Am J Transl Res 2018;10(2):432-443
www.ajtr.org /ISSN:1943-8141/AJTR0067615

Original Article

Mdivi-1 pretreatment mitigates isoflurane-induced cognitive deficits in developmental rats

Jie Gao, Ailin Luo, Jing Yan, Xi Fang, Xiaole Tang, Yilin Zhao, Shiyong Li

Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, Hubei, China

Received October 20, 2017; Accepted January 6, 2018; Epub February 15, 2018; Published February 28, 2018

Abstract: Accumulating evidence indicates that general anesthetics can cause acute neuroapoptosis and long-term cognitive deficit in models exposed to anesthetics during the brain growth-spurt period. Anesthetics-induced imbalance of mitochondrial fusion and fission preceded and contributed to developmental neuroapoptosis. Accordingly, the imbalance was accompanied by activation of dynamin-related protein (Drp)1 which was closely associated with synaptic degeneration in neurodegenerative diseases. Based on the neuroprotective role of mitochondrial division inhibitor-1 (mdivi-1) in neurodegeneration and stroke, we set out to examine whether mdivi-1 can mitigate developmental neurotoxicity induced by isoflurane. In the present study, we showed that 2% isoflurane exposure for 2 h triggered Drp1 dephosphorylation at serine 656 and increased translocation of Drp1 and Bax from cytosol to mitochondria, concomitant with cytochrome C leakage into the cytosol. Remarkably, pretreatment with mdivi-1 not only alleviated isoflurane-induced disturbed mitochondrial translocation of Drp1 and Bax and almost restored morphological changes, but also inhibited cytochrome C release, caspase9 and caspase3 activation in hippocampi. Furthermore, mdivi-1 mitigated the loss of synaptic proteins and long-lasting cognitive deficit in later life of rats neonatally exposed to isoflurane. Taken together, isoflurane-induced Drp1 activation and translocation led to excessive mitochondrial fission and subsequently contributed to the synaptic injury and long-term cognitive impairment. However, mdivi-1 pretreatment prevented Drp1-dependent excessive mitochondrial fission and mitigated neuroapoptosis and synaptic injury, and improved the long-term cognitive function. Thus mdivi-1 holds far-reaching insight for prophylaxis of developmental neurotoxicity induced by isoflurane.

Keywords: Mdivi-1, isoflurane, Drp1, mitochondria, Bax

Introduction

Recent experimental evidence and emerging clinical trial data indicate that extensively-used general anesthetics in pediatric surgery could be detrimental to the developmental brain, especially at the burst of brain growth [1-5]. The neurodegenerative effects are present as acute neuronal apoptosis and long-lasting alteration in synaptogenesis and cognitive impairment [4, 6-8]. Therefore, most of the recent investigations are engaged in elucidating the precise mechanism of anesthesia-induced developmental neurotoxicity so that a targeted and efficient prophylactic measurement can be contrived.

Based on published data, mitochondria are likely to be the integrated hub of general anesthetics-induced harmful signals. It seems this organelle is the initial and the most vulnerable organelle impaired by anesthesia in the brain of neonatal rat [7, 9, 10]. Sole or combined general anesthetics caused excessive mitochondrial fission even fragmentation, which may contribute to anesthetics-induced neurodegenerative alterations in the developmental brain by disrupting the balance between mitochondrial fusion and fission [6, 11, 12]. Further investigation showed that general anesthetics promoted dynamin-related protein (Drp)1 oligomerization and translocation from cytosol to mitochondria, which is associated with a disturbed mithochondrial shape [4]. It is noteworthy that Drp1-mediated disproportionate mitochondrial fission is widely involved in neuronal damage in stroke, traumatic brain injury, seizure as well as neurodegenerative diseases [13-18]. Based on these evidence, targeting Drp1 is likely to pharmaceutically beneficial in clinical practice.

Theoretically, measurements that can inactivate Drp1 or prevent Drp1 activation and/or translocation might be effective, at least partially, in mitigating anesthesia-induced developmental neurotoxicity. Mitochondrial division inhibitor-1 (mdivi-1) is a highly selective and efficient inhibitor of Drp1. Mdivi-1 bound to an allosteric site of Drp1 and impeded its conformational change that was required for self-assembly and GTP hydrolysis so that prevents mitochondria fission and formed inter-connected net-like mitochondria [19]. It was further demonstrated that mdivi-1 mitigated apoptosis via inhibiting Drp1 activity and mitochondrial outer membrane permeabilization (MOMP) and hence retarded the release of cytochrome C into cytosol. Recent investigations found that mdivi-1 provided widespread protection against various insults to brain, heart and kidney [17, 18, 20-22]. Furthermore, it is proven that mdivi-1 mitigated mitochondrial dysfunction and synaptic impairment in Alzheimer's disease, Parkinson's disease, and Huntington's disease [23-25]. Interestingly, a current study revealed that mdivi-1 inhibited propofol- or sevoflurane-induced neurotoxicity in vitro [6, 26]. These evidence paved mdivi-1 an attractively therapeutic avenue for clinical treating cerebral injuries, and also inspire us to examine the influence of mdivi-1 on the long-lasting cognitive dysfunction induced by isoflurane (an inhalational anesthetic, more neurotoxic than sevoflurane to the developmental brain [27]).

Therefore, we hypothesize that mdivi-1 alleviates isoflurane-induced cognitive dysfunction by inhibiting Drp1-mediated excessive mitochondrial fission and subsequently neuroapoptosis and synaptic injury. In this study, we found that isoflurane exposure downregulated the phosphorylation level of Drp1 (ser656) and facilitated the translocation of Drp1 and Bax to mitochondria. However, mdivi-1 pretreatment inhibited Drp1 activation and translocation, mitigated mitochondrial apoptotic pathway and improved the long-term cognitive performance during later life of rats neonatally exposed to isoflurane.

Materials and methods

Animals/experimental design

The experiments were approved by the Ethical Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, China. All experimental procedures were performed following the Guide for the National Science Council of the Republic of China. Efforts were made to minimize the number of animals used.

This study was conducted using postnatal day 7 (PND7) Sprague-Dawley rat, as it was established that PND7 rats were most susceptible to neuronal insult induced by general anesthetics [3]. Pups were purchased from Center of Experimental Animal (Tongji Medical College), Pups were cross-fostered and housed under standard laboratory conditions (room temperature: 23±1°C, relative humidity: 60±5%, 12 h light/dark cycle).

Isoflurane anesthesia and drug administration

Mdivi-1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The protocol for mdivi-1 dissolution and administration referred to that in Cui's work [9, 21].

