

Combination of isoflurane and propofol as general anaesthesia during orthopaedic surgery of perioperative cerebral hypoperfusion rats to avoid cognitive impairment Anaesthesia during perioperative cerebral hypoperfusion

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Abstract

Background: Perioperative cerebral hypoperfusion (CH) is common, although the underlying mechanism of cognitive impairment that results due to perioperative cerebral hypoperfusion remains to be determined. Isoflurane anaesthesia induces neuronal injury via endoplasmic reticulum (ER) stress, whereas a sub-anaesthetic dose of propofol improves postoperative cognitive function. However, the effects of the combination of isoflurane plus propofol, which is a common aesthetic combination administered to patients, on ER stress and cognition remain unknown. Methods: We sought to determine the effects of isoflurane plus propofol on ER stress and cognitive function in rats insulted by cerebral hypoperfusion. Ligation of the bilateral common carotid arteries (CCA) was adopted to develop the cerebral hypoperfusion rat model. A second surgery, open reduction and internal fixation (ORIF), requiring general anaesthesia, was performed 30 days later so that the effects of anaesthetics on the cognitive function of CH rats could be assessed. 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). Behavioural studies (fear conditioning [FC] test), histological analyses (Nissl staining) and biochemical analyses (western blotting of the harvested rat brain tissues) were employed. Results: The combination of 1% isoflurane plus 20 mg·kg⁻¹·h⁻¹ propofol did not aggravate cognitive impairment or ER stress in ageing rats with CH that were further subjected ORIF surgery. Conclusions: These data suggest that ER stress contributes to the underlying mechanism of cognitive impairment and that the combination of isoflurane and propofol did not aggravate cognitive impairment and ER stress in ageing rats with CH that were further subjected 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 orthopaedic 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 $\geq 50\%$ occurring in 7% of men and 5% of women in this age group [4]. CAS is an independent risk factor for chronic CH [5], which reduces tissue oxygen levels and leads to oxidative stress and endothelial injury [6]. In rodents, experimental chronic CH can be initiated by occlusion of the major arterial supply. This way chronic CH 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. Oxidative injury to vascular endothelial cells, glia, and neurons also impair vascular function and neurovascular coupling, which may result in a vicious cycle that further reduces cerebral perfusion [7]. Taking all these factors into account, ageing orthopaedic patients with preoperative carotid stenosis make up a population that needs to be treated carefully. Special caution on the selection of anaesthetic drugs is needed to protect cognitive function.

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

γ -aminobutyric acid (GABA) is the main inhibitory neurotransmitter [15]. The major subtype of GABA_A receptor (GABA_AR) contains the $\alpha 1$ subunit. According to a previous study, GABA receptors in the central nervous system are divided into three types: A, B, and C. Among the three receptors, GABA_A receptor (GABA_AR) is the earliest expressed and most widely distributed, found mainly in the hippocampus, the prefrontal cortex and the striatum. Studies have confirmed that most GABAergic synaptic transmission in the mammalian brain is mediated by GABA_AR. It is also an important target receptor for central nervous system (CNS) general anaesthetics, such as propofol and isoflurane. GABA_AR consists of five subunits embedded in the cell membrane of neurons. At the centre, a 0.5 nm diameter GABA-gated Cl⁻ channel is formed. When GABA binds to GABA_AR, the Cl⁻ channel of the postsynaptic membrane is opened, and Cl⁻ enters the cell due to the concentration gradient. The potential increases to produce hyperpolarization, which in turn causes neuronal inhibition [16, 17]. In 2014, Labrakakis et al. confirmed that the postsynaptic membrane GABA_AR subunit composition determines the heterogeneity of inhibitory postsynaptic potential (IPSP), namely, GABA_AR function [18]. The native GABA_ARs present in the mammalian brain are mainly composed of α , β and γ subunits. The most common configuration is a transmembrane pentamer composed of $2\alpha_1 2\beta_2 \gamma_2$, accounting for 43% of all GABA_AR configurations and representing the most abundant configuration in the hippocampus and the cerebral cortex [18]. The GABA_AR $\alpha 1$ subunit, which is related to cognition, is the most widely distributed in the mammalian brain, [19, 20]. Its main function is to maintain CNS arousal and the sensitivity of the receptor to sedative hypnotics (propofol, isoflurane, etc.). Mutation of the M2 domain Ser270 and the M3 domain Ala291 in the $\alpha 1$ subunit affects the potency of isoflurane and propofol on GABA_ARs [21]. Kelley et al. confirmed that cerebral ischaemia 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 $\alpha 1$ subunit expression [22]. This finding suggests that the change in GABA_AR activity is triggered by a decrease in the expression of its functional subunit $\alpha 1$. Furthermore, our previous study showed that a sub-anaesthetic dose ($20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of propofol exerts post-treatment brain protection

by activating the KCC2-GABA_AR pathway. Propofol post-treatment can reverse the decrease in 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]. It remains unknown whether or not cerebral ischaemia triggers the expression change and structural regulation of GABA_AR functional subunit protein. Is there any upstream mechanism other than KCC2 that regulates the GABA_AR structure, thereby affecting its function? To answer these questions, we chose the GABA_AR $\alpha 1$ subunit as a target of research in this study.

