Combination of isoflurane and propofol, a means for general anesthesia in the orthopedic surgery of perioperative cerebral hypoperfusion rats to avoid cognitive impairment Anesthesia of perioperative cerebral hypoperfusion

Xinyue Bu
Tianjin Third Central Hospital
Tang Li
Tianjin Third Central Hospital
Haiyun Wang (why819@126.com)
https://orcid.org/0000-0002-0084-2342
Zhengyuan Xia
University of Hong Kong
Di Guo
Tianjin Third Central Hospital
Jinxin Wang
Tianjin Third Central Hospital
Yi Sun
Tianjin Third Central Hospital
Chenyi Yang
Tianjin Third Central Hospital
Guoqiang Liu
Tianjin Third Central Hospital
Ji Ma
Tianjin Third Central Hospital
Zhuo Yang
Nankai University
Guolin Wang
Tianjin Medical University General Hospital

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Abstract

Background: Perioperative cerebral hypoperfusion often occurs. However, the underlying mechanism of cognitive impairment resulting when perioperative cerebral hypoperfusion occurs remain mostly to be determined. Anesthetic isoflurane induces neuronal injury via endoplasmic reticulum (ER) stress, whereas sub-anesthetic dose of propofol improves postoperative cognitive function. However, the effects of the combination of isoflurane plus propofol, which is a common combination of anesthesia for patient, on ER stress and the associated cognitive function remain unknown. Methods: We therefore set out to determine the effects of isoflurane plus propofol on the ER stress and cognitive function in the rats insulted by cerebral hypoperfusion. A ligation of bilateral common carotid arteries (CCA) surgery was adopted to prepare rats as cerebral hypoperfusion (CH) animal model. A second surgery, open reduction and internal fixation (ORIF), requiring general anesthesia, was operated 30 days later so that the effects of anesthetics on cognitive function of these CH rats could be assessed. The rats received isoflurane alone (1.9%), propofol alone (40 mg·kg -1 ·h -1) or a combination of isoflurane and propofol (1% and 20 mg·kg -1 ·h -1 or 1.4% and 10 mg·kg -1 ·h -1). Behavior studies (Fear Conditioning test), histological analyses (Nissl staining) and biochemical analyses (western blotting for the harvested rat brain tissues) were employed in the studies. Results: We found that the combination of 1% isoflurane plus 20 mg·kg -1 ·h -1 propol did not aggravate the cognitive impairment and the ER stress in aging rats with cerebral hypoperusion and being subjected to an ORIF surgery. Conclusions: These data suggest that ER stress contributes to the underlying mechanism of cognitive impairment and the combination of isoflurane and propofol did not aggravate the cognitive impairment and the ER stress in aging rats with cerebral hypoperfusion and being subjected to an ORIF surgery.

Background

Perioperative neurocognitive disorders (PND) have become the most common complications after routine surgical procedures, particularly in the elderly [1, 2]. Following surgery (e.g., common orthopedic procedures), up to 50% of patients experience cognitive disturbances that can lead to serious complications, including poorer prognosis and a higher 1-year mortality rate in subjects with pre-existing neurodegeneration [3]. Carotid artery stenosis (CAS) can be detected in 75% of men and 62% of women aged ≥65, with a stenosis extent of ≥50% occurring in 7% of men and 5% of women in this age group [4]. CAS is an independent risk factor for chronic cerebral hypoperfusion (CCH) [5], which reduces tissue oxygen levels leading to oxidative stress and endothelial injury [6]. In rodents, experimental CCH can be initiated by occlusion of the major arterial supply. This way CCH brings about mitochondrial dysfunction and protein synthesis inhibition. These effects may destroy the balance of anti-oxidases and reactive oxygen species (ROS) and produce oxidative damage. At the same time, oxidative injury to vascular endothelial cell, glia, and neuron impairs vascular function and neurovascular coupling, which may result in a vicious cycle of further reduction of cerebral perfusion [7]. Taking all these factors into account, aging patient with carotid stenosis preoperatively in orthopedics department is a population need to be

treated carefully. Special caution on the selection of anesthetic drugs is needed to protect cognitive function.

We and others [8-10] previously reported that the two commonly used anesthetics, isoflurane and propofol, at certain doses, have opposite effects on cognitive function. Isoflurane induces neuronal injury upon prolonged exposure to isoflurane at high dose [11], with an underlying mechanism linked to endoplasmic reticulum (ER) stress. By contrast, propofol at a sub-anesthetic dosage protects against neuronal damage of cerebral ischemia reperfusion injury, and such protective effects were not observed at a higher dose [12]. We, therefore, tested the effect of using sub-anesthetic dose of propofol to partially replace isoflurane (a combined usage of isoflurane and propofol) on cognitive function of rats with CH in the current studies. Previous studies showed that 1.9% isoflurane, equivalent to 1.3 minimum alveolar concentration (MAC), was sufficient to induce general anesthesia in rats [13], while to induce general anesthesia in rats using propofol alone, the minimal infusion rate at 40 $mg \cdot kg^{-1} \cdot h^{-1}$ was required [14]. Therefore, in our study, doses were carefully selected combining isoflurane and propofol (1% and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ or 1.4% and 10 $mg \cdot kg^{-1} \cdot h^{-1}$) to ensure the required depth of general anesthesia.

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter [15]. The major subtype of $GABA_A$ receptor ($GABA_AR$) contains α1 subunit. Based on previous study, the GABA receptors in the central nervous system are divided into three types: A, B, and C. Among them, $GABA_A$ receptor ($GABA_AR$) is the earliest and most widely distributed among the three receptors, mainly in the hippocampus, prefrontal cortex and striatum. Studies have confirmed that most GABAergic synaptic transmission in mammalian brain is mediated by $GABA_AR$. It is also an important target receptor for general anesthesia of central nervous system (CNS) mediators such as propofol and isoflurane. It consists of five subunits embedded in the cell membrane of neurons. At the center, a 0.5 mm diameter GABA-gated Cl^- channel is formed. When GABA binds to $GABA_AR$, the Cl^- channel of the postsynaptic membrane is open, and the Cl^- concentration-gradient enters the cell. The potential increases to produce hyperpolarization, which in turn causes neuronal inhibition [16, 17]. In 2014, Labrakakis et,al. confirmed that the post-synaptic membrane $GABA_AR$ subunit composition determines the heterogeneity of inhibitory postsynaptic potential (IPSP), namely $GABA_AR$ function [18]. The native $GABA_ARs$ present in mammalian brain are mainly composed of α, β and γ subunits. The most common configuration is transmembrane pentamer composed of $2\alpha_1 2\beta_2\gamma_2$, accounting for 43% of all $GABA_AR$ configurations, which is the most abundant in hippocampus and cerebral cortex [18]. The $GABA_AR$ α1 subunit is the most widely distributed in the mammalian brain, which is related to cognition [19, 20]. Its main function is to maintain the CNS arousal and maintain the sensitivity of the receptor to sedative hypnotics (propofol, isoflurane, etc.). Mutation of the M2 domain Ser270 and the M3 domain Ala291 in α1 subunit affects the potency of isoflurane and propofol on $GABA_ARs$ [21]. Kelley et,al. confirmed that cerebral ischemia can induce miniature inhibitory postsynaptic current (mIPSC) reduction and GABA-activated current inhibition [22]. Further studies found that mIPSC frequency and kinetic parameters did not change, only amplitude decreased, while oxygen-glucose deprivation (OGD) inhibited neuronal $GABA_AR$ α1 subunit expression [22]. It suggests that the change in