All the pups were randomly divided into four groups: control (CON), isoflurane (ISO), mdivi-1+isoflurane (MDV+ISO), mdivi-1 (MDV). Rats in the MDV and MDV+ISO groups received a dose of 20 mg/kg mdivi-1 by intraperitoneal injection 4 h before exposure to isoflurane anesthesia. Rats in the CON and ISO groups were intraperitoneally injected with an identical volume of saline containing 0.25% DMSO. The isoflurane anesthesia procedure was performed as before. Briefly, rats in ISO and MDV+ISO were exposed to 2% isoflurane for 2 hours while rats in CON and MDV received vehicle gas (40% O_2+60% N_2). Rats were put in anesthesia-induction chambers which were kept in homeothermic incubator to maintain the environmental temperature at 33°C. At the end of anesthesia, five rats in each group were randomized to perform arterial blood analysis. Other rats were sent to their home dams until they were fully awake. Twelve hours after anesthesia ended, fifteen rats in each group were randomized to be sacrificed and the hippocampi were dissected for electron microscope (EM) analysis (n=5 in each group), mitochondrial purification (n=5 in each group), and western blotting (n=5 in each group). The left (n=10 in each group) were raised for Morris water maze (MWM) test and weaned from their mothers at PND21.

Arterial gas analysis

Immediately the isoflurane treatment ended, five rats in each group were allocated to perform arterial gas analysis with an ABL-800FLEX analyzer. Blood was aspirated percutaneously from the left cardiac ventricle.

Electron microscopy and mitochondrial ultrastructural analysis

Processing hippocampal CA1 area for electron microscopy was referred to the detailed protocol described previously [28, 29]. Ultrathin sections were cut with the ultramicrotome and scanned through a transmission electron microscopy (FEI Tecnai G12, America). The ultrastructural analysis was carried out according to the protocol used before [30-32]. Briefly, three random and non-overlapping electron micrographs were taken in each neuron. Five neurons in each animal were selected for electron microscopy. The length and size of mitochondrial were calculated by ImagePro Plus 6.0 software (Media Cybernetics Inc, Rockville, MD).

Mitochondria purification

Mitochondria isolation kit (Sigma-Aldrich Corporation, St. Louis, MO) was used to isolated mitochondria in hippocampi tissue following the instruction provided by the manufacturer. In brief, the dissected hippocampi were cut into small piece after washed twice with 2 volumes of Extraction Buffer and homogenized with 10 volumes of Extraction Buffer on the ice. The homogenate was transferred to a 2 ml Eppendorf tube and centrifuged at 600× g for 5 minutes. The supernatant liquid carefully collected into a new tube and Centrifuged at 11000× g for 10 minutes. And then the pellet was suspended with storage buffer. The supernatant was further centrifuged at 100,000 g for 1 h, and the supernatant was harvested as the cytosol fraction. Samples were kept in -80°C refrigerator for further detection.

Western blotting (WB) analysis

The protocol of this test performed as previously mentioned [33, 34]. The total protein was extracted from the dissected hippocampi using RIPA Lysis Buffer Kit (Beyotime Institute Biotechnology, Haimen, China). The concentration of total protein was assayed by the BCA kit (Boster, Wuhan, China). Sodium dodecyl sulfate polyacrylamide gels were used to separate the total protein which was transferred to 0.22 μm polyvinylidene difluoride (Millipore, Bedford, MA, USA) by electrophoresis. The membranes were blocked with 5% bovine serum albumin for 30 min at room temperature and then incubated overnight at 4°C with anti-Drp1 antibody, anti Drp1 (ser656) antibody, anti-Bax antibody, anti-cytochrome C antibody (Cell Signaling Technology, Beverly, MA), anti-active caspase9 antibody, anti-active caspase 3 antibody, anti-synapsin I antibody, anti-PSD95 antibody (Abcam, Cambridge, MA), and anti-β-actin antibody (Boster, Wuhan, China). Next day, HRP-conjugated secondary antibody (anti-mouse or anti-rabbit (1:7500, Abcam, Cambridge, MA) was used to incubate with the membranes. Predicted bands were stained and irradiated with SuperSignal West Pico (Thermo Scientific, Rockford, IL). The blots were scanned and quantified with Image Lab™ Software Systems (BIO-RAD, Hercules, CA).

Morris water maze (MWM) test

On PND60, rats underwent Morris water maze test. The detailed procedure was employed as previously described [34]. In brief, a platform (10 cm diameter) was submerged in a circular tank (120 cm diameter; 50 cm high) filled with warm (22°C) opaque water mixed homogeneously with carbonic ink. Training was administered twice each day with 7 h interval and last for four days. Each training session was comprised of three trials. At the beginning of each trial, the rat was released from pseudorandomly assigned quadrants that did not contain the platform in the tank. Each rat was given 60 s to mount the hidden platform could stay on the platform for 15 s after it was located. If rats failed to locate the hidden platform in the given time, they were guided to the platform and removed from the platform after 15 s. The time spent on locating the platform (latency) was recorded and analyzed using the EthoVision tracking system (Noldus Information Technology, Wageningen, the Netherlands).

To assess memory retention for the hidden platform location on the fifth day, a probe trial, with the platform being removed from the pool, was performed. Rats were placed in a quad-

Table 1. Effect of isoflurane exposure on physiological parameters of arterial blood gas analysis

	CON	ISO	MDV+ISO	MDV
pH	7.34±0.02	7.29±0.06	7.30±0.04	7.37±0.02
$PaCO_2$ (mmHg)	35.1±2.3	38.1±4.5	37.2±4.2	38.5±2.7
PaO_2 (mmHg)	110±14	107±13	101±7	106±4
Glucose (mmol/L)	4.5±0.4	4.5±0.7	4.9±0.7	4.3±0.3
SaO_2 (%)	98±1	96±0.9	97±0.9	99±0.5

The pH, $PaCO_2$, PaO_2, Glucose and SaO_2 levels did not differ significantly in four groups. Results were represented as mean ± SEM (n=5). PaO_2, arterial oxygen tension; $PaCO_2$, arterial carbon dioxide tension; SaO_2, arterial oxygen saturation.

rant that did not contain the platform and allowed to swim freely for 60 s. The percentage of time spent in the target quadrant was considered an indicator of memory retention.

Statistical analysis

Data were represented as mean ± SEM, and analyzed by SPSS 16.0. Results collected from the spatial acquisition trials were analyzed using a two-way ANOVA (treatments as between groups factors and time as repeated measures factor) followed by a post hoc Bonferroni multiple comparison test. All remaining data were analyzed by one-way ANOVA and followed by Tukey's post-hoc test to compare four groups. $P<0.05$ was considered significant.

Results

Arterial blood analysis

In the present study, parameters of arterial blood analysis including pH, $PaCO_2$, PaO_2, glucose and arterial oxygen saturation (SaO_2) were kept with physiological range and did not change significantly among four groups, as shown in **Table 1**.

Mdivi-1 improved spatial memory of rats undergoing neonatal exposure to isoflurane

To examine whether mdivi-1 provided neuroprotective effect against neonatal isoflurene anesthesia, the test of MWM was explored to assess the spatial learning memory at PND60 after drug treatment. As shown in **Figure 1**, isoflurane exposure damaged spatial as shown by longthening escape latency at training day 3 (**Figure 1A**, $P<0.05$), training day 4 (**Figure 1A**, $P<0.05$) and training day 5 (**Figure 1A**, $P<0.05$) compared with CON group. As expected, mdivi-1 premedication mitigated isoflurane-induced defect in spatial memeory as indicated by shortening escape latency (**Figure 1A**, $P<0.05$) at training day 4 (**Figure 1A**, $P<0.05$) and training day 5 (**Figure 1A**, $P<0.05$) compared with ISO group.