GABA_AR undergoes post-synthesis modification and folding in the ER. Prolonged ER stress has been well known to be 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 (approximately 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 and is required to alleviate ER stress [26]. Once ER stress occurs, BiP binds to unfolded proteins and activates 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 stress, UPR attempts to increase protein-folding capacity and remove misfolded and unfolded proteins. If homeostasis is inadequately restored under chronic ER stress, terminal UPR will trigger apoptosis through abundant signalling 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 levels of BiP, CHOP and the GABA_AR $\alpha 1$ subunit were used to evaluate the cellular mechanisms accounting for the neural substrate conditions that allow normal cognitive functions in this study.

The objective of the current study was to explore general anaesthetics for rats with CH that are subjected to ORIF surgery to protect cognitive function. By using behavioural and biochemical analyses, we tested the hypothesis that a combination of isoflurane and propofol better protects cognitive function than isoflurane or propofol administered alone during ORIF surgery.

Results

Combination treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol protected cognitive function in ageing 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 FC test was performed on the first and seventh days after ORIF. The percentage of freezing time in Group C and Group IP₁ was not significantly different on the first day (C vs IP₁: 44.23 ± 6.60 vs 42.86 ± 7.12, *P* = 1.00) or the seventh day (C vs IP₁: 35.70 ± 5.21 vs 34.85 ± 5.02, *P* = 1.000) after ORIF (Fig. 1A). However, in Groups IP₂, I and P, the percentage of freezing time was significantly reduced compared with Group C on day 1 (C vs IP₂: 44.23 ± 6.60 vs 31.55 ± 5.68; C vs I: 44.23 ± 6.60 vs 22.86 ± 3.53; C vs P: 44.23 ± 6.60 vs 21.32 ± 3.42; all *P* < 0.05) and day 7 (C vs IP₂: 35.70 ± 5.21 vs 28.48 ± 2.54; C vs I: 35.70 ± 5.21 vs 21.34 ± 2.12; C vs P: 35.70 ± 5.21 vs 22.16 ± 2.74; all *P* < 0.05) (Fig. 1A). The results suggest that the combination of 1% isoflurane and 20 mg·kg⁻¹·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 ageing rats with CH that were subjected to ORIF surgery.

Hippocampal slices were stained with cresyl violet (Nissl staining) to investigate potential neuronal damage caused by anaesthetics on days 1 and 7 after ORIF. Compared with Group C, the number of surviving 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 surviving neurons in Groups I and P was significantly lower than 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 changes were found in Groups IP₁ and IP₂ on day 1 (C vs IP₁: 193.13 ± 23.94 vs 179.75 ± 26.60, *P* = 0.923; C vs IP₂: 193.13 ± 23.94 vs 175.75 ± 35.94, *P* = 0.799) or day 7 (C vs IP₁: 187.38 ± 19.86 vs 179.13 ± 19.96, *P* = 0.975; C vs IP₂: 187.38 ± 19.86 vs 177.25 ± 26.02, *P* = 0.940) (Fig. 1 B-C).

Combination treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol maintained the expression level of cell GABA_AR α1 in the hippocampus.

As described 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 the GABA_AR α1 subunit. There was no difference in the expression of GABA_AR α1 between Group C and Group IP₁ on day 1 (C vs IP₁: 100.00 ± 18.48 vs 91.86 ± 15.45, *P* = 0.629) or day 7 (C vs IP₁: 100.00 ± 14.72 vs 112.39 ± 20.17, *P* = 0.261) after ORIF. The expression of GABA_AR α1 was downregulated after ORIF in Groups IP₂, I and P compared with Group C on day 1 (C vs IP₂: 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 IP₂: 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·kg⁻¹·h⁻¹ propofol protected neurons from apoptosis

To analyse ER-related apoptosis, the expression of CHOP was evaluated by western blotting. There was no difference between Group C and Group IP₁ on day 1 (C vs IP₁: 100.00 ± 13.63 vs 76.93 ± 13.74, *P* = 0.409) or day 7 (C vs IP₁: 100.00 ± 20.70 vs 82.77 ± 11.96, *P* = 0.876). Compared with Group C, the expression of CHOP in Group IP₂ did not markedly change on the first day (C vs IP₂: 100.00 ± 13.63 vs 136.70 ± 17.07, *P* = 0.058) but increased markedly on the seventh day after ORIF (C vs IP₂: 100.00 ± 20.70 vs 191.85 ± 37.16, *P* < 0.001). The expression of CHOP was significantly upregulated in Groups I and P on 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·kg⁻¹·h⁻¹ propofol protect neurons by elevating the expression of BiP

The expression levels of BiP in Groups IP₁, IP₂, I and P were all upregulated compared with that in Group C on day 1 (C vs IP₁: 100.00 ± 18.58 vs 442.86 ± 69.09, C vs IP₂: 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 expression level was found in Group IP₁, and the lowest expression level was found in Group I. On day 7, the expression of BiP fell in all four groups, and there was no significant difference between Group I and Group C (C vs I: 100.00 ± 13.91 vs 142.57 ± 18.70, *P* = 0.053). However, the expression of BiP in Groups IP₁, IP₂ and P was significantly higher than that in Group C (C vs IP₁: 100.00 ± 13.91 vs 268.27 ± 46.51, C vs IP₂: 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 expression was observed in Group IP₁ (Fig. 4).