$GABA_AR$ activity is triggered by a decrease in the expression of its functional subunit α1. Furthermore, our previous study showed that subanesthetic dose (20 $mg \cdot kg^{-1} \cdot h^{-1}$) propofol exerts post-treatment brain protection by activating the KCC2-$GABA_AR$ pathway. Propofol post-treatment can reverse the decrease of hippocampal IPSCs after OGD injury, promote KCC2 expression, and maintain the normal function of $GABA_AR$. However, administration of KCC2 antagonists only partially reversed the effect of propofol on mIPSC [23]. Whether cerebral ischemia triggers the expression change and structural regulation of $GABA_AR$ functional subunit protein or not? Is there any other upstream mechanism other than KCC2 to regulate the $GABA_AR$ structure, thereby affecting its function? To explain these issues, we chose $GABA_AR$ α1 subunit as one of the parameters in this article.

$GABA_AR$ undergoes post-synthesis modification and folding in the ER. Prolonged ER stress has been well known related with neurodegenerative diseases [24, 25]. The unfolded protein response (UPR) triggered by ER stress is an important quality control system for maintaining protein homeostasis (Proteostasis). Proteostasis refers to an equilibrium state of specific protein synthesis, folding and unfolding, modification and degradation in the intracellular proteome at a specific time point. The ER of the cell is a site for the folding and post-translational processing of secreted proteins and membrane proteins (about 1/3 of the human proteome). Binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 (GRP78), is an ER chaperone protein whose expression is part of the UPR, is required to alleviate ER stress [26]. Once ER stress occurs, Bip binds to unfolded proteins and activates the downstream receptor proteins, increasing molecular chaperone expression, reducing global protein translation, and increasing unfolded/misfolded proteins. It degrades and reduces ER stress and protects cells through endoplasmic reticulum-associated degradation (ERAD).

The expression of C/EBP homologous protein (CHOP), a transcriptional factor, is also induced by ER stress but indirectly regulates apoptosis [27]. During a stress situation, UPR attempts to increase protein-folding capacity and remove misfolded and unfolded proteins. If the remedy is inadequate to restore homeostasis under chronic ER stress, terminal UPR will trigger apoptosis through abundant signaling mechanisms, mainly mediated by CHOP, c-Jun N-terminal kinase (JNK), and caspase-12, with CHOP as the most widely studied [28].

Thus, the expression of BiP, CHOP and $GABA_AR$ α1 subunit was used to evaluate cellular mechanisms accounting for neural substrate conditions allowing normal cognitive functions in this study.

The objective of the current studies is to explore ways of general anesthesia for rats with CH and being subjected to an ORIF surgery to protect cognitive function. We tested a hypothesis that combination of isoflurane and propofol is better to protect cognitive function than single usage of isoflurane or propofol during ORIF surgery by using behavior studies and biochemical analyses.

Results

Combination treatment with 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol protected cognitive function in aging rats with CH and being subjected to an ORIF surgery.

To observe the effects of different dosages of isoflurane and propofol on cognitive function, a contextual fear conditioning test was performed on the first and seventh days after ORIF. The percentage of freezing time in Group C and Group IP_1 was not significantly different on both the first day (C vs IP_1: 44.23 ± 6.60 vs 42.86 ± 7.12, P = 1.00) and seventh day (C vs IP_1: 35.70 ± 5.21 vs 34.85 ± 5.02, P = 1.000) after ORIF (Fig. 1A). However, in Group IP_2, I and P, the percentage of freezing time was significantly reduced compared to Group C on Day 1 (C vs IP_2: 44.23 ± 6.60 vs 31.55 ± 5.68; C vs I: 44.23 ± 6.60 vs 22.86 ± 3.53; C vs I: 44.23 ± 6.60 vs 21.32 ± 3.42; all P < 0.05) and Day 7 (C vs IP_2: 35.70 ± 5.21 vs 28.48 ± 2.54; C vs I: 35.70 ± 5.21 vs 21.34 ± 2.12; C vs I: 35.70 ± 5.21 vs 22.16 ± 2.74; all P < 0.05) (Fig. 1A). The results suggest that combination of 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol could protect cognitive function while other dosages could not.

Treatments with isoflurane or propofol alone were not able to prevent CA1 neuronal death in aging rats with cerebral hypoperfusion and being subjected to an ORIF surgery.

Hippocampal slices were stained with cresyl violet (Nissl-staining method) to investigate potential neuronal damage caused by anesthetics on days 1 and 7 after ORIF. Compared with group C, the number of survival neurons decreased one day after ORIF only in Group I (C vs I: 193.13 ± 23.94 vs 150.88 ± 20.19, P = 0.039, Fig. 1 B-C). On the seventh day after ORIF, the number of survival neurons in groups I and P was significantly reduced compared to that in group C (C vs I: 187.38 ± 19.86 vs 146.75 ± 16.70, P= 0.008; C vs P: 187.38 ± 19.86 vs 148.13 ± 18.39, P = 0.011). No significant change was found in Group IP_1 and IP_2 on both Day 1 (C vs IP_1: 193.13 ± 23.94 vs 179.75 ± 26.60, P= 0.923; C vs IP_2: 193.13 ± 23.94 vs 175.75 ± 35.94, P= 0.799) and Day 7 (C vs IP_1: 187.38 ± 19.86 vs 179.13 ± 19.96, P= 0.975; C vs IP_2: 187.38 ± 19.86 vs 177.25 ± 26.02, P= 0.940). (Fig. 1 B-C)

Combination treatment with 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol maintained the expression level of cell $GABA_AR$ α1 in the hippocampus

As mentioned above, $GABA_AR$ α1 is a key functional component of the neural substrate involved in cognitive functions. Therefore, western blotting was performed on the first and seventh days after ORIF to evaluate the expression of $GABA_AR$ α1 subunit. There was no difference in the expression of $GABA_AR$ α1 between group C and group IP_1 on day 1 (C vs IP_1: 100.00 ± 18.48 vs 91.86 ± 15.45, P = 0.629) or day 7 (C vs IP_1: 100.00 ± 14.72 vs 112.39 ± 20.17, P = 0.261) after ORIF. The expression of $GABA_AR$ α1 were down-regulated after ORIF in Group IP_2, I and P compared with group C on Day 1 (C vs IP_2: 100.00 ± 18.48 vs 57.57 ± 8.39, P < 0.005; C vs I: 100.00 ± 18.48 vs 18.02± 3.07, P < 0.001; C vs P: 100.00 ± 18.48 vs 16.90 ±3.45, P < 0.001;) and Day 7 (C vs IP_2: 100.00 ± 14.72 vs 56.23 ± 8.12, P < 0.001; C vs I: 100.00 ± 14.72 vs 27.92 ± 4.39, P < 0.001; C vs P: 100.00 ± 14.72 vs 24.71 ±4.01, P < 0.001;) (Fig. 2).