In the probe trial, isoflurane exposure decreased the percentaget ime in the target quadrant (**Figure 1B**, $P<0.01$) compared with CON group. However, mdivi-1 pretreatment significantly improved spatial cognition performance as revealed by greater percentage time in the target quadrant (**Figure 1B**, $P<0.05$) compared with ISO group. Taken together, our results suggested that mdivi-1 pretreatment attenuated isoflurane-induced long-time cognitive injury.

Mdivi-1 inhibited Drp1 phosphorylation and translocation from cytoplasm to mitochondria

Previous reports found that de phosphoryaliton of Drp1 at ser656 resulted in its activation while phosphorylation inhibited mitochondrial fission [24]. In our study, we determined the effect of isoflurane on Drp1 phosphorylation. Western blot analysis demonstrated that isoflurane anesthesia decreased the level of ser-656 phosphorylation of cytoplasmic Drp1 but did not affect the protein level of total Drp1 (**Figure 2A**, $P<0.01$). Importantly, isoflurane exposure promoted translocation of Drp1 and Bax from cytosol to mitochondria (**Figure 2B** and **2C**, $P<0.01$). However, mdivi-1 pretreatment increased the level of Drp1 phosphorylation and inhibited its translocation while did not induce any change of basic level of Drp1 in cytoplasm. In summary, mdivi-1 pretreatment reversed isoflurane-induced Drp1 dephosphorylation and migration.

Mdivi-1 pretreatment attenuated isoflrane-induced the release of cytochrome C and activation of the mitochondrial apoptotic cascade

Based on previous findings that cytochrome C release was an indicator of permeability of the mitochondrial membrane, we attempted to examine whether mdivi-1 pretreatment would

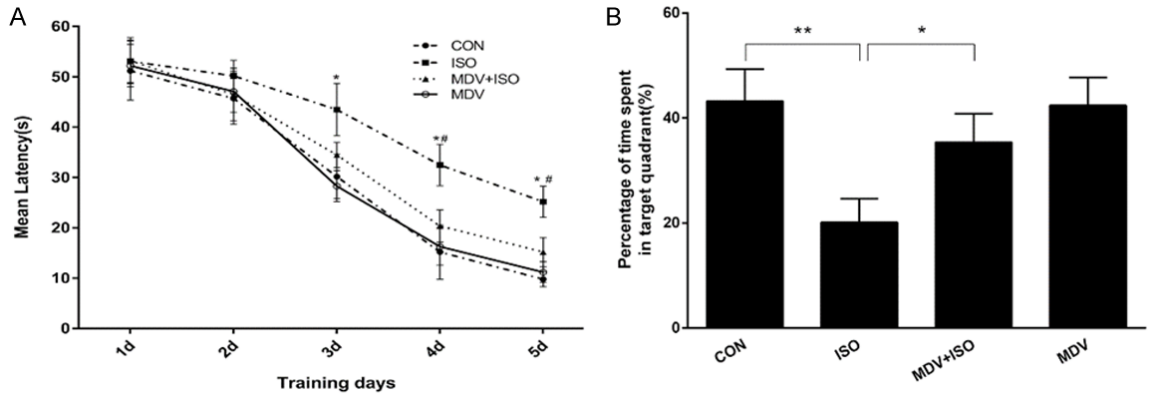

Figure 1. Mdivi-1 (20 mg/kg, i.p.) pretreatment mitigated cognitive deficit induced by neonatal exposure to isoflurane (2% for 2 h). A. Mean latency plotted against the training days in the spatial acquisition trials. Repeated measures ANOVA followed by a post hoc Bonferroni multiple comparison test (n=10 per group). ISO VS MDV+ISO, $P<0.05$. B. Percentage of time spent in target quadrant in probe test. Data are presented as mean ± SEM (n=10 per group) CON: control group; ISO: isoflurane group; MDV+ISO: mdivi-1+isoflurane group; MDV: mivi-1 group. *denotes $P<0.05$ CON vs ISO, #denotes $P<0.05$ ISO vs ISO+MDV or **denotes $P<0.01$ CON vs ISO.

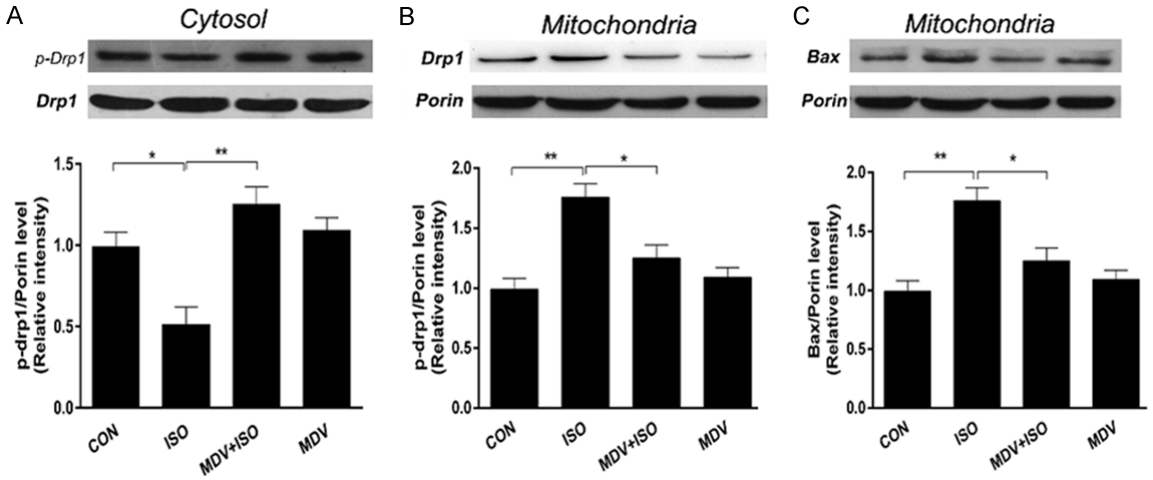

Figure 2. Mdivi-1 pretreatment inhibited isoflurane-induced Drp1 dephosphorylation (ser656) and mitochondrial translocation of Drp1 and Bax. (A) A representative graph of western blot analysis of p-Drp1 expressions in hippocampi. The relative protein level of cytosolic p-Drp1 (ser656) in hippocampi was normalized to the total cytosolic Drp1. The statistical analysis showed that application of mdivi-1 (20 mg/kg, i.p.) inhibited the isoflurane-induced dephosphorylation of cytoplasmic Drp1 in the hippocampi of neonatal rats. Relative levels of mitochondrial Drp1 (B) and Bax (C) in hippocampi of developmental rats, normalized to the internal reference Porin. The statistical analysis showed that administration of mdivi-1 (20 mg/kg, i.p.) significantly attenuated isoflurane-induced up-regulation of mitochondrial Drp-1 and Bax in hippocampi of neonatal rats. Data are presented as mean ± SEM (n=5 per group) *denotes $P<0.05$, **denotes $P<0.01$.

mitigate the change. As displayed in **Figure 3A** ($P<0.05$), isoflurane resulted in burgeoning release of cytochrome C into cytoplasm, but mdivi-1 pretreatment reduced the leak of cytochrome C from mitochondria. Moreover, mdivi-1 pretreatment curbed the activation of caspase9 (**Figure 3B**, $P<0.05$) and the executioner of mitochondrial apoptotic pathway active caspase3 (**Figure 3C**, $P<0.001$).