Discussion

In our study, ageing (16-18 month) rats were chosen as test subjects. All rats received ligation of the bilateral CCA to mimic the pathological process of CAS. Thirty days after ligation surgery, ORIF surgery was performed under different anaesthesia regimens according to the group. After ORIF surgery, behavioural experiments (FC test) were carried out to evaluate the cognitive function of the rats. Histological analyses (Nissl staining) were performed to explore neuronal damage, and biochemical analyses (western blotting) of harvested rat brain tissues were performed to detect molecular changes.

The first consideration that must be discussed is the selection and intervention of the test subject. The incidence of PND in orthopaedic patients varies from 16% to 45%, although it can be as high as 72% [29], and it has been proven that ageing is a risk factor [30]. Therefore, we chose ageing rats as the test subjects. CH has been reported to be a key factor 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 ageing patients with perioperative CH require more attention to be paid to the selection of surgery and anaesthesia. Moreover, CAS has been detected in 75% of men and 62% of women older than 65 years, with a prevalence of ≥50% stenosis of 7% in men and 5% in women [33]. Taking incidence into account, we therefore used ligation of the CCA to induce CH in

ageing rats in this study. As it is difficult to separate clinical anaesthesia and surgery, and our main purpose was to explore the combined effects of the two factors, no separate anaesthesia group was used, which is consistent with most recent studies [34-36].

The FC test is a very sensitive and effort-independent test of learning and memory [37]. To eliminate effects on motor ability caused by tibial fracture, the FC test was chosen to inspect cognitive function after ORIF surgery. Isoflurane has been reported to suppress learning in a dose-dependent fashion. Hence, we trained animals before surgery and anaesthesia to remove the influence of the acquisition phase on the assessment of memory postoperatively [38]. After ORIF surgery and anaesthesia, the rats were placed in the same chamber that was used during the FC training phase. No tone was delivered while the rats were in the chamber. In this circumstance, freezing behaviours rely on hippocampal memory. On the other hand, the freezing behaviour of rats in a different chamber and exposed to the tone stimulus relies on hippocampal-independent memory. We found that the freezing time of rats was significantly shorter in Groups I, P and IP₂ than in Group C, while there was no obvious difference between Groups C and IP₁. The only difference in the intervention among Groups I, P, IP₂ and IP₁ was the anaesthesia method. Our results suggest that hippocampal-dependent memory impairment was not exhibited by the rats anaesthetized with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol, in contrast to all other groups of rats. Such an obvious difference aroused our interest in evaluating the state of related anatomic structures.

The hippocampal CA1 area is crucial for context-specific memory retrieval and spatial memory. After CA1 lesions, both recent and remote memory are impaired [39]. Furthermore, this area is vulnerable to ischaemia injury [40]. Thus, we chose the hippocampal CA1 area to measure the number of survival neurons and the expression of certain protein. On day 1, the number of neurons in Group I decreased obviously compared with Group C, and on day 7, the number of neurons in Groups I and P were markedly decreased compared with Group C. The difference between Group C and the combined anaesthesia groups was not significant. Thus, we can draw the conclusion that, compared with the combination groups, the high dose of isoflurane or propofol alone can cause irreversible damage to the nervous system.

The 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 to be significantly downregulated in rats with chronic ischaemic encephalopathy [41]. In our study, the expression of GABA_AR containing the α1 subunit decreased in all but one group (the 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol group). This trend was coincident with the change in freezing time, indicating that improper usage of anaesthetics could aggravate cognitive impairment even though neurons are alive.

A previous study confirmed that isoflurane affects cognitive function by ER stress [42]. The 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·kg⁻¹·h⁻¹ propofol did not cause CHOP activation one or seven days after administration, while other modes of general anaesthesia increased the expression of CHOP to various degrees. This finding suggests that the

three modes of general anaesthesia caused severe or chronic ER stress that was far beyond what UPR could buffer, thus leading to cell apoptosis. BiP normally alleviates the UPR and is anti-apoptotic [26]. In our experiments, the expression of BiP was the highest in rats anaesthetized with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol among all four general anaesthesia groups. The results indicate that higher BiP expression may be the key as to how 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol protects cognitive function from deterioration.

Based on previous studies and our experiments, we can make the following inferences. Anaesthetics stimulate neurons and cause accumulation of unfolded or misfolded proteins in the ER, thereby inducing ER stress. BiP dissociates with transducers of UPR to transport unfolded/misfolded proteins to the 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, as observed for Group IP₁ in this study. Otherwise, prolonged or excess expression of CHOP will lead to apoptosis [44], as observed for Groups I, P and IP₂. Different anaesthetic schedules alter various neural substrate components (GABA_AR α1 subunit) that play roles in cognitive functions. Although the stimulation may not be severe enough in some cases to cause apoptosis, cognitive impairment may still be aggravated (or induced) or prevented (Fig. 5).

Conclusion

In conclusion, 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol is a more favourable aesthetic combination to avoid further damage to cognitive function of ageing rats with CH during orthopaedic 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 anaesthesia, was operated 30 days later so that the effects of anaesthetics on cognitive function of these CH rats could be assessed.

Animals

Male Wistar rats, 16-18 months of age and 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 housing environment was maintained at a temperature of 20-22°C and a humidity of 45%~65% under 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 were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University. Rats were housed individually per cage 3 days before ligation of the CCA and fasted 12 h before surgery a normally supply of drinking water. After surgery, rats were also housed individually per cage for recovery.