Combination treatment with 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol protected neurons from apoptosis

To analyze ER related apoptosis, expression of CHOP was valued by western blotting. There was no difference between group C and group IP_1 on day 1 (C vs IP_1: 100.00 ± 13.63 vs 76.93 ± 13.74, $P = 0.409$) and day 7 (C vs IP_1: 100.00 ± 20.70 vs 82.77 ± 11.96, $P = 0.876$). Compared with Group C, the expression of CHOP in group IP_2 did not change obviously on the first day (C vs IP_2: 100.00 ± 13.63 vs 136.70 ± 17.07, $P = 0.058$) but increased markedly on the seventh day after ORIF (C vs IP_2: 100.00 ± 20.70 vs 191.85 ± 37.16, $P < 0.001$). The expression of CHOP was significantly up-regulated in the I and P groups on both Day 1 (C vs I: 100.00 ± 13.63 vs 256.72 ± 33.15, $P < 0.001$; C vs P: 100.00 ± 13.63 vs 270.81 ± 40.61, $P < 0.001$) and Day 7 (C vs I: 100.00 ± 20.70 vs 277.16 ± 50.77, $P < 0.001$; C vs P: 100.00 ± 20.70 vs 304.08 ± 45.71, $P < 0.001$) after ORIF. (Fig. 3)

1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ protects neurons by heightening expression of BiP

The expression of BiP in Group IP_1, IP_2, I and P was all up-regulated compared with Group C on Day 1 (C vs IP_1: 100.00 ± 18.58 vs 442.86 ± 69.09, C vs IP_2: 100.00 ± 18.58 vs 248.02 ± 35.15, C vs I: 100.00 ± 18.58 vs 165.13 ± 25.53, C vs P: 100.00 ± 18.58 vs 188.54 ± 27.90,all $P < 0.05$). The highest was Group IP_1 and the lowest was Group I. On Day 7, the expression of BiP fell down in all four groups and there was no difference between Group I and Group C (C vs I: 100.00 ± 13.91 vs 142.57 ±18.70, $P = 0.053$). However, compared to Group C, the expression of BiP in Group IP_1, IP_2 and P was significantly increased (C vs IP_1: 100.00 ± 13.91 vs 268.27 ± 46.51, C vs IP_2: 100.00 ± 13.91 vs 199.47 ±31.66, C vs P: 100.00 ± 13.91 vs 154.64 ± 27.93, all $P < 0.05$, Fig. 4). The highest one was still Group IP_1. (Fig. 4)

Discussion

In our study, aging (16-18 month) rats were chosen as the test subject. All the rats received ligation of the bilateral CCA to mimic the pathological process of CAS. 30 days after the ligation surgery, the ORIF surgery was operated and different anesthesia was given according to the group. After the ORIF surgery, behaviour experiments (FC test) were carried out to evaluate cognitive function of rats. Histological analyses (Nissl staining) were used to explore neuronal damage and biochemical analyses (western blotting) for the harvested rat brain tissues were performed to detect molecular changes.

The first thing that needs to be argued is the selection and intervention of the test subject. The incidence of PND in orthopedic patients varies from 16% to 45%, although it can be as high as 72% [29] and it has been proved that aging is a risk factor [30]. That's why we chose aging rats to be the test subject. CH has been reported to be one of the key factors in the development of cognitive impairment [31]. The underlying mechanism could be hypoxia-induced white matter damage, microvascular inflammation and neuro-glio-vascular dysfunction [32]. We deem that aging patients with perioperative CH need more attention on the selection of surgery and anesthesia. Moreover, CAS detected in population older than 65 is 75% for men and 62% for women, with prevalence of stenosis ≥50% in this population 7% for men and 5% for women [33]. Taking incidence into account, we therefore used ligation of CAA induced CH aging

rats in this study as the subject. Since it is difficult to separate clinical anesthesia and surgery, and our main purpose is to explore the combined effects of the two factors, no separate anesthesia group was set up in this study, which is consistent with most current studies [34-36].

FC test is a very sensitive and effort-independent test of learning and memory [37]. In order to eliminate effects on motor ability caused by tibial fracture, FC test was chosen to inspect cognitive function after surgery of ORIF. Isoflurane has been reported to suppress learning in a dose-dependent fashion. Hence, we trained animals before surgery and anesthesia to remove the influence of the acquisition phase on assessment of memory postoperatively [38]. After the ORIF surgery and anesthesia, the rats were placed in the same chamber as the one during the FC training phase. No tone was made during the rats were in the case. Under this circumstance, freezing behaviors rely on hippocampal memory. On the other hand, freezing behavior from rats exposed in the different chamber and given tone stimulus rely on hippocampal independent memory. In the current study, compared to group C, freezing time in group I, P and IP_2 all shortened significantly, while there was no obvious difference between group C and IP_1. The only difference intervention between group I, P, IP_2 and IP_1 was anesthesia method. Our results suggest that hippocampal dependent memory was not impaired only in rats anesthetized with 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol. Such obvious difference aroused our interests in detecting the state of related anatomic structures.

Hippocampal CA1 area is crucial for context-specific memory retrieval and spatial memory. After CA1 lesions, both recent and remote memory is impaired [39]. More than that, this area is vulnerable to ischemia injury [40]. Thus, we chose hippocampal CA1 area to measure the number of survival neurons and expression of certain protein. On the Day 1, numbers of neurons in group I decreased obviously compared to group C and on the Day 7 numbers of neurons in group I and P decreased obviously compared to group C. The difference between group C and combination anesthesia groups had not statistical significance. Thus, we can draw a conclusion from the results that, compared to combination groups, the high dose of isoflurane or propofol alone can cause irreversible damage to the nervous system.

$GABA_AR$ α1 subunit has also been linked to brain cognitive functions [41]. More recently the expression level of $GABA_AR$ α1 in the hippocampal CA1 region was found significantly down-regulated in rats with chronic ischemic encephalopathy [41]. In our study, expression of $GABA_AR$ contains α1 subunit decreased in all but one group (1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol). The trend was coincident with the change of freezing time. It indicates that improper usage of anesthetics could aggravate cognitive impairment even though neurons are alive.

Previous study has confirmed that isoflurane affects cognitive function by ER stress [42]. Expression of BiP and CHOP are evidence of heightened ER stress [11]. Prolonged or excess CHOP expression has been accepted as key to ER stress-related apoptosis [27]. In our study, 1% isoflurane and 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol did not cause CHOP activation on neither one nor seven days after administration of anesthetics, while other ways of general anesthesia increased expression of CHOP variously. It suggests

that these three ways of general anesthesia have caused severe or chronic ER stress that was far more beyond what UPR could buffer and led to cell apoptosis. BiP normally alleviates the UPR and is anti-apoptotic [26]. In our experiments, the expression of BiP in rats anesthetized with 1% isoflurane and 20 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ propofol was the highest among all these four general anesthesia groups. The results indicate that highly increased should be the key for 1% isoflurane and 20 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ propofol to protect cognitive function from deterioration.