Midiv-1 restored isoflurane-induced excessive mitochondrial fission

Several delicate studies showed that anesthetics-triggered Drp1 activation led to excessive mitochondrial division even fragmentation. Therefore, we set out to determine whether mdivi-1 pretreatment circumvented the pathological morphological changes. As shown in

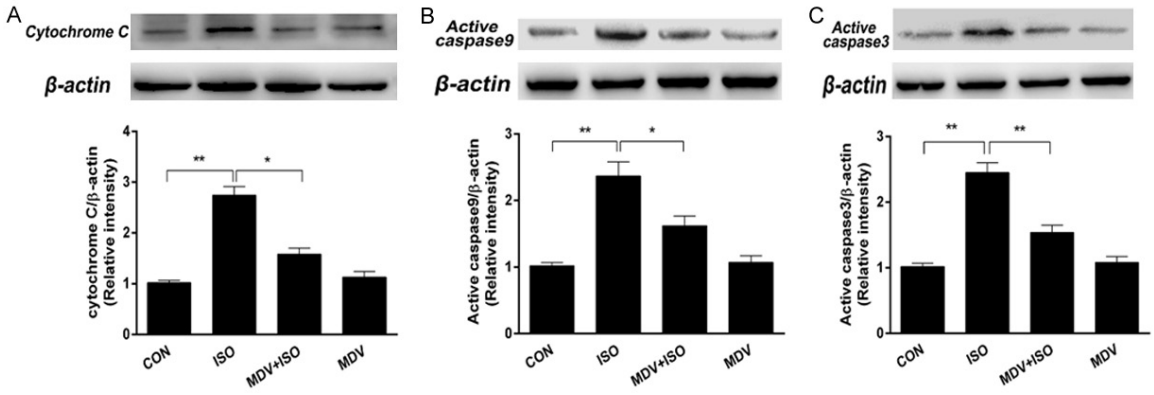

Figure 3. Mdivi-1 pretreatment attenuated isoflurane-induced the release of cytochrome C and activation of mitochondrial apoptotic cascade. A. The representative immunoblots (the upper) and quantitative graphs (the lower) of cytochrome C. Pretreatment with mdivi-1 (20 mg/kg, i.p.) significantly attenuated isoflurane-induced the release of cytochrome C in the hippocampus of neonatal rats. B. Pretreatment with mdivi-1 (20 mg/kg, i.p.) significantly reduced isoflurane-induced up-regulation of active caspase9 in the hippocampus of neonatal rats. C. Application of mdivi-1 (20 mg/kg, i.p.) significantly reduced isoflurane-induced enhancement of active caspase3 in hippocampi of neonatal rats. Data are presented as mean ± SEM (n=5 per group) *denotes $P<0.05$ or **denotes $P<0.01$.

Figure 4A ($P<0.01$), mitochondria in ISO group was markedly condensed and round-like compared to that in CON group and MDV group, while mdivi-1 pretreatment inhibited these changes in MDV+ISO group. As shown in **Figure 4B** ($P<0.05$) and **4C** ($P<0.05$), mdivi-1 pretreatment increased mitochondrial length and size that was reduced by isoflurane exposure.

Mdivi-1 mitigated isoflurane-induced synaptic protein loss

Anesthetics induced synaptic disturbance contributed to the long-term cognitive lesion. Hence we attempted to examine whether mdivi-1 pretreatment mitigated isoflurane-induced synaptic protein loss. Our data showed that isoflurane anesthesia downregulated the expression of PSD95 (**Figure 5A**, $P<0.05$) and synapsin I (**Figure 5B**, $P<0.05$) in hippocmapi. However, mdivi-1 pretreatment significantly inhibited downregulated levels of PSD95 and synapsin I, as shown in **Figure 5A** ($P<0.05$) and **Figure 5B** ($P<0.05$).

Discussion

This study indicated that neonatal isoflurane exposure promoted Drp1 dephosphorylation, an indispensable step in Drp1 conformational change and activation, and translocation from cytoplasm to mitochondria. Furthermore, isoflurane anesthesia facilitated Drp1 and Bax translocation to mitochondria. This alteration resulted in increased MOMP and promoted cytochrome C leakage into cytosol and initiated the mitochondria-dependent apoptosis. Importantly, pretreatment with mdivi-1, a highly efficient and selective blocker of Drp1, attenuated these acute conversions and long-lasting cognitive impairment of rats exposed to isoflurane. The neuroprotective effect is associated with preventing dephosphorylation and translocation of Drp1 and the following uncontrolled mitochondrial division and increased MOMP.

As known, mitochondria are dynamic organelles that keep constant fusion and fission and change their shape and location in response to cellular activity. The fusion event is mediated by Opa1, mitofusin (mfn) 1, and mfn 2. The fission process is mainly mediated by Drp1 [35, 36]. The balance of fusion and fission and the proper distribution of mitochondria in axonal growth cones and synapse is critical for neuronal function such as synaptogenesis, while disturbed mitochondrial dynamics and distribution may compromise the synaptic plasticity [14, 37-39]. In light of the great importance of mitochondrial fission in neuronal activity, tremendous effort during the last two decades has been made to decipher the physiological role in neuronal development and growth and pathophysiological mechanism in neurodegenerative diseases. It was well documented that Drp1 interacted with hyperphos-