Ligation of the CCA

Rats were first anaesthetized with intraperitoneal (i.p.) injection of 10% thiobutabarbital (100 ml/kg). After disappearance of body motion and the righting reflex, the rat was fixed on the operation platform. The surgical field was maintained sterile throughout the entire procedure. 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 the 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 surgery, a heating lamp was used to help maintain the body temperature of anaesthetized rats at $37 \pm 0.5^\circ\text{C}$ [44].

Anaesthesia and ORIF surgery

During ORIF surgery, rats were administered isoflurane via inhalation or propofol through tail vein injection. For the induction phase of anaesthesia, the rat was 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 rat's righting reflex disappeared, the chamber was replaced by a mask. Each rat was then assigned to one of the following 5 groups (n=32/group) and administered the respective anaesthesia as maintenance: (1) Group C: local administration of anaesthesia 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 $40 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ propofol and inhalation with air containing 40% oxygen via the mask for 3 h; (4) Group IP₁: venous transfusion with $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ propofol and inhalation with air containing 40% oxygen and 1% isoflurane for 3 h; and (5) Group IP₂: venous transfusion with $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ 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 surgery.

ORIF surgical model: Under different modes of general anaesthesia, the rats underwent an open tibial fracture of the left hind paw with intramedullary fixation. Supplemental analgesia was provided using less than 1 ml buprenorphine (0.3 mg/kg in saline) administered intraperitoneally [46]. Surgery was carried out via aseptic techniques. The left hind paw of the rat was shaved and disinfected with iodine tincture. After the skin was incised, a 0.38 mm pin was inserted into the intramedullary canal. Once the tibia was internally fixated, the bone was fractured at the middiaphysis (tibial, midshaft) using surgical pliers. The skin was sutured with 8/0 Prolene sutures. In Group C, only the skin was incised and sutured. During surgery, a heating lamp was used to help maintain the body temperature of the anaesthetized rats at $37 \pm 0.5^\circ\text{C}$. Postintervention rats were moved to heated pads for recovery and then returned to their home cage supplied with sufficient food and water. 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 FC test was utilized to evaluate cognitive function [38]. The FC test consisted of a training phase at 24 h prior to ORIF surgery 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, a 20 s 70-dB tone (conditional stimulus) was delivered, followed by an interval of 25 s. After the interval, an 0.70 mA electrical foot shock was delivered to the rat for 2 s (unconditional stimulus). After six pairs of conditional-unconditional stimuli, the rats learned the association and had 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 placement of the next 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 behaviour (without any movements) was analysed by using the ANY-Maze Video Tracking System (Stoelting, Illinois, USA). The percentage of time spent exhibiting freezing behaviour was calculated using the formula of $100 \times f / 5 \text{ min}$, where f was the total of freezing time within 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 ($n=8/\text{group}$) were first anaesthetized with 10% thiobutabarbital (100 ml/kg, i.p.). Rats were perfused with saline before the heart stopped, 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 was obtained from each slice, and the sections were stained with cresyl violet [49]. For each brain, five sections at the dorsal hippocampus located at coordinates -3.14 from the bregma to -4.52 from bregma were analysed for Ammon's horn pyramidal cell counts [50]. Sections were examined by an observer who was blinded to the experimental conditions under light microscopy at a magnification of 200x. The number of surviving neurons in a 30,000 μm^2 area of the CA1 was counted in each section. Only pyramidal neurons showing normal morphology with distinct cytoplasmic and nuclear outlines and a visible nucleolus were counted. Analysis of the data was performed by using Image Pro Plus 6.0 software (Media Cybernetics Co., USA).

Western blotting

On days 1 and 7 after ORIF, rats ($n=8/\text{group}$) were sacrificed with sodium pentobarbital (240 mg/ml, Department of Pharmacy, Tianjin Medical University General Hospital, i.p., 800 mg/kg) [51]. After ensuring that the heart of the rat had stopped, the brain was removed, and the hippocampal tissue was

separated. The hippocampus was homogenized in RIPA solution (Biomart, Beijing, China) buffer and then centrifuged at 4°C at 12000r/min for 10 min (Sigma 3-30KS, Sigma Laboratory Centrifuges, Germany). The quantity of protein in the supernatant was determined using a bicinchoninic acid (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, the membranes were blocked by 5% skim milk Tris-buffered saline containing 0.1% Tween (TBST) buffer for 90 min and washed with TBST buffer for 5 min. The membranes were incubated with the following primary antibodies: anti-GABA_AR α 1 (1:1,000, Abcam, Cambridge, UK), anti-BiP (1:1,000; Abcam), anti-pan-cadherin (1:2,000, Sigma, St. Louis, MO, USA), and anti- β -actin (1:10,000, Proteintech, Wuhan, China) overnight at 4°C. After washing with TBST 5 times (each for 5 min), the 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. The membranes were again washed 5 times (each for 5 min) 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 the expression of anti-pan-cadherin (1:2,000, Sigma) and anti- β -actin (1:10,000, Proteintech).

