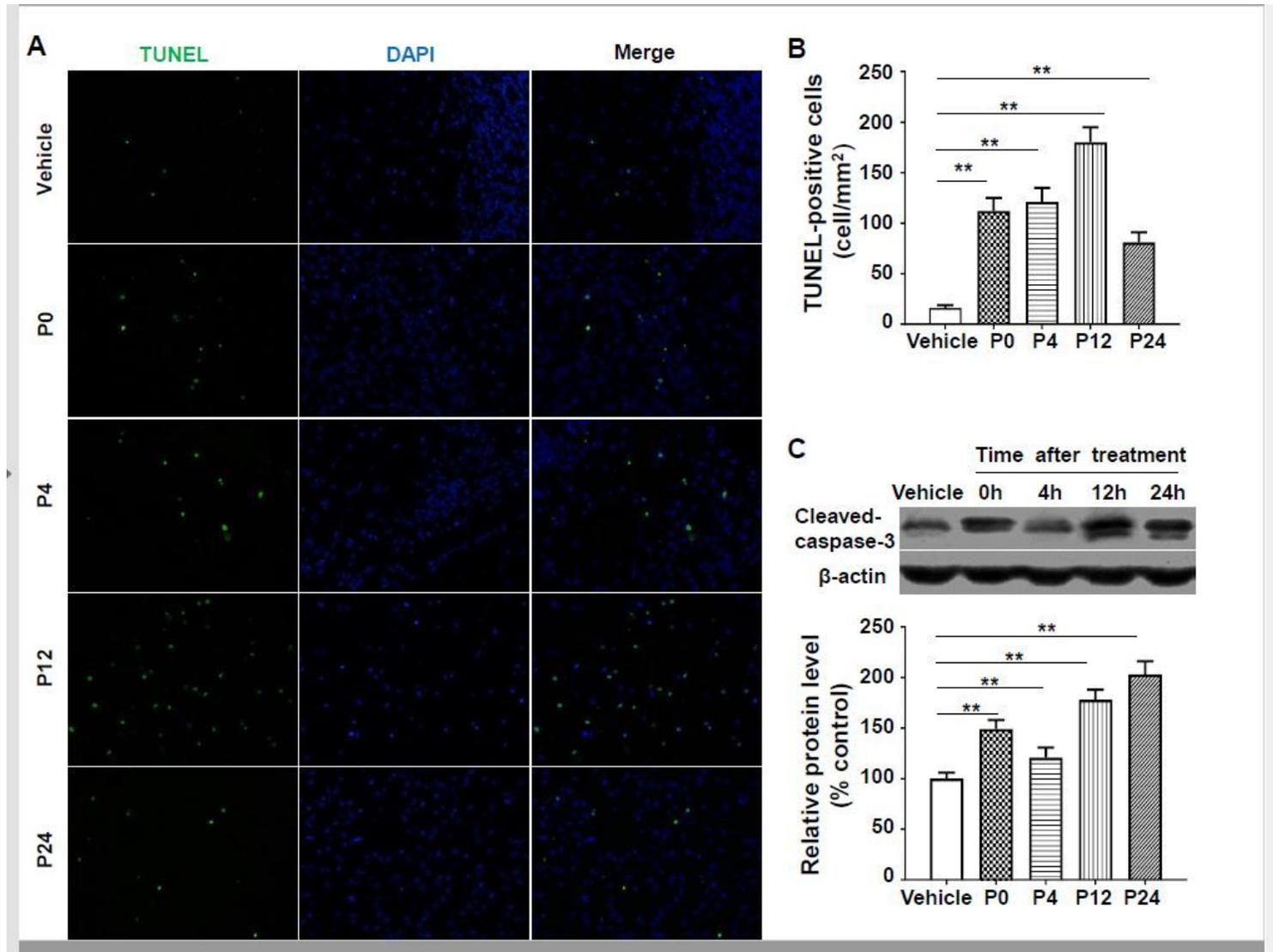


Figure 2

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 SEM (n = 6), **P < 0.01. Vehicle = control intralipid; PND = postnatal days; TUNEL = terminal deoxynucleotidyl transferase nick-end labeling; Vehicle = intralipid control; P0–24 = 0–24 h after propofol treatment.