Based on previous studies and our experiments, we can get the following inference. Anesthetics stimulate neurons and cause accumulation of unfolded or misfolded proteins in ER, thereby induce ER stress. BiP dissociates with transducers of UPR to transport unfolded/misfolded proteins to cytoplasm and trigger ERAD [43]. Meanwhile, the transcription and translation of proteins within neurons except for UPR downstream biomarkers are inhibited. The expression of BiP and CHOP increases due to UPR. If the increased expression of BiP is sufficient to bind unfolded/misfolded proteins, then ER stress could be alleviated and the neuron could adapt, like what group IP_1 did in this study. If not, prolonged or excess expression of CHOP will lead to apoptosis [44] , like what group I, P and IP_2 did in this study. Different anesthetic schedules alter neural substrate components ($GABA_AR$ α1 subunit) accounting for cognitive functions, even though in some cases the stimulation is not severe enough to cause apoptosis, it still may either aggravate (or induce), but also prevent, cognitive impairment. (Fig.5)

Conclusion

In conclusion, we can draw a conclusion that 1% isoflurane and 20 mg·kg-1·h-1 propofol is the best way to avoid further damage to cognitive function of aging rats with CH during orthopedic surgery. The underlying mechanism of this phenomenon is related to alleviation of ER stress.

Methods

In our study, a ligation of bilateral CCA surgery [44] was adopted to prepare rats as CH animal model [45]. A second surgery, ORIF [46], requiring general anesthesia, was operated 30 days later so that the effects of anesthetics on cognitive function of these CH rats could be assessed.

Animals

Male Wistar rats, 16-18 months of age, 450-570 g in weight, were purchased from the Academy of Military Medical Science of the Chinese People's Liberation Army, and housed in groups of six per cage with *ad libitum* access to food and water. The environment temperature was 20-22℃ and humidity was 45%~65% with a 12 h light/dark cycle. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals [47] and approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Rats were housed alone per cage 3 days before the ligation of CCA and fasted 12 h before the surgery with normally supplied drinking water. After surgeries, rats were also housed alone per cage for recovery.

Ligation of CCA

The rats were first anaesthetized with intraperitoneal (i.p.) injection of 10% thiobutabarbital (100 ml/kg). After the body motion reaction and righting reflex disappeared, rat was fixed on the operation platform. Throughout the whole procedure, the surgical field was maintained sterile. The skin of the rat's neck was shaved and disinfected with iodine tincture. A median incision of approximately 2-3 cm was made in the neck. The muscles and surrounding tissues were separated to expose CCA. The CCA and a blunt end syringe needle (0.45 mm in diameter, 1 cm in length) were ligated tightly at the proximal side 1.5 cm from the bifurcation of the internal and external carotid arteries. The slipknot was firmly fixed and the needle was carefully removed. The wound was sutured and disinfected. During the surgery, a heating lamp was used to help maintaining the body temperature of anesthetized rats at 37 ± 0.5°C. [44]

Anesthesia and surgery of ORIF

During the ORIF surgery, rats were administered isoflurane inhalation or propofol through the tail vein. For the induction phase of anesthesia, rats were placed in a transparent chamber (W 25 cm × D 15 cm × H 10 cm) connected to a vaporizer and anaesthetized with 5% isoflurane and 40% oxygen. When the rats' righting reflex disappeared, the chamber was replaced by a mask. Then all the rats were separated into 5 groups (n=32/group) and given different anesthesia maintenance methods. (1) Group C: local infiltration anesthesia with 2% Lidocaine and inhalation with air containing 40% oxygen via the mask for 3 h. (2) Group I: inhalation with air containing 40% oxygen and 1.9% isoflurane for 3 h. (3) Group P: venous transfusion with 40 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol and inhalation with air containing 40% oxygen via the mask for 3 h. (4) Group IP_1: venous transfusion with 20 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol and inhalation with air containing 40% oxygen and 1% isoflurane for 3 h. (5) Group IP_2: venous transfusion with 10 $mg \cdot kg^{-1} \cdot h^{-1}$ propofol and inhalation with air containing 40% oxygen and 1.4% isoflurane for 3 h. The concentration of isoflurane was detected continuously by a gas monitor (Puritan-Bennett; Tewksbury, MA, USA) during the process of surgery.

ORIF surgical model: under different general anesthesia, rats received an open tibial fracture on the left hind paw with an intramedullary fixation. Supplemental analgesia was provided by buprenorphine (0.3 mg/kg in saline) intraperitoneally in less than 1 ml [46]. The surgery was carried out with aseptic techniques. The left hind paw of surgical rats was shaved and disinfected with iodine tincture. After the skin was incised, an insertion of a 0.38 mm pin was performed in the intramedullary canal. Once the tibia was internally fixated, the bone was fractured in the middiaphysis (tibial, midshaft) using surgical pliers. The skin was sutured with 8/0 Prolene sutures. Only skin incisions and sutures were performed on rats in group C. During the surgery, a heating lamp was used to help maintaining the body temperature of anesthetized rats at 37 ± 0.5°C. Postintervention rats were moved to heated pads for recovery and then delivered back to their own cage, where food and water were sufficient. For post-procedural pain relief, the rats were administered buprenorphine (0.05 mg/kg, subcutaneous) twice daily for 3 days [48].

Fear conditioning test

The fear conditioning (FC) test was carried on to estimate cognitive function [38]. The FC test consisted of a training phase at 24 h prior to ORIF operation and an evaluation phase on days 1 and 7 after ORIF when hippocampal-dependent memory was assessed.

During the training phase, rats were placed in a chamber (Ugo Basile, Italy) and allowed to adapt to the environment for 120 s. After adaption, they were stimulated by a 20 s 70-dB tone (conditional stimulus). Then there was an interval of 25 s. After the interval, rats were stimulated by a 0.70 mA electrical foot-shock for 2 s (unconditional stimulus). After six pairs of conditional-unconditional stimuli, the rats learned the association and established long-term memory. The pairs of conditional-unconditional stimuli were separated by 60 s inter-training intervals. Each training chamber was cleaned with 95% ethyl alcohol before the placement of a rat and was illuminated only with a 10 W bulb in a dark experimental room.

During the evaluation phase, rats were placed again in the training chamber for 5 min without tone and foot shock. Each animal's freezing behavior (without any movements) was scored by the ANY-maze video tracking system (Stoelting, Illinois, USA). The percentage of time in freezing behavior was calculated using the formula of 100*f/5min, where f was the total of freezing time in the 5 min. Freezing time measured during exposure to the known context, or after a conditional stimulus in the known context, reflects hippocampal-dependent memory whereas assessment during delivery of the conditional stimulus (tone) assesses hippocampal-independent memory [38]. Thus the results in this experiment were used to assess hippocampus-dependent memory.