Statistical analysis

The data were analysed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Data are presented as the mean \pm standard deviation (SD). Behavioural data were tested using a two-way analysis of variance (ANOVA) with repeated measures. Other data were analysed 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

CNS, central nervous system

ER, endoplasmic reticulum

ERAD, endoplasmic reticulum-associated degradation

FC, fear conditioning

GABA, γ -aminobutyric acid

GABA_AR, γ -aminobutyric acid A type receptor

MAC, minimum alveolar concentration

mIPSC, miniature inhibitory postsynaptic current

OGD, oxygen-glucose deprivation

ORIF, open reduction and internal fixation

PND, perioperative neurocognitive disorders

ROS, reactive oxygen species

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

All authors declare that they content to publish on BMC Neuroscience.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Figures

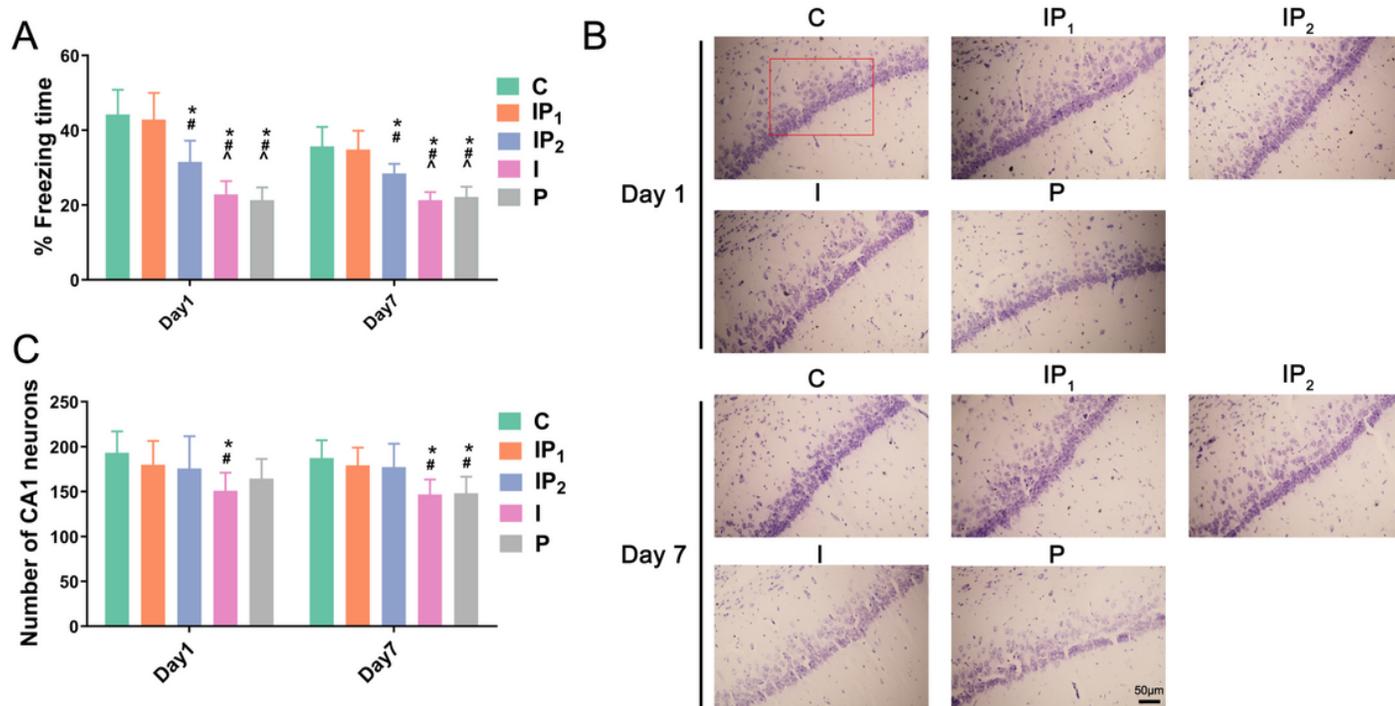


Figure 1

Combined treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol protected cognitive function and survival neurons in CH rats. (A) Hippocampus-dependent memory was evaluated as the percentage of

freezing time on day 1 and day 7 after ORIF. Data are expressed as the mean \pm SD (n=8/group). Note that ORIF resulted in a significant reduction in the time of freezing behaviour in the CA1 in Groups IP2, I and P, which was prevented by the anaesthetic schedule in Group IP1. (B) Nissl staining images of the hippocampal CA1 region were used to evaluate neuronal damage on day 1 and day 7 after ORIF. Note that ORIF resulted in a significant reduction in the number of remaining pyramidal neurons in the CA1 in Groups IP2, I and P, which was prevented by the anaesthetic schedule in Group IP1. (C) Quantification of surviving neurons in the CA1 on day 1 and day 7 after ORIF. Data are expressed as the mean \pm 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 μ m.