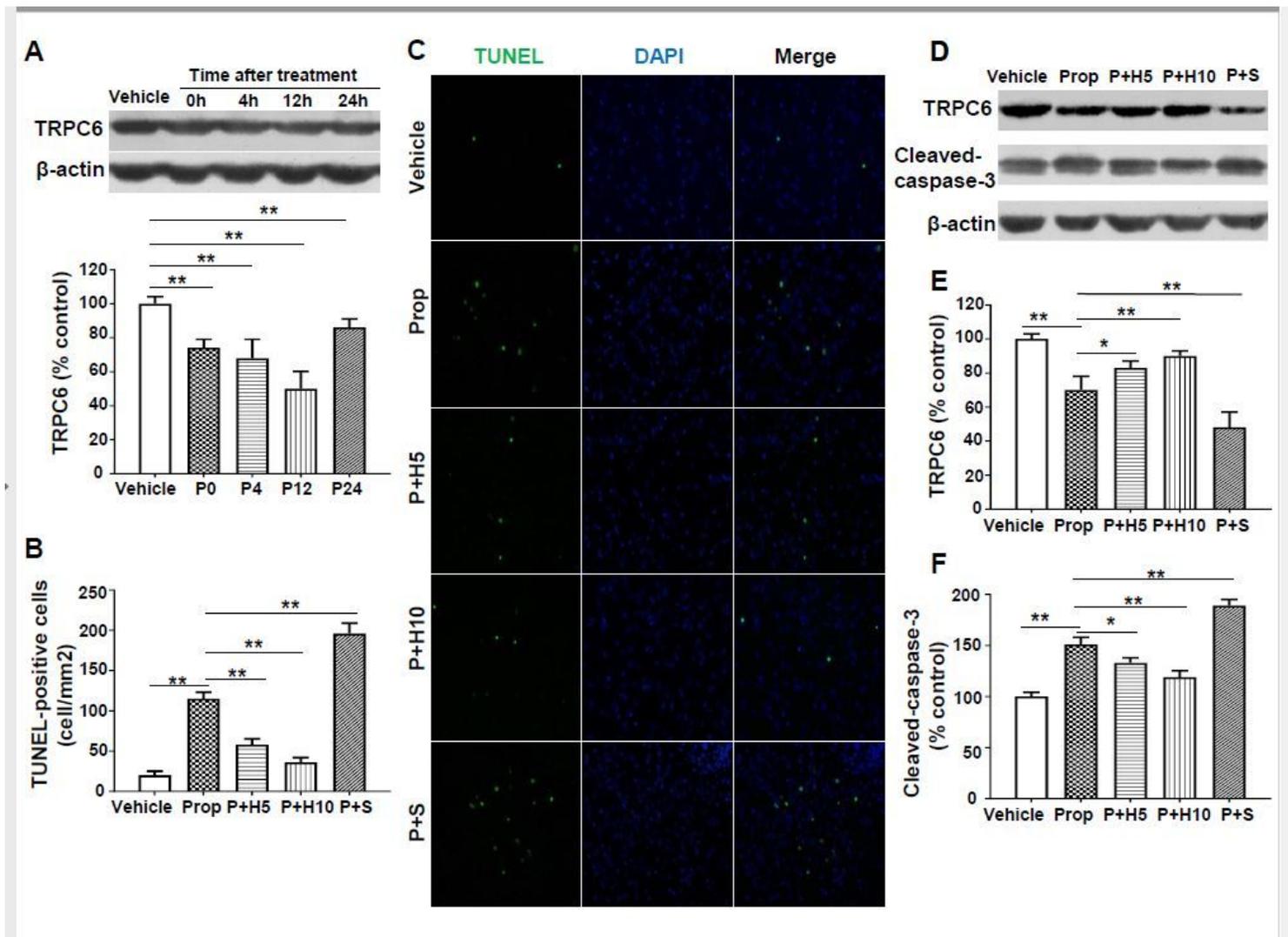


Figure 3

TRPC6 contributes to caspase-3 expression and number of TUNEL-positive cells induced by neonate 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 SEM ($n = 6$), * $P < 0.05$, ** $P < 0.01$. TUNEL = terminal deoxynucleotidyl transferase nick-end labeling; Vehicle = control intralipid; P0–24 = 0–24 h after propofol treatment, Prop = propofol; P+H5 = propofol + pretreatment with hyperforin 5 μ M; P+H10 = propofol + pretreatment with hyperforin 10 μ M; P+S=propofol + pretreatment with SKF96365 20 μ M.