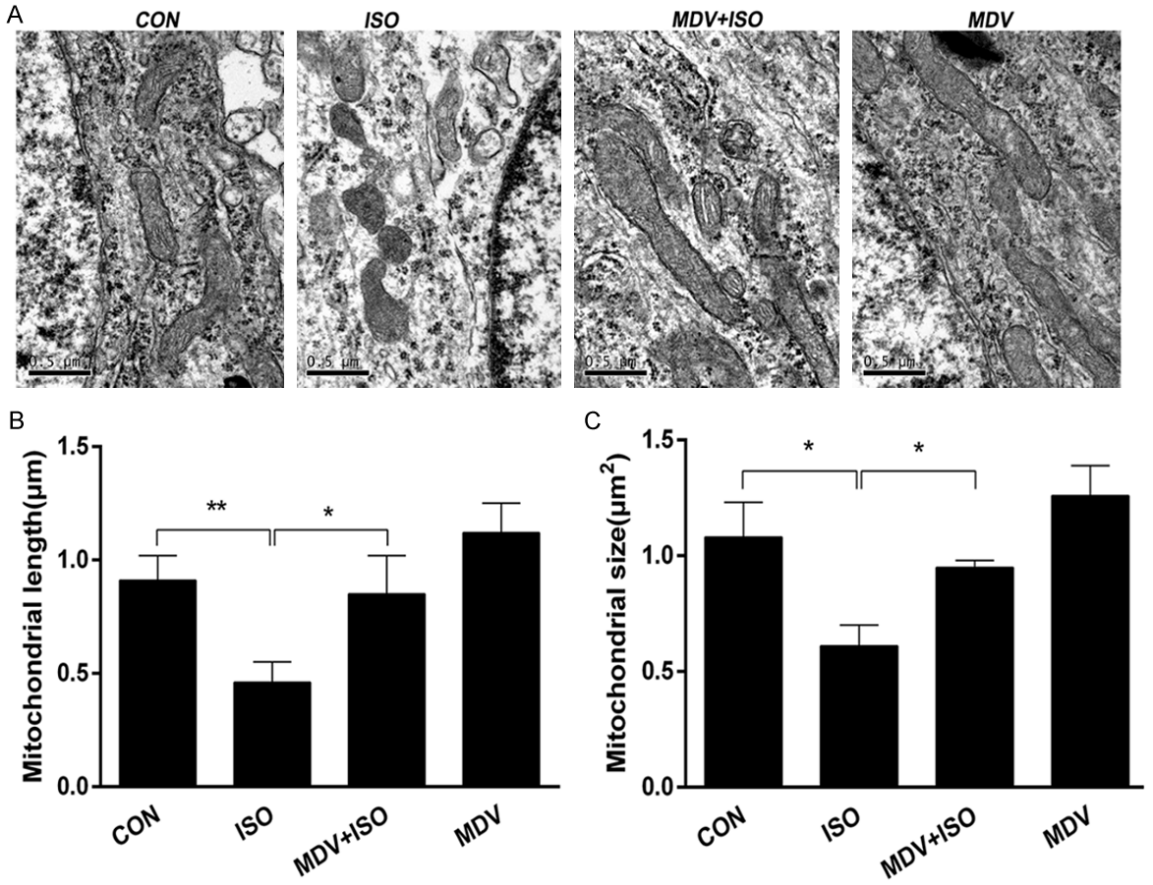

Figure 4. Mdivi-1 pretreatment alleviated isoflurane-induced mitochondrial morphological changes. A. The representative images of mitochondrial morphological changes of each group in hippocampi of developmental rats. B. Isoflurane exposure induced a significant decrease of the mitochondria length in hippocampi, whereas the decrease was reversed by the delivery of mdivi-1 (20 mg/kg, i.p.). C. Pretreatment with mdivi-1 (20 mg/kg, i.p.) significantly alleviated isoflurane-induced decrease of the mitochondrial average size in hippocampi of neonatal rats. Data are presented as mean ± SEM (n=5 per group), five neurons in each rats was scanned by transmission electron microscopy. *denotes $P<0.05$ or **denotes $P<0.01$.

phorylated-tau, amyloid beta, Parkin, and mutant huntingtin and promoted neuronal damage and synaptic degeneration [13, 16, 40, 41]. This implicates that Drp1-mediated mitochondrial fission may be a crucial event in these neurodegenerative diseases.

Although there is no direct clinical evidence that general anesthesia administered to infant can cause toxic effect on the brain, extensive data gained from rodents and primates experiments showed that neonatal exposure to general anesthetic results in acute neuronal injury in the vulnerable brain area such as hippocampus and long-term learning and memory defect in the later life [1-3, 10, 42, 43]. In fact, it has been demonstrated that general anesthetics-activated mitochondrial apoptotic pathway seemed to be the integrated step in vivo and in vitro [3, 7, 9, 10]. Further investigations found that anesthetics cocktail (isoflurane-nitrous oxide-midazolam) or sole anesthetics (sevoflurane, desflurane, or propofol) administered to the developing neurons generated neuronal ultrastructural changes, including synaptic disturbance, mitochondrial excessive division and abnormal distribution [5, 6, 11, 12, 26, 31]. This raises the interesting, although speculative, notion whether activation of factors regulating mitochondrial excessive fission could be the initial event in this neuronal injury. In this regard, several recently published investigation indicated that drp1 activation and translocation preceded the initiation of mitochondrial apoptotic cascade. Moreover, it was proposed that general anesthetics induced imbalance of

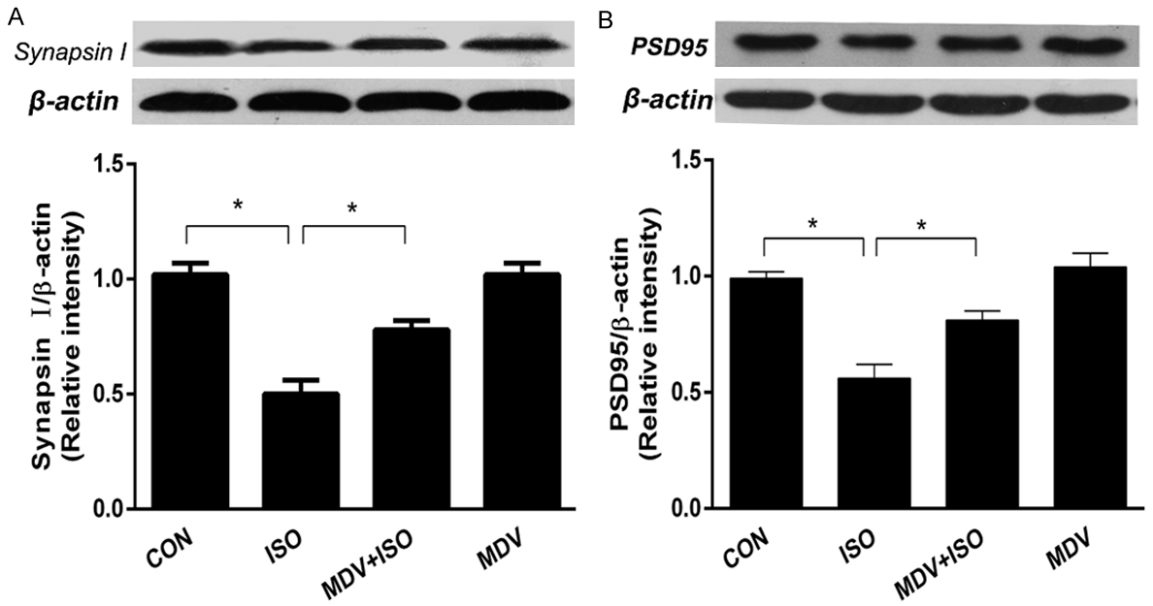

Figure 5. Mdivi-1 pretreatment mitigated isoflurane-induced synaptic protein loss in hippocampi of neonatal rat. A. Pretreatment with mdivi-1 (20 mg/kg, i.p.) increased the hippocampal expression of synapsin I that was reduced by isoflurane exposure. B. Prophylactic application with mdivi-1 (20 mg/kg, i.p.) upregulated the expression of PSD95 that was weakened by isoflurane anesthesia in hippocampi. Data are represented as mean ± SEM (n=5 per group), *denotes $P<0.05$.