Nissl staining

On days 1 and 7 after ORIF, rats (n=8/group) were first anaesthetized with 10% thiobutabarbital (100 ml/kg, i.p.). Perfusion with saline was given to the rats before the heart stops, followed by perfusion with 4% paraformaldehyde solution. Then the brain was taken out and fixed in 4% paraformaldehyde for 24 h. Coronal slices (3.0-mm thick) from each brain containing the dorsal hippocampus and the medial dorsal prefrontal cortex were dehydrated and embedded in paraffin. A series of 10 μm thick coronal sections were obtained from each slice and stained with Cresyl violet [49]. For each brain, five sections at the dorsal hippocampus located between bregma -3.14 through bregma -4.52 were analyzed for Ammon's horn pyramidal cell counts [50]. Sections were examined by an observer unaware of the experimental condition, under light microscopy at a magnification of 200x. The number of surviving neurons in a 30,000 μm^2 area of CA1 was counted in each section. Only pyramidal neurons showing normal morphology with distinct cytoplasmic and nuclear outlines and a visible nucleolus were counted. The analysis of data was performed through Image Pro Plus 6.0 software (Media Cybernetics Co., USA).

Western blotting

On days 1 and 7 after ORIF, rats (n=8/group) were sacrificed with sodium pentobarbital (240mg/ml, Department of Pharmacy, Tianjin Medical University General Hospital, i.p., 800 mg/kg) [51]. After ensuring that the heart of rat stopped, the brain was taken out and hippocampus tissue was separated. Hippocampus was homogenized in RIPA solution (Biomart, Beijing, China) buffer and then centrifuged at

4℃ at 12000r/min for 10 min (Sigma 3-30KS, Sigma Laboratory Centrifuges, Germany). The quantity of protein in the supernatants was determined using a BCA protein assay kit (Beyotime Biotechnology, Beijing, China). Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Then membranes were blocked by 5% skim milk Tris-buffered saline containing 0.1% Tween (TBST) buffer for 90 min and then washed by TBST buffer for 5 min. The membranes were incubated with primary antibodies: anti-$GABA_AR$ α1 (1:1,000, Abcam, Cambridge, UK), anti-BiP (1:1,000; Abcam), anti-pan-cadherin (1:2,000, Sigma, St. Louis, MO, USA), anti-β-actin (1:10,000, Proteintech, Wuhan, China) overnight at 4℃. After washing with TBST 5 times (each for 5 min), membranes were incubated with a secondary polyclonal antibody conjugated to horseradish peroxidase, anti-rabbit immunoglobulin G (IgG) (1:5000, KPL, Gaithersburg, MD), and anti-mouse IgG (1:5000, KPL) at room temperature for 1 h. Then the membranes were washed 5 times (each for 5 min) again and treated with an enhanced chemiluminescence detection kit (EMD Millipore, Billerica, MA, USA). The intensity of each band was quantified by densitometry using a gel image analysis software (Image Pro Plus, Media Cybernetics, USA). Relative expression was normalized to that of anti-pan-cadherin (1:2,000, Sigma) and anti-β-actin (1:10,000, Proteintech).

Statistical analysis

The data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Data were presented as mean ± standard deviation (SD). Behavioral data were tested using a two-way analysis of variance (ANOVA) with repeated measures. Other data were analyzed using a one-way ANOVA with Tukey *post hoc* comparisons. $P < 0.05$ was the criterion for statistical significance.

Abbreviations

BiP, binding immunoglobulin protein;

CCA, common carotid arteries;

CH, Cerebral hypoperfusion

CHOP, C/EBP homologous protein;

ER, endoplasmic reticulum;

ERAD, endoplasmic reticulum associated degradation

EC, fear conditioning

MAC, minimum alveolar concentration;

ORIF, open reduction and internal fixation

GABA, γ-aminobutyric acid

$GABA_AR$, γ-aminobutyric acid A type receptor

UPR, unfolded protein response

Declarations

Ethics approval and consent to participate

All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals [47] and approved by the Institutional Animal Care and Use Committee of Tianjin Medical University.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Name: Haiyun Wang, Ph.D.

Contribution: This author helped with conception and design; acquisition, analysis, and interpretation of data; critically revising the article; and giving final approval.

Name: Xinyue Bu, M.S.

Contribution: This author helped with conception and design; acquisition, analysis, and interpretation of data; drafting and critically revising the article; and giving final approval.

Name: Tang Li, M.S.

Contribution: This author helped with conception and design; acquisition, analysis, and interpretation of data; drafting and critically revising the article; and giving final approval.

Name: Zhengyuan Xia, Ph.D.

Contribution: This author helped with critically revising the article; and giving final approval.

Name: Di Guo, M.S.

Contribution: This author helped with analysis and interpretation of data acquisition, analysis, and interpretation of data; critically revising the article; and giving final approval.

Name: Jinxin Wang, M.S.

Contribution: This author helped with conception and design; analysis and interpretation of data; critically revising the article; and giving final approval.

Name: Yi Sun, M.S.

Contribution: This author helped with conception and design; analysis and interpretation of data; critically revising the article; and giving final approval.

Name: Chenyi Yang, M.S.

Contribution: This author helped with conception and design; analysis and interpretation of data; critically revising the article; and giving final approval.

Name: Guoqiang Liu, M.S.

Contribution: This author helped with analysis and interpretation of data; critically revising the article; and giving final approval.

Name: Ji Ma, M.S.

Contribution: This author helped with analysis and interpretation of data; critically revising the article; and giving final approval

Name: Zhuo Yang, Ph.D.

Contribution: This author helped with critically revising the article; and giving final approval

Name: Guolin Wang, Ph.D.

Contribution: This author helped with critically revising the article; and giving final approval