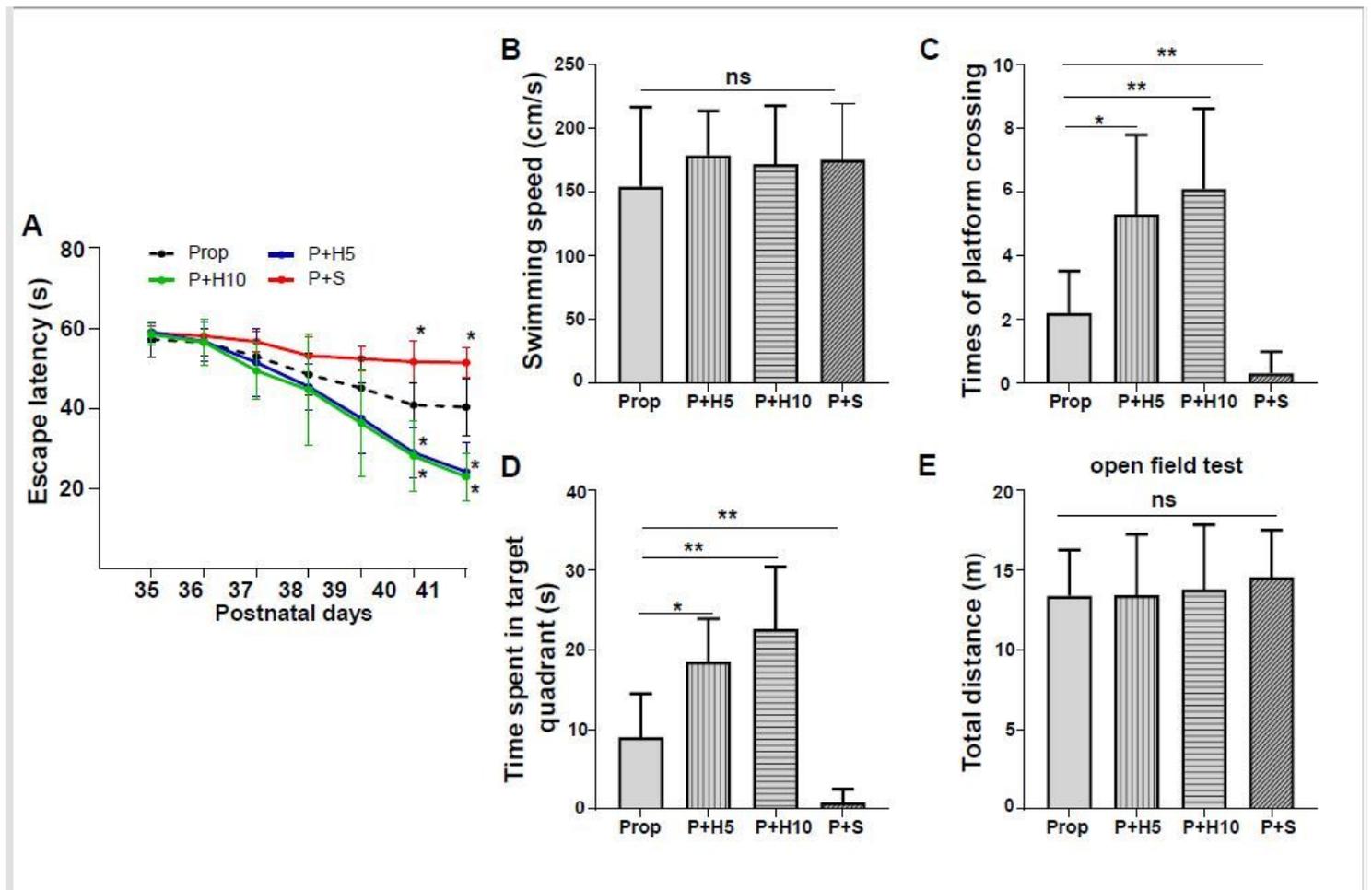


Figure 4

TRPC6 contributes to long-term spatial memory and learning impairment induced by neonate exposure to propofol. Neonate rats were administered 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 SEM (n = 10), *P < 0.05, **P < 0.01. Vehicle = control intralipid; Prop = propofol; P+H5 = propofol + pretreatment with hyperforin 5 μ M; P+H10 = propofol + pretreatment with hyperforin 10 μ M; P+S = propofol + pretreatment with SKF96365 20 μ M.