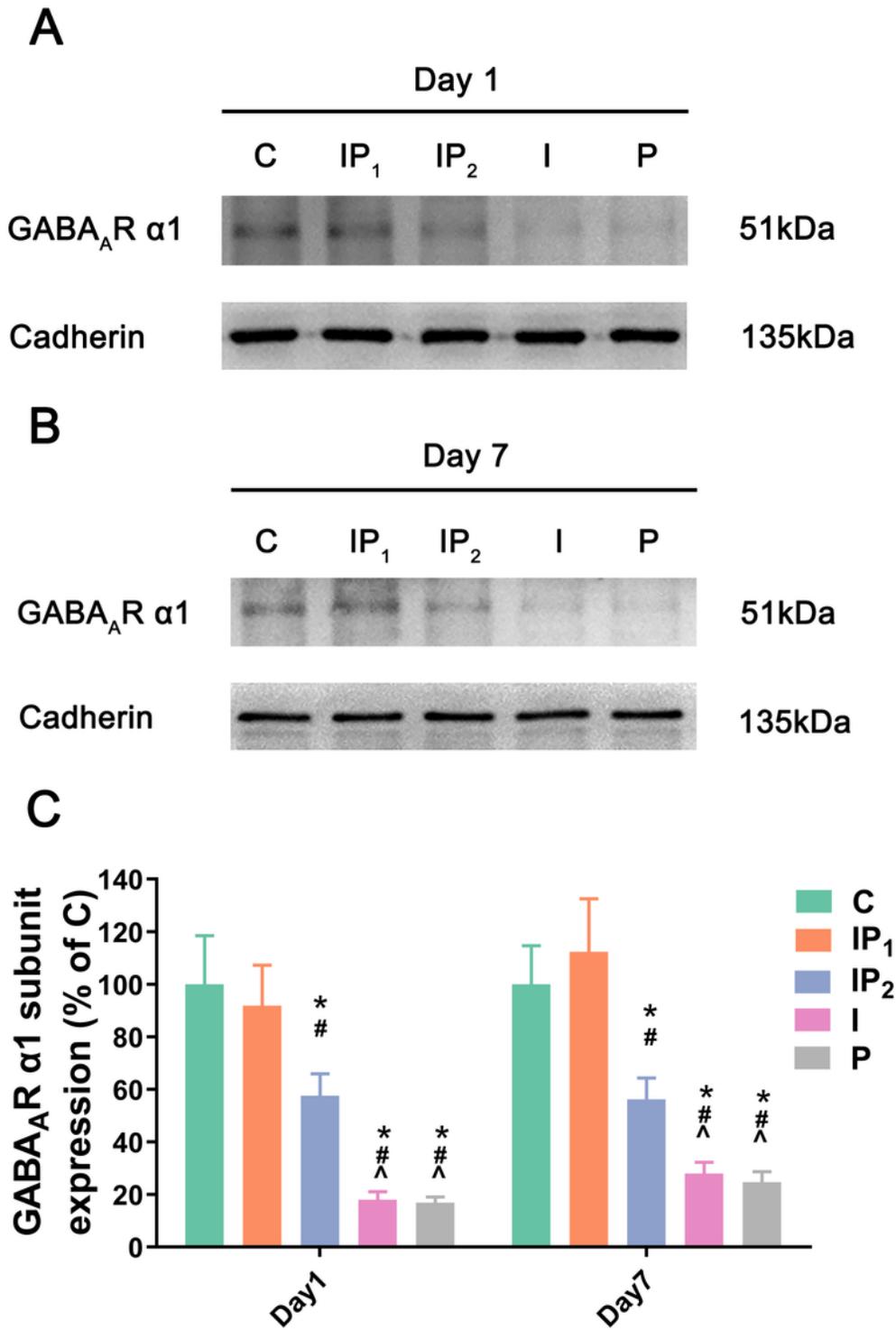


Figure 2

Combined treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol maintained the expression of the GABAAR α1 subunit. (A, B) The expression of the GABAAR α1 subunit in the hippocampus was determined by western blotting on day 1 and day 7 after ORIF. (C) Statistical graph of the expression of the GABAAR α1 subunit on day 1 and day 7 after ORIF. Data are expressed as the mean ± SD (n=8/group). Note that ORIF resulted in a significant reduction in the expression of the GABAAR α1

subunit in the CA1 in Groups IP2, I and P, which was prevented by the anaesthetic schedule in the Group IP1. * P < 0.05 compared with Group C; # P < 0.05 compared with Group IP1; ^ P < 0.05 compared with Group IP2.