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Figures

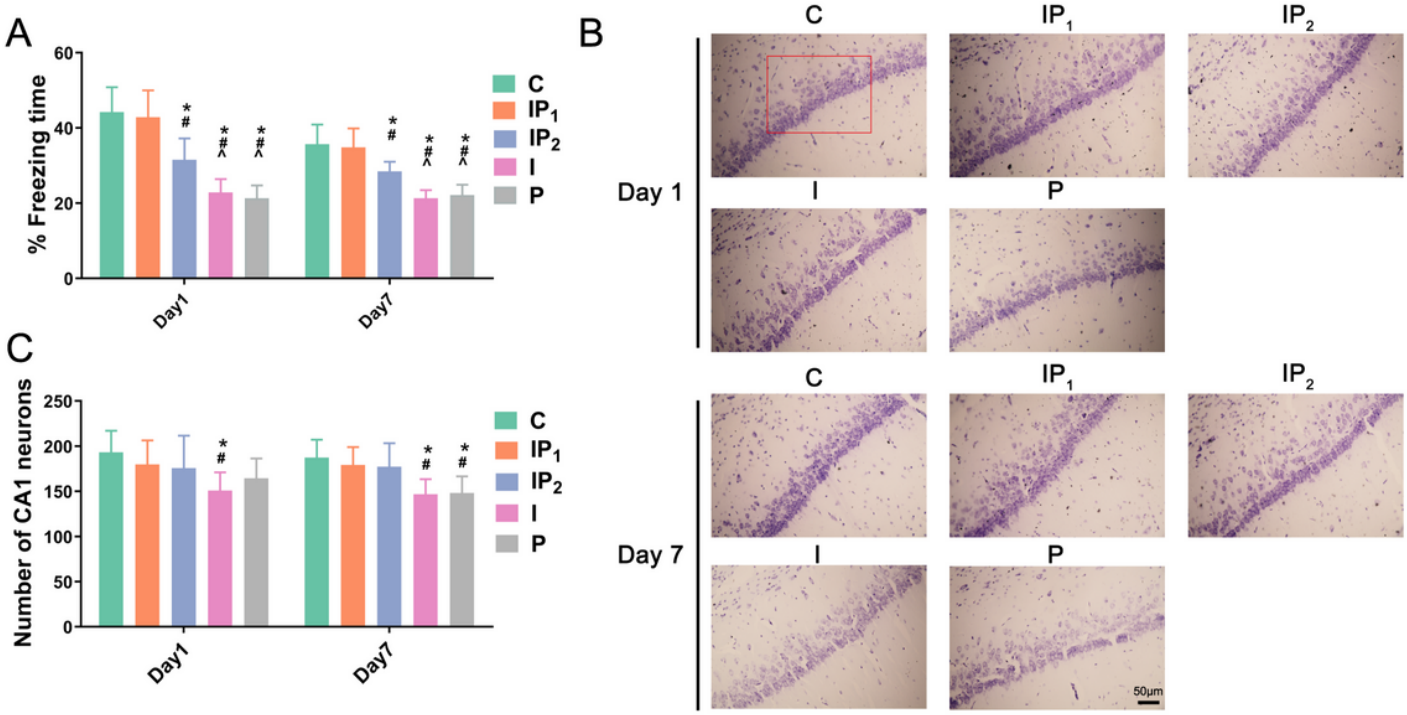

Figure 1

Combination treatment with 1% isoflurane and 20 mg·kg-1·h-1 propofol protected cognitive function and survival neurons of CH rats. (A)Hippocampus-dependent memory was evaluated as the percentage of freezing time on Day 1 and Day 7 after ORIF. Data was expressed as mean ± SD (n=8/group). Note that ORIF resulted in a significant reduction of the time of freezing behavior in CA1 in IP2, I and P groups, which was prevented by the anesthetic schedule in the IP1 group. (B) Nissl staining images for hippocampal CA1 were shown to evaluate neuronal damage on Day 1 and Day 7 after ORIF. Note that ORIF resulted in a significant reduction of the number of remaining pyramidal neurons in CA1 in IP2, I and P groups, which was prevented by the anesthetic schedule in the IP1 group. (C) Quantification of CA1 survival neurons on Day 1 and Day 7 after ORIF. Data was expressed as mean ± SD (n=8/group). * P < 0.05 compared with group C; # P < 0.05 compared with group IP1; ^ P < 0.05 compared with group IP2. Scale bars = 50 μm.

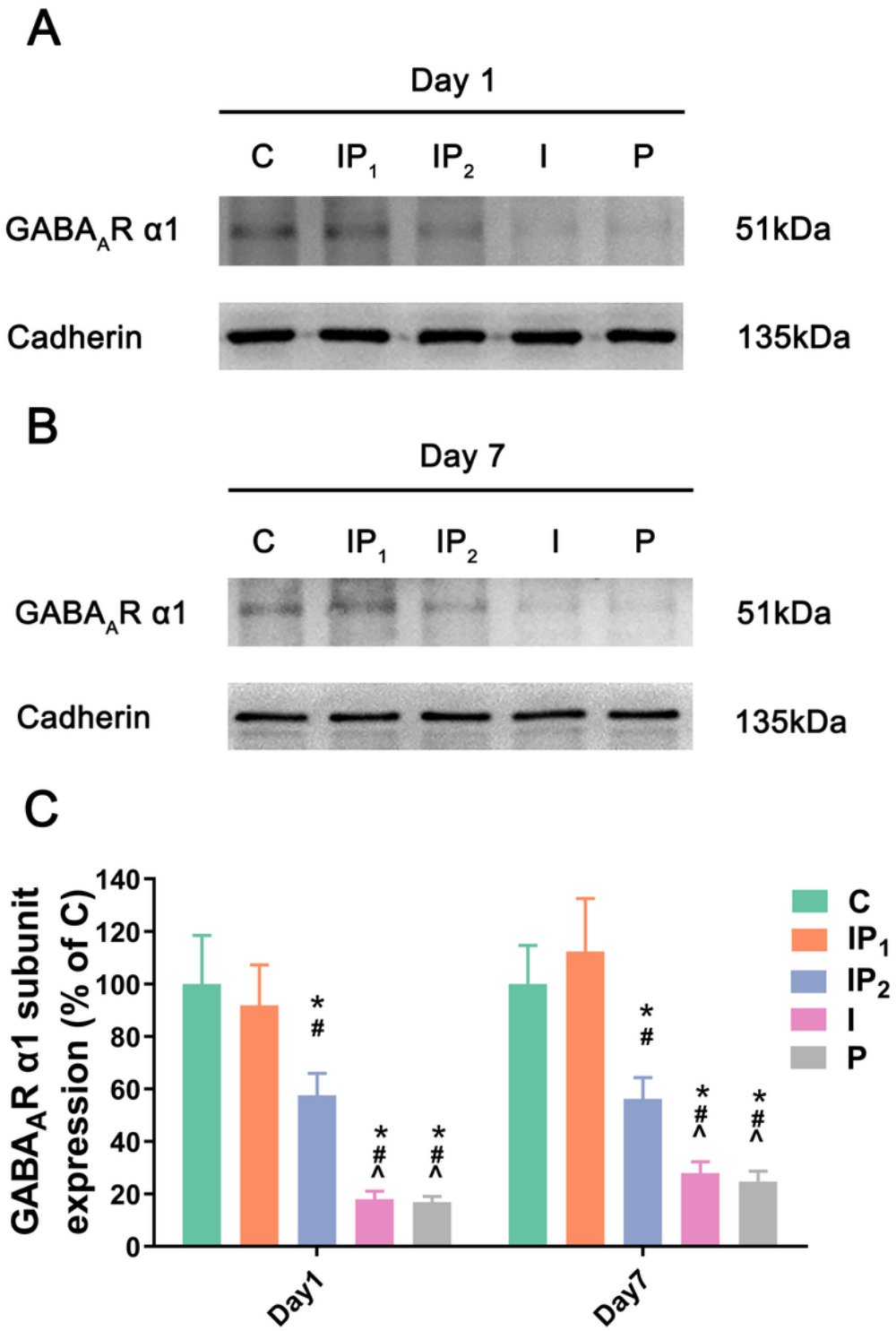

Figure 2

Combination treatment with 1% isoflurane and 20mg·kg-1·h-1 propofol maintain the expression of GABAAR α1 subunit (A,B) The expression of GABAAR α1 subunit in hippocampus was determined by western blotting on Day 1 and Day 7 after ORIF. (C) Statistical graph of expression of GABAAR α1 subunit on Day 1 and Day 7 after ORIF. Data was expressed as mean ± SD (n=8/group). Note that ORIF resulted in a significant reduction of expression of GABAAR α1 subunit in CA1 in IP2, I and P groups, which was

prevented by the anesthetic schedule in the IP1 group. * P < 0.05 compared with group C; # P < 0.05 compared with group IP1; ^ P < 0.05 compared with group IP2.