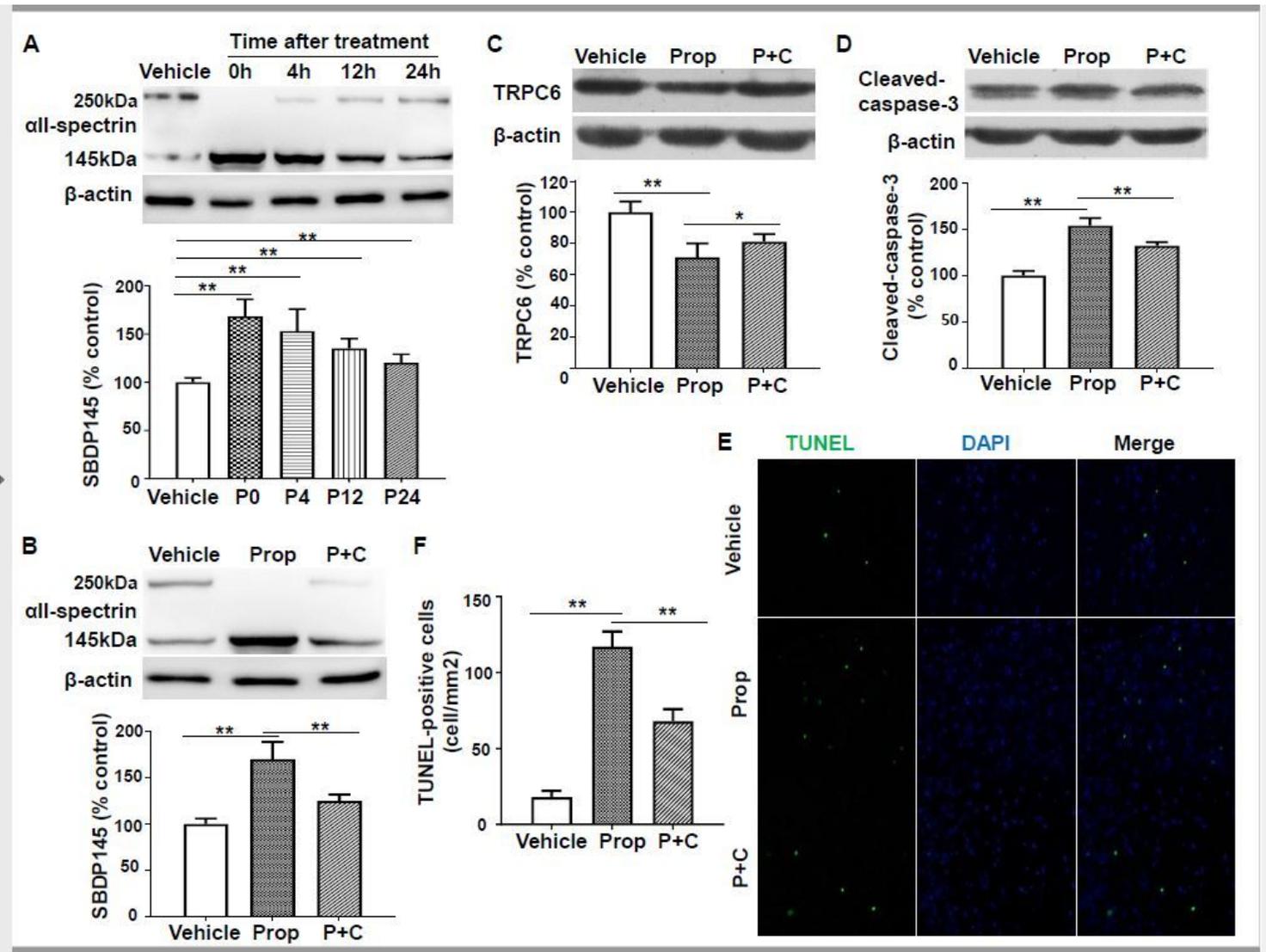


Figure 5

Calpeptin counteracts calpain activity, TRPC6 expression, cleaved-caspase-3 expression, and number of TUNEL-positive cells induced by neonate 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 SEM (n = 6), *P < 0.05, **P < 0.01. Vehicle = control intralipid, P0–24 = 0–24 h after propofol treatment. Prop = propofol, P+C = propofol + pretreatment with ICV injection of calpeptin 20 μ M.