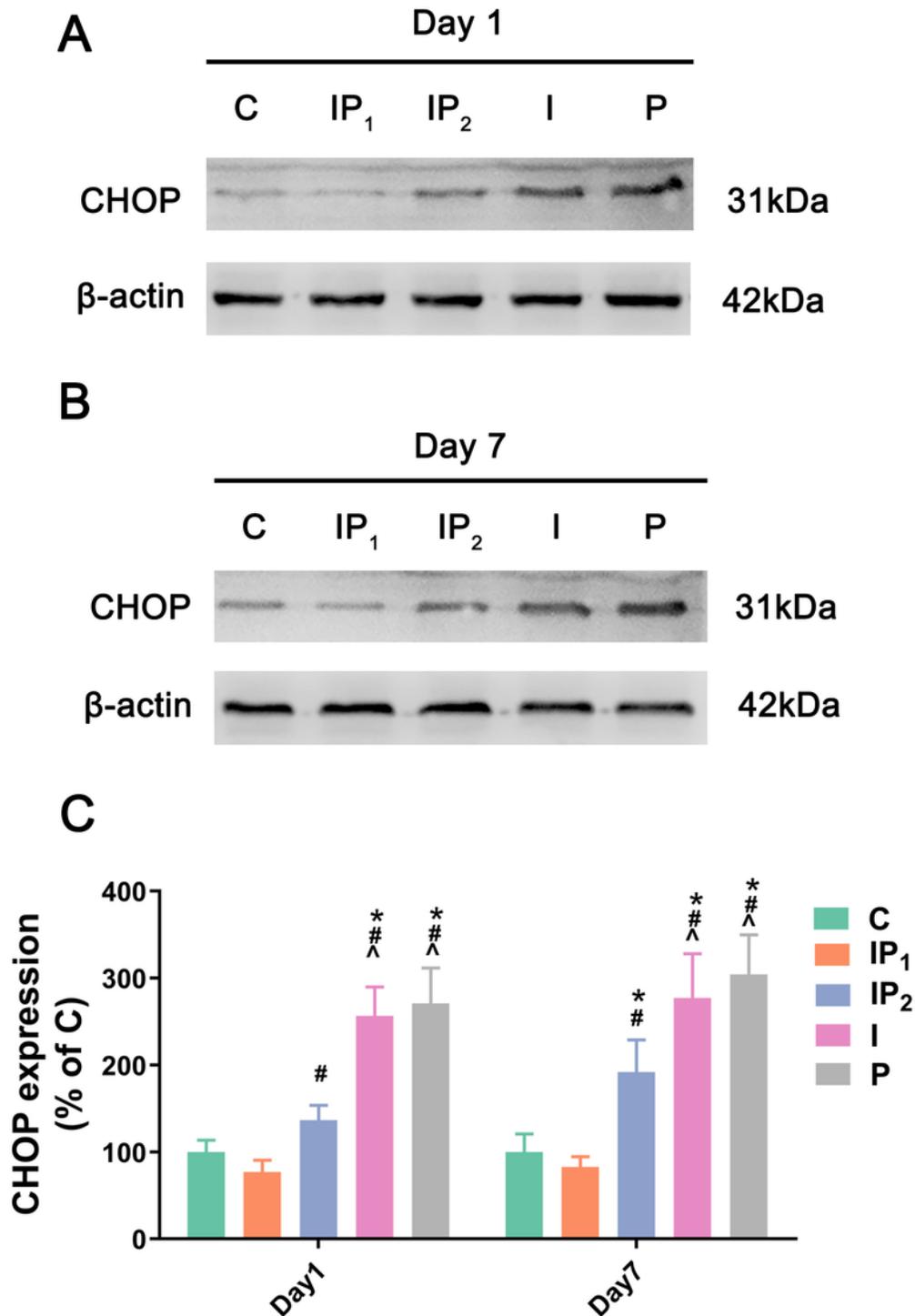


Figure 3

Combined treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol prevented ER-related apoptosis. (A, B) The expression of CHOP in the hippocampus was determined by western blotting on day 1 and day 7

after ORIF. (C) Statistical graph of the expression of CHOP on day 1 and day 7 after ORIF. Data are expressed as the mean \pm SD (n=8/group). Note that ORIF resulted in a significant increase in the expression of CHOP in the CA1 in Groups IP2, I and P, which was prevented by the anaesthetic schedule in Group IP1. * P < 0.05 compared with Group C; # P < 0.05 compared with Group IP1; ^ P < 0.05 compared with Group IP2.