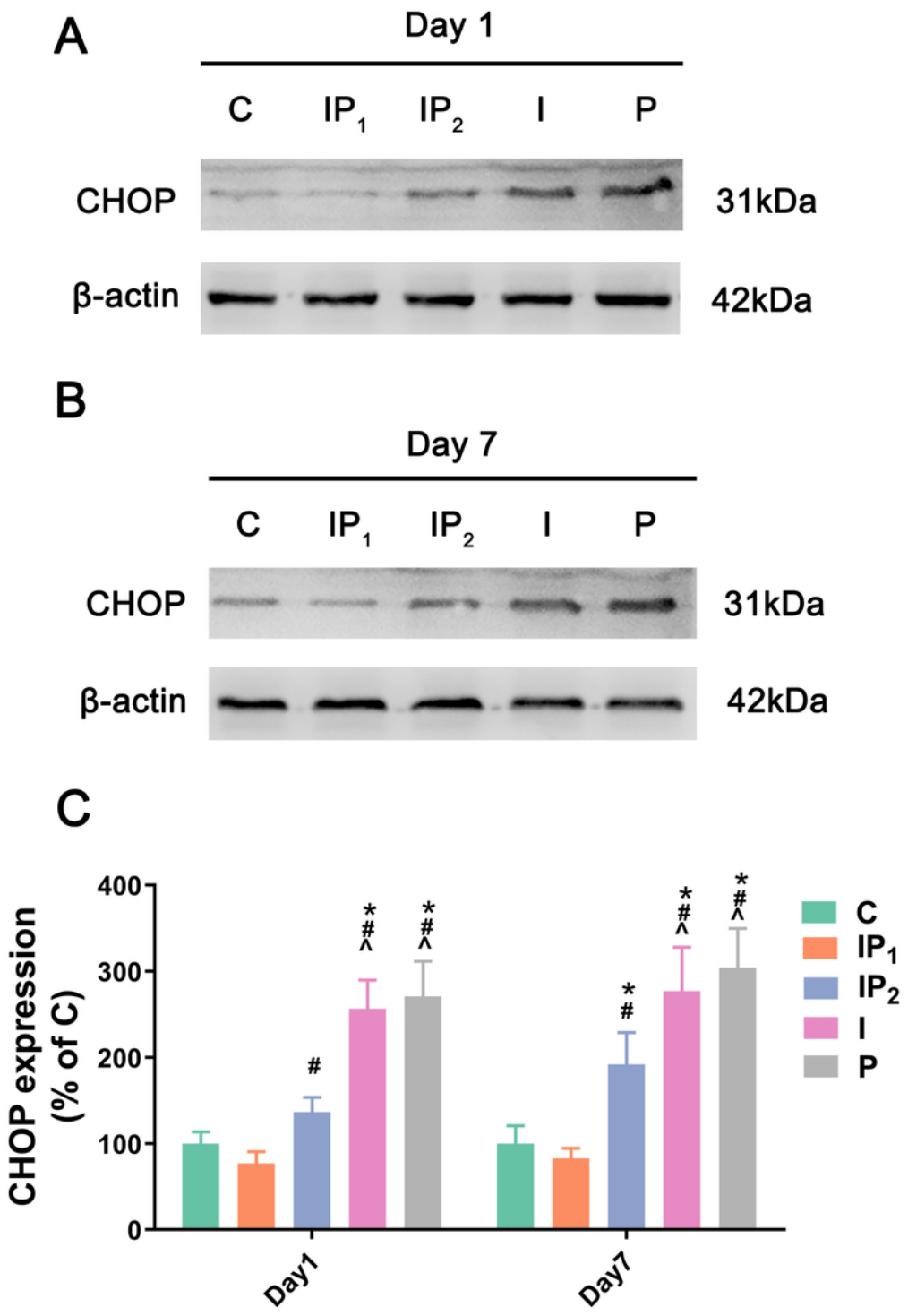

Figure 3

Combination treatment with 1% isoflurane and 20mg·kg-1·h-1 propofol avoided ER related apoptosis (A,B) The expression of CHOP in hippocampus was determined by western blotting on Day 1 and Day 7 after ORIF. (C) Statistical graph of expression of CHOP on Day 1 and Day 7 after ORIF. Data was

expressed as mean ± SD (n=8/group). Note that ORIF resulted in a significant increase of the number of expression of CHOP in CA1 in IP2, I and P groups, which was prevented by the anesthetic schedule in the IP1 group. * P < 0.05 compared with group C; # P < 0.05 compared with group IP1; ^ P < 0.05 compared with group IP2.