mitochondrial ultrastructure accompanied by Drp1 oligomerization [4, 5]. Consistent with previous results, our study found that isoflurane, a clinically used inhalational anesthetic, repressed expression of phosphorylated Drp1 (ser656) and promoted Drp1 and Bax mitochondrial translocation in hippocampi of developing rats. Unexpectedly, we found that Drp1 and Bax formed complex in cytosol using co-immunoprecipitation. We proposed that this combination might facilitate Bax mitochondrial shift as inhibiting Drp1 dephosphorylation decreased Bax translocation. More importantly, we found that mdivi-1 treatment prior to isoflurane anesthesia remarkably inhibited the Drp1 phosphorylation, translocation, and the interaction with Bax. Mdivi-1 pretreatment also reduced the leakage of cytochrome C into cytosol and the expression of active caspase9 and active caspase3. This implied that drp1 dephosphorylation at ser656 was involved in isoflurane-induced neuroapoptosis and inhibition of Drp1 activation may offer neuroprotection against isoflurane-induced developmental neurotoxicity.

Dephosphorylation of Drp1 at serine656 promoted excessive mitochondrial fission even fragmentation and augmented the susceptibility to apoptotic stimuli [44]. Some studies argued that Drp1-mediated mitochondrial fission did not inevitably cause apoptosis, but it had been proven an important step in apoptosis [45, 46]. The phosphorylation of Drp1 is regulated by a few kinases in the different context [47]. However, it remains elusive which signaling pathways are involved in isoflurane-induced Drp1 dephosphorylation. The pro-apoptotic factor Bax plays an essential role in execution of apoptotic cascade. However, it was reported that Drp1-dependent mitochondrial fission is dispensable for Bax-dependent apoptosis [48, 49], while Drp1-mediated apoptotic mitochondrial fission is independent of Bax translocation to mitochondria [50]. These two factors seem irrelevant to each other in the apoptotic program. In fact, recent studies found that Drp1 is involved in Bax shift to mitochondria in response to apoptotic stress [51, 52]. In line with these results, we found that Drp1 bound with Bax in cytosol. This may imply that Drp1 facilitates Bax mitochondrial translocation in hippocampi after isoflurane exposure.

Mdivi-1 is an efficient and selective inhibitor of Drp1. Evidence suggested that mdivi-1 produced rapid, reversible, and inter-net-like mitochondria in mammalian cells [19, 53]. In addi-

tion, observation demonstrated that mdivi-1 inhibited the activity of Drp1 by directly binding to an allosteric site so that prevented the conformational change and subsequent Drp1 oligomerization and GTP hydrolysis [19]. The anti-apoptotic characteristic of mdivi-1 makes it an attractive pharmaceutical measurement for neurodegenerative diseases and stroke. For instance, mdivi-1 protected hippocampal neurons from epilepsy-induced apoptosis [22] and traumatic brain injury [54]. Strikingly, it had demonstrated that mdivi-1 prevented neuronal damage in Alzheimer's disease, Parkinson's disease, and Huntington's disease [23-25]. Recent studies also found that mdivi-1 provided neuroprotective effect against ischemic brain injuries and reduces the cognitive injuries [18, 21, 29, 55]. Moreover, mdivi-1 offered anti-apoptotic effect in cardiac ischemic models and renal injury models [17, 20].

In accordance with these observations, we found that mdivi-1 pretreatment mitigated isoflurane-induced ultrastructural change of mitochondria and inhibited neuroapoptosis in hippocampus. In addition, mdivi-1 inhibited isoflurane-induced release of cytochrome C confined in mitochondrial cristae and downregulated the expression of active caspase9 and active caspase3 in hippocampi. More importantly, we found that mdivi-1 alleviated, not completely inhibited, long-term cognitive impairment in rats receiving isoflurane anesthesia on PND7. As aforementioned, synaptic degeneration is another demonstration of developmental neurotoxicity induced by isoflurane. In our study, the expression of two representative proteins of synaspsin I and PSD95 were both reduced after isoflurane exposure. However, mdivi-1 pretreatment effectively mitigated this change. This indicates that mdivi-1 also can attenuate isoflurane-induced synaptic impairment.

In present study, we found mdivi-1 pretreatment almost completely inhibited Drp1 dephosphorylation and mitochondrial translocation and did mitigate, but not fully restore, isoflurane-induced neuroapoptosis, synaptic injury, and cognitive deficit. In our opinion, this phenomenon might be attributed to several factors. First of all, besides phosphorylation, the activity of Drp1 is regulated in several post-translational modification manners including ubiquitination, SUMOylation, and S-nitrosylation. It is unclear whether mdivi-1 could repress drp1 activity in all ways. We cannot rule out the possibility that isoflurane activates Drp1 in other ways except phosphorylation. An alternative explanation might be that other paralleled components in maintaining the equilibrium between mitochondrial fusion and fission is dysregulated and involved in the neurotoxicity. A recent study found that Drp1 mediated the acute neuronal injury while degradation of mfn2, located on mitochondrial out membrane and involved in mediating the process of mitochondrial fusion, contributed to delayed excitotoxic damage [56]. Previous investigations showed that anesthetic altered the expression of mfn2 in developing brain and cultured neurons [4, 26]. However, it needs to be further examined whether the disturbed mfn2 expression is related to neurotoxicity induced by anesthetics in developmental brain.

Conclusion

Data from this study showed that mdivi-1 pretreatment mitigated cognitive impairment of rats exposed to isoflurane at PND7 by preventing Drp1 phosphorylation and translocation to mitochondria and synaptic protein loss. The neuroprotective effect of mdivi-1 is associated with inhibiting activation of mitochondria-dependent apoptotic cascade and synaptic degeneration. Mdivi-1 hold potential to be an efficient prophylactic measurement for isoflurane-induced acutely developmental neurotoxicity and long-lasting cognitive dysfunction.

Acknowledgements

The present work was supported by grants from the National Natural Science Foundation of China (grant No. 81400882 to Shiyong Li, grant Nos. 81771159, 81571047 and 81271233 to Ailin Luo, grant No. 8150051085 to Yilin Zhao), Science and Technology Projects of Wuhan (grant number 2015060101010036 to Ailin Luo) and also supported by 2010 Clinical Key Disciplines Construction Grant from the Ministry of Health of China (grant to Anesthesiology Disciplines of tongji medical college).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shiyong Li, Department of Anesthesiology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, Hubei, China. Tel: +86-27-83665480; E-mail: syli@tjh.tjmu.edu.cn; shiyongli@hust.edu.cn

References

[1] Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, Dissen GA, Creeley CE and Olney JW. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. Anesthesiology 2010; 112: 834-841.