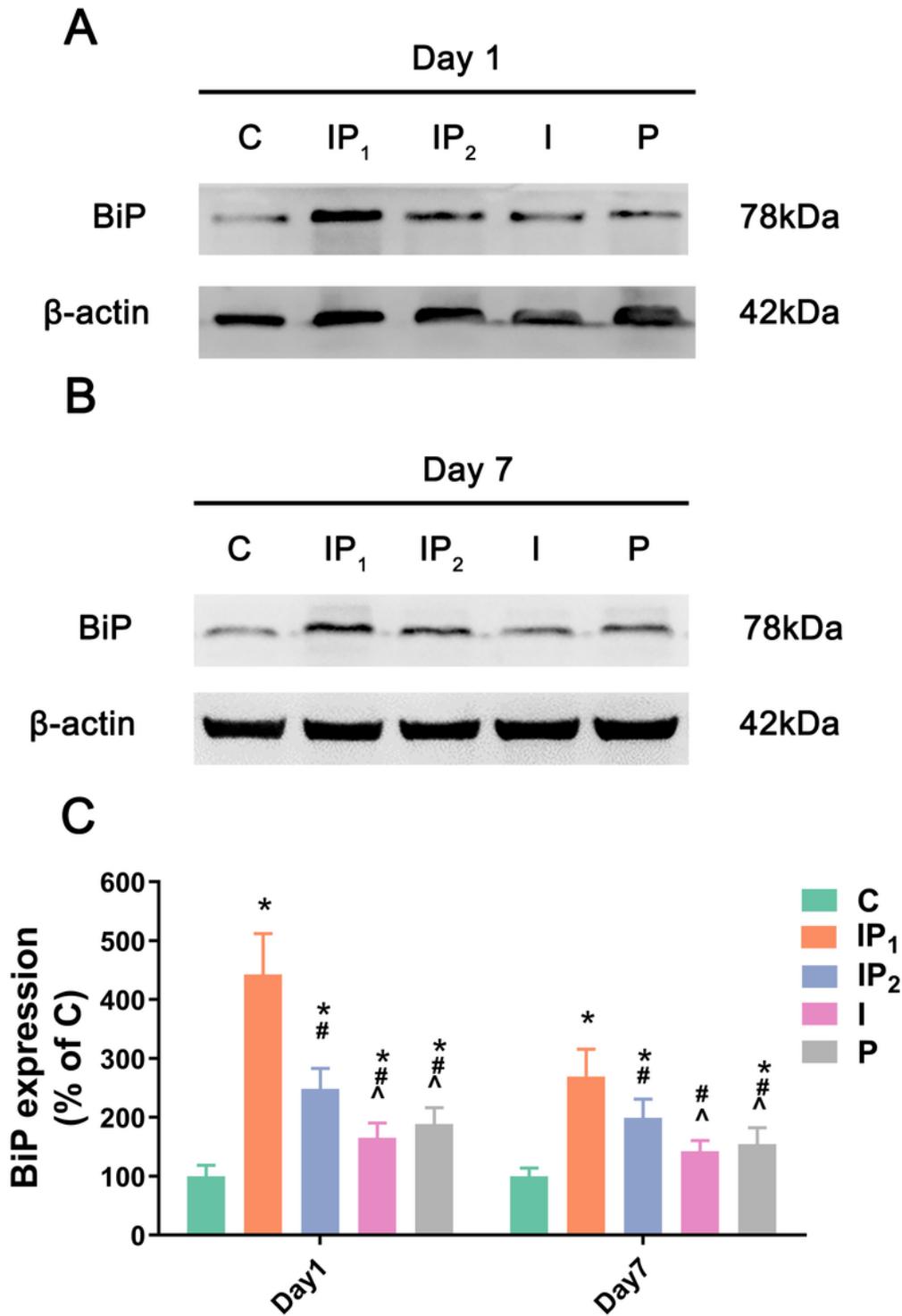


Figure 4

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

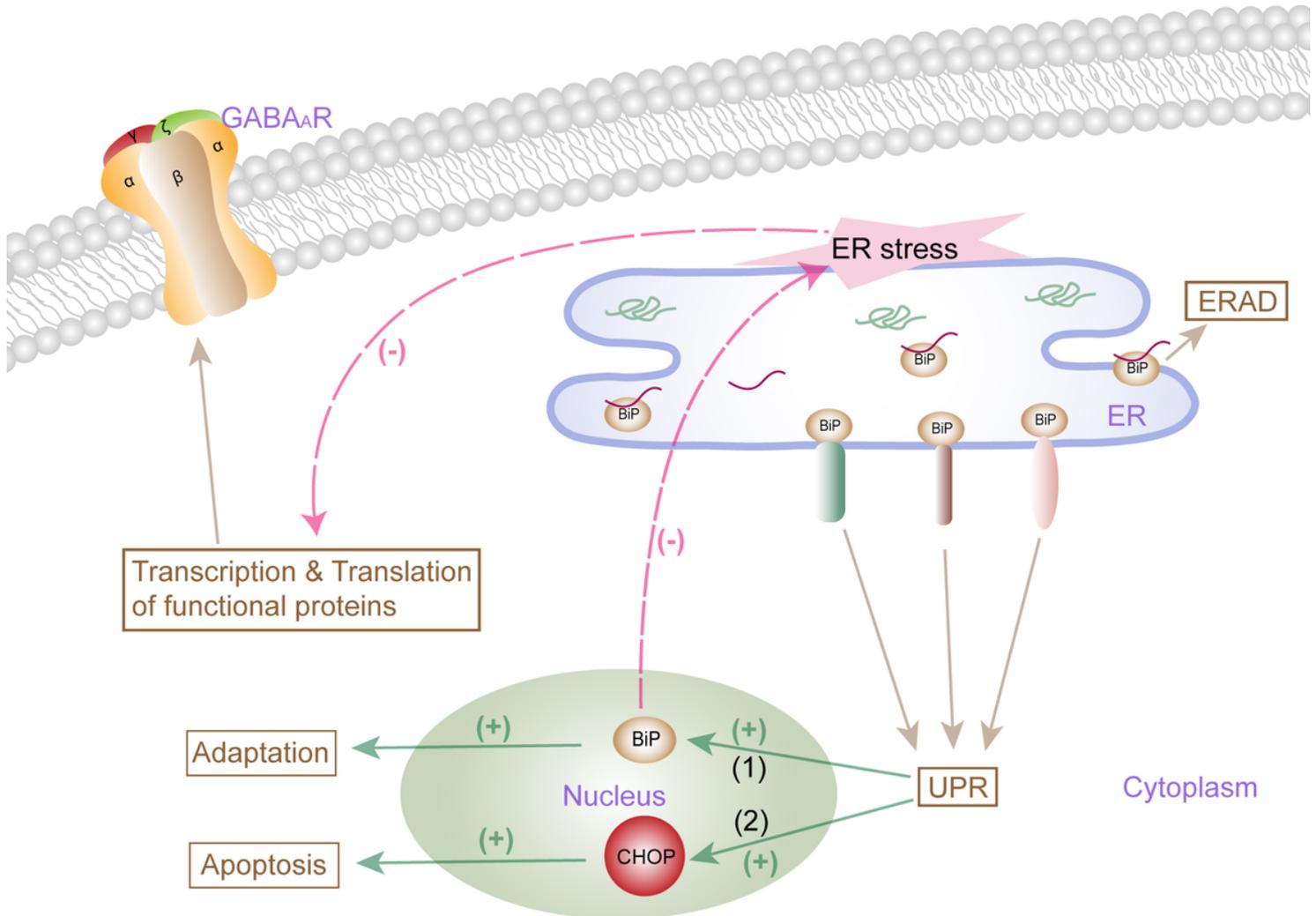


Figure 5

A proposed schematic model of the molecular mechanism that occurs under ER stress, showing either activation (1) or inhibition (2) by treatment with 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol. (1) 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol elevated the expression of BiP compared with the other groups. (2) 1% isoflurane and 20 mg·kg⁻¹·h⁻¹ propofol inhibited the expression of CHOP compared with the other groups.

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