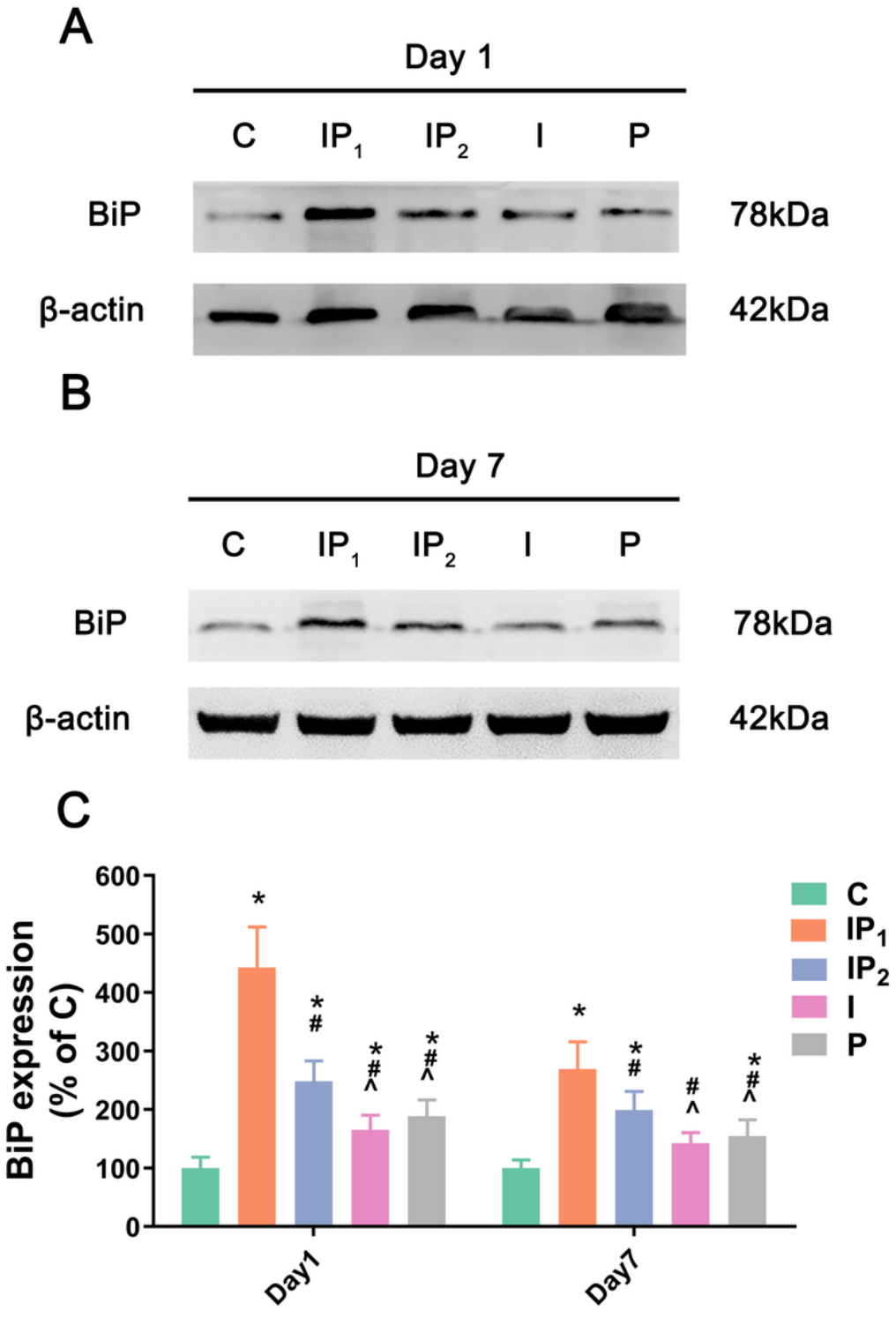

Figure 4

Combination treatment with 1% isoflurane and 20mg·kg-1·h-1 propofol maintained neurons adaptive ability by increasing expression of BiP (A,B) The expression of BiP in hippocampus was determined by western blotting on Day 1 and Day 7 after ORIF. (C) Statistical graph of expression of BiP on Day 1 and Day 7 after ORIF. Data was expressed as mean ± SD (n=8/group). Note that ORIF resulted in a significant increase of the number of expression of BiP in CA1 in IP2, I and P groups, which was prevented by the anesthetic schedule in the IP1 group. * P < 0.05 compared with group C; # P < 0.05 compared with group IP1; ^ P < 0.05 compared with group IP2.

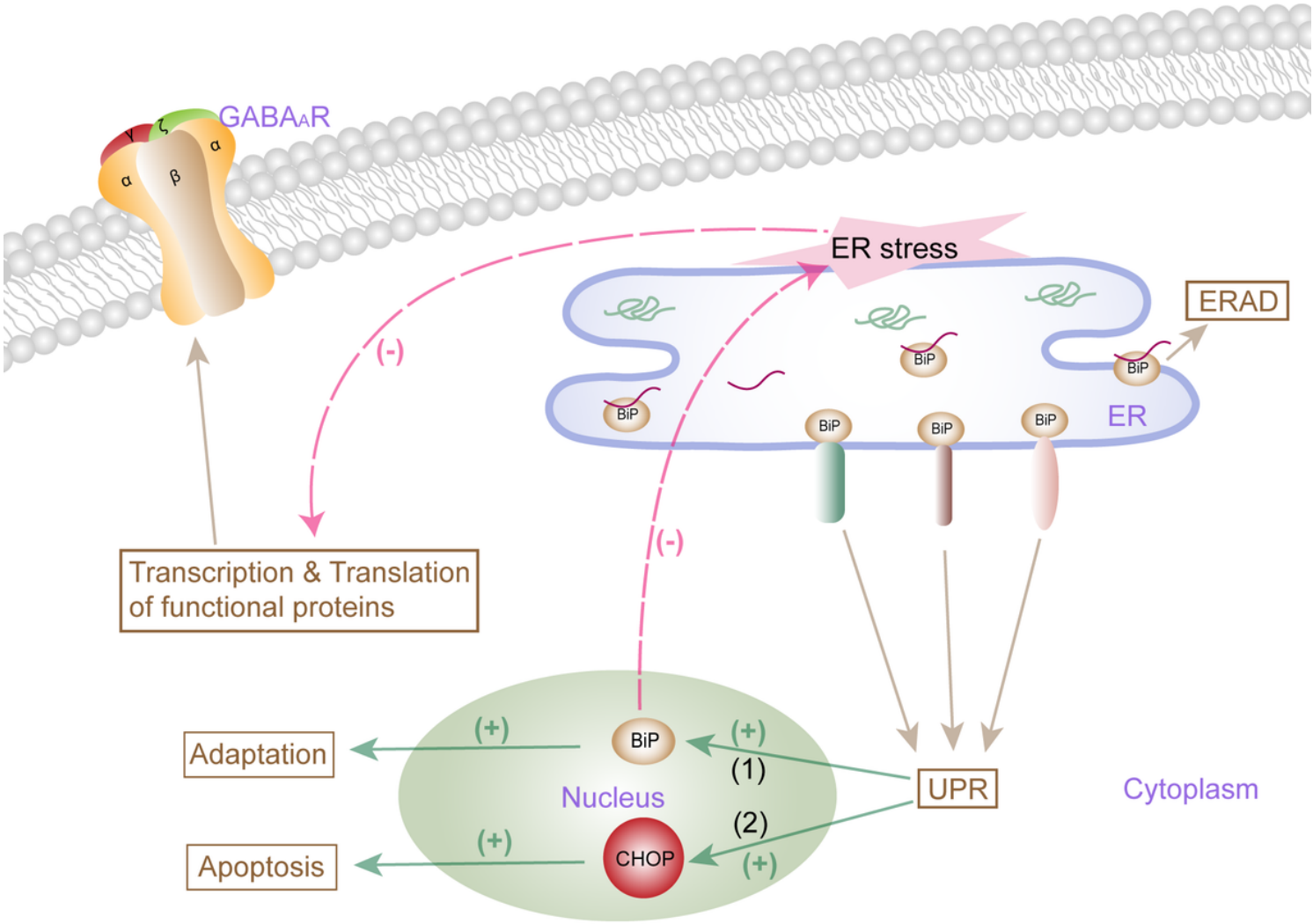

Figure 5

A proposed schematic model of molecular phenomena occurring under ER stress, that are either activated (1) or inhibited (2) by treatment with 1% isoflurane and 20mg·kg-1·h-1 propofol. (1) 1% isoflurane and 20mg·kg-1·h-1 propofol activated expression of BiP than other groups. (2) 1% isoflurane and 20mg·kg-1·h-1 propofol inhibited expression of CHOP while other groups did.

Supplementary Files

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