[2] Culley DJ, Baxter MG, Yukhananov R and Crosby G. Long-term impairment of acquisition of a spatial memory task following isoflurane-nitrous oxide anesthesia in rats. Anesthesiology 2004; 100: 309-314.

[3] Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW and Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23: 876-882.

[4] Boscolo A, Milanovic D, Starr JA, Sanchez V, Oklopcic A, Moy L, Ori CC, Erisir A and Jevtovic-Todorovic V. Early exposure to general anesthesia disturbs mitochondrial fission and fusion in the developing rat brain. Anesthesiology 2013; 118: 1086-97.

[5] Zhang Y, Xu Z, Wang H, Dong Y, Shi HN, Culley DJ, Crosby G, Marcantonio ER, Tanzi RE and Xie Z. Anesthetics isoflurane and desflurane differently affect mitochondrial function, learning, and memory. Ann Neurol 2012; 71: 687-98.

[6] Xu F, Armstrong R, Urrego D, Qazzaz M, Pehar M, Armstrong JN, Shutt T and Syed N. The mitochondrial division inhibitor Mdivi-1 rescues mammalian neurons from anesthetic-induced cytotoxicity. Mol Brain 2016; 9: 35.

[7] Zhang Y, Dong Y, Wu X, Lu Y, Xu Z, Knapp A, Yue Y, Xu T and Xie Z. The mitochondrial pathway of anesthetic isoflurane-induced apoptosis. J Biol Chem 2010; 285: 4025-4037.

[8] Zhao YL, Xiang Q, Shi QY, Li SY, Tan L, Wang JT, Jin XG and Luo AL. GABAergic excitotoxicity injury of the immature hippocampal pyramidal neurons' exposure to isoflurane. Anesth Analg 2011; 113: 1152-1160.

[9] Zhang G, Dong Y, Zhang B, Ichinose F, Wu X, Culley DJ, Crosby G, Tanzi RE and Xie Z. Isoflurane-induced caspase-3 activation is dependent on cytosolic calcium and can be attenuated by memantine. J Neurosci 2008; 28: 4551-4560.

[10] Yon JH, Daniel-Johnson J, Carter LB and Jevtovic-Todorovic V. Anesthesia induces neuronal cell death in the developing rat brain via the intrinsic and extrinsic apoptotic pathways. Neuroscience 2005; 135: 815-827.

[11] Sanchez V, Feinstein SD, Lunardi N, Joksovic PM, Boscolo A, Todorovic SM and Jevtovic-Todorovic V. General anesthesia causes long-term impairment of mitochondrial morphogenesis and synaptic transmission in developing rat brain. Anesthesiology 2011; 115: 992-1002.

[12] Lunardi N, Ori C, Erisir A and Jevtovic-Todorovic V. General anesthesia causes long-lasting disturbances in the ultrastructural properties of developing synapses in young rats. Neurotox Res 2010; 17: 179-188.

[13] Manczak M and Reddy PH. Abnormal interaction between the mitochondrial fission protein Drp1 and hyperphosphorylated tau in Alzheimer's disease neurons: implications for mitochondrial dysfunction and neuronal damage. Hum Mol Genet 2012; 21: 2538-2547.

[14] DuBoff B, Gotz J and Feany MB. Tau promotes neurodegeneration via DRP1 mislocalization in vivo. Neuron 2012; 75: 618-632.

[15] Song W, Chen J, Petrilli A, Liot G, Klinglmayr E, Zhou Y, Poquiz P, Tjong J, Pouladi MA, Hayden MR, Masliah E, Ellisman M, Rouiller I, Schwarzenbacher R, Bossy B, Perkins G and Bossy-Wetzel E. Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. Nat Med 2011; 17: 377-382.

[16] Wang DB, Garden GA, Kinoshita C, Wyles C, Babazadeh N, Sopher B, Kinoshita Y and Morrison RS. Declines in Drp1 and parkin expression underlie DNA damage-induced changes in mitochondrial length and neuronal death. J Neurosci 2013; 33: 1357-1365.

[17] Ong SB, Subrayan S, Lim SY, Yellon DM, Davidson SM and Hausenloy DJ. Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. Circulation 2010; 121: 2012-2022.

[18] Grohm J, Kim SW, Mamrak U, Tobaben S, Cassidy-Stone A, Nunnari J, Plesnila N and Culmsee C. Inhibition of Drp1 provides neuroprotection in vitro and in vivo. Cell Death Differ 2012; 19: 1446-1458.

[19] Cassidy-Stone A, Chipuk JE, Ingerman E, Song C, Yoo C, Kuwana T, Kurth MJ, Shaw JT, Hinshaw JE, Green DR and Nunnari J. Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Dev Cell 2008; 14: 193-204.

[20] Brooks C, Wei Q, Cho SG and Dong Z. Regulation of mitochondrial dynamics in acute kidney injury in cell culture and rodent models. J Clin Invest 2009; 119: 1275-1285.

[21] Cui M, Ding H, Chen F, Zhao Y, Yang Q and Dong Q. Mdivi-1 protects against ischemic brain injury via elevating extracellular adenosine in a cAMP/CREB-CD39-dependent manner. Mol Neurobiol 2016; 53: 240-253.
[22] Qiu X, Cao L, Yang X, Zhao X, Liu X, Han Y, Xue Y, Jiang H and Chi Z. Role of mitochondrial fission in neuronal injury in pilocarpine-induced epileptic rats. Neuroscience 2013; 245: 157-165.
[23] Gan X, Huang S, Wu L, Wang Y, Hu G, Li G, Zhang H, Yu H, Swerdlow RH, Chen JX and Yan SS. Inhibition of ERK-DLP1 signaling and mitochondrial division alleviates mitochondrial dysfunction in Alzheimer's disease cybrid cell. Biochim Biophys Acta 2014; 1842: 220-231.
[24] Dagda RK, Gusdon AM, Pien I, Strack S, Green S, Li C, Van Houten B, Cherra SR and Chu CT. Mitochondrially localized PKA reverses mitochondrial pathology and dysfunction in a cellular model of Parkinson's disease. Cell Death Differ 2011; 18: 1914-1923.
[25] Cui M, Tang X, Christian WV, Yoon Y and Tieu K. Perturbations in mitochondrial dynamics induced by human mutant PINK1 can be rescued by the mitochondrial division inhibitor mdivi-1. J Biol Chem 2010; 285: 11740-11752.
[26] Twaroski DM, Yan Y, Zaja I, Clark E, Bosnjak ZJ and Bai X. Altered mitochondrial dynamics contributes to propofol-induced cell death in human stem cell-derived neurons. Anesthesiology 2015; 123: 1067-1083.
[27] Ramage TM, Chang FL, Shih J, Alvi RS, Quitoriano GR, Rau V, Barbour KC, Elphick SA, Kong CL, Tantoco NK, Ben-Tzur D, Kang H, McCreery MS, Huang P, Park A, Uy J, Rossi MJ, Zhao C, Di Geronimo RT, Stratmann G and Sall JW. Distinct long-term neurocognitive outcomes after equipotent sevoflurane or isoflurane anaesthesia in immature rats. Br J Anaesth 2013; 110 Suppl 1: i39-46.
[28] Graham L and Orenstein JM. Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat Protoc 2007; 2: 2439-2450.
[29] Zuo W, Zhang S, Xia CY, Guo XF, He WB and Chen NH. Mitochondria autophagy is induced after hypoxic/ischemic stress in a Drp1 dependent manner: the role of inhibition of Drp1 in ischemic brain damage. Neuropharmacology 2014; 86: 103-115.
[30] Sasaki S. Determination of altered mitochondria ultrastructure by electron microscopy. Methods Mol Biol 2010; 648: 279-290.
[31] Boscolo A, Starr JA, Sanchez V, Lunardi N, DiGruccio MR, Ori C, Erisir A, Trimmer P, Bennett J and Jevtovic-Todorovic V. The abolishment of anesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: the importance of free oxygen radicals and mitochondrial integrity. Neurobiol Dis 2012; 45: 1031-1041.
[32] Boscolo A, Milanovic D, Starr JA, Sanchez V, Oklopcic A, Moy L, Ori CC, Erisir A and Jevtovic-Todorovic V. Early exposure to general anesthesia disturbs mitochondrial fission and fusion in the developing rat brain. Anesthesiology 2013; 118: 1086-1097.
[33] Li SY, Xia LX, Zhao YL, Yang L, Chen YL, Wang JT and Luo AL. Minocycline mitigates isoflurane-induced cognitive impairment in aged rats. Brain Res 2013; 1496: 84-93.
[34] Wang W, Chen X, Zhang J, Zhao Y, Li S, Tan L, Gao J, Fang X and Luo A. Glycyrrhizin attenuates isoflurane-induced cognitive deficits in neonatal rats via its anti-inflammatory activity. Neuroscience 2016; 316: 328-336.
[35] Detmer SA and Chan DC. Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol 2007; 8: 870-879.
[36] Liesa M, Palacin M and Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiol Rev 2009; 89: 799-845.
[37] Verstreken P, Ly CV, Venken KJ, Koh TW, Zhou Y and Bellen HJ. Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. Neuron 2005; 47: 365-378.
[38] Ishihara N, Nomura M, Jofuku A, Kato H, Suzuki SO, Masuda K, Otera H, Nakanishi Y, Nonaka I, Goto Y, Taguchi N, Morinaga H, Maeda M, Takayanagi R, Yokota S and Mihara K. Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. Nat Cell Biol 2009; 11: 958-966.
[39] Sheng ZH and Cai Q. Mitochondrial transport in neurons: impact on synaptic homeostasis and neurodegeneration. Nat Rev Neurosci 2012; 13: 77-93.
[40] Manczak M, Calkins MJ and Reddy PH. Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: implications for neuronal damage. Hum Mol Genet 2011; 20: 2495-2509.
[41] Shirendeb UP, Calkins MJ, Manczak M, Anekonda V, Dufour B, McBride JL, Mao P and Reddy PH. Mutant huntingtin's interaction with mitochondrial protein Drp1 impairs mitochondrial biogenesis and causes defective axonal transport and synaptic degeneration in Huntington's disease. Hum Mol Genet 2012; 21: 406-420.
[42] Creeley CE, Dikranian KT, Dissen GA, Back SA, Olney JW and Brambrink AM. Isoflurane-induced apoptosis of neurons and oligodendro-

cytes in the fetal rhesus macaque brain. Anesthesiology 2014; 120: 626-638.
[43] Loepke AW, Istaphanous GK, McAuliffe JJ 3rd, Miles L, Hughes EA, McCann JC, Harlow KE, Kurth CD, Williams MT, Vorhees CV and Danzer SC. The effects of neonatal isoflurane exposure in mice on brain cell viability, adult behavior, learning, and memory. Anesth Analg 2009; 108: 90-104.
[44] Cribbs JT and Strack S. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep 2007; 8: 939-944.
[45] Lee YJ, Jeong SY, Karbowski M, Smith CL and Youle RJ. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell 2004; 15: 5001-5011.
[46] Jagasia R, Grote P, Westermann B and Conradt B. DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature 2005; 433: 754-760.
[47] Reddy PH, Reddy TP, Manczak M, Calkins MJ, Shirendeb U and Mao P. Dynamin-related protein 1 and mitochondrial fragmentation in neurodegenerative diseases. Brain Res Rev 2011; 67: 103-118.
[48] Clerc P, Ge SX, Hwang H, Waddell J, Roelofs BA, Karbowski M, Sesaki H and Polster BM. Drp1 is dispensable for apoptotic cytochrome c release in primed MCF10A and fibroblast cells but affects Bcl-2 antagonist-induced respiratory changes. Br J Pharmacol 2014; 171: 1988-1999.
[49] Park JH, Ko J, Hwang J and Koh HC. Dynamin-related protein 1 mediates mitochondria-dependent apoptosis in chlorpyrifos-treated SH-SY5Y cells. Neurotoxicology 2015; 51: 145-157.
[50] Oettinghaus B, D'Alonzo D, Barbieri E, Restelli LM, Savoia C, Licci M, Tolnay M, Frank S and Scorrano L. DRP1-dependent apoptotic mitochondrial fission occurs independently of BAX, BAK and APAF1 to amplify cell death by BID and oxidative stress. Biochim Biophys Acta 2016; 1857: 1267-1276.
[51] Grosse L, Wurm CA, Bruser C, Neumann D, Jans DC and Jakobs S. Bax assembles into large ring-like structures remodeling the mitochondrial outer membrane in apoptosis. EMBO J 2016; 35: 402-413.
[52] Xu W, Jing L, Wang Q, Lin CC, Chen X, Diao J, Liu Y and Sun X. Bax-PGAM5L-Drp1 complex is required for intrinsic apoptosis execution. Oncotarget 2015; 6: 30017-30034.
[53] Lackner LL and Nunnari J. Small molecule inhibitors of mitochondrial division: tools that translate basic biological research into medicine. Chem Biol 2010; 17: 578-583.
[54] Wu Q, Xia SX, Li QQ, Gao Y, Shen X, Ma L, Zhang MY, Wang T, Li YS, Wang ZF, Luo CL and Tao LY. Mitochondrial division inhibitor 1 (Mdivi-1) offers neuroprotection through diminishing cell death and improving functional outcome in a mouse model of traumatic brain injury. Brain Res 2016; 1630: 134-143.
[55] Zuo W, Yang PF, Chen J, Zhang Z and Chen NH. Drp-1, a potential therapeutic target for brain ischaemic stroke. Br J Pharmacol 2016; 173: 1665-1677.
[56] Martorell-Riera A, Segarra-Mondejar M, Munoz JP, Ginet V, Olloquequi J, Perez-Clausell J, Palacin M, Reina M, Puyal J, Zorzano A and Soriano FX. Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death. EMBO J 2014; 33: 2388-2407.