Dexmedetomidine reduced isoflurane neurotoxicity by preventing cytoskeletal depolymerization via the BDNF/RhoA signal pathway

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Research Article

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Abstract

Background

Dexmedetomidine (Dex) has a significant neuroprotective effect in isoflurane-induced neurotoxicity during the critical period of synaptogenesis. However, the mechanisms by which Dex protects developing neurons are not clear. This research evaluated the protective effect of Dex against neuronal damage induced by isoflurane using a mouse model.

Methods

Neonatal Swiss mice at postnatal day 7 (PND7) were injected intraperitoneally with 15μg/kg, 20μg/kg, or 25μg/kg Dex, or normal saline, and then treated with 2% isoflurane for 2h. The results showed that 20μg/kg Dex could reduce isoflurane-induced neurocognitive deficits as assessed using the Morris water maze. The mechanisms by which Dex protected neurons were investigated using hippocampal neurons isolated from PND4-7 mice and exposed to 2% isoflurane (2h). Dex prevented cytoskeletal depolymerization that was induced by isoflurane.

Results

15μg/ml Dex significantly reduced the percentage of apoptotic neurons. Dex reduced expression of activated caspase-3 in neurons compared to isoflurane only exposed mice. Using primary cell cultures from neonatal mouse hippocampi, we determined that dexmedetomidine could reverse isoflurane-induced RhoA (a small

Background

Isoflurane is a volatile general inhalation anesthetic, which is commonly used in clinical medicine due to its capacity for rapid induction and ability to stabilize the hemodynamic status of patients. However, studies have reported the occurrence of neurodegeneration and cognitive deficits in the brains of rodents when they are exposed to isoflurane during synaptogenesis [1–3]. Brain et al.(2011)reported recently that isoflurane-mediated neurotoxicity is caused by cytoskeletal depolymerization as a result of RhoA activation. Dexmedetomidine is a highly selective, α_2-agonist sedative drug, which possesses several properties that might additionally benefit critically ill patients who require short term sedation and analgesia [4]. Dex can reduce the hemodynamic response to intubation and extubation, attenuate the stress response to surgery, and induce a sedative response that exhibits properties similar to natural sleep [5, 6]. In recent years, Dex has been reported to have neuroprotective effects [7] and alleviated isoflurane-induced neurotoxicity in aged rats [8]. Sanders et al reported that Dex attenuated isoflurane-induced neurocognitive impairment and neuronal apoptosis in neonatal rats [9, 10]. Su *et al* (2017) reported that Dex could mitigate neuronal apoptosis induced by exposure to isoflurane, and reverse down-regulation of

brain-derived neurotrophic factor (BDNF) expression in fetal rats during the second trimester of pregnancy. Su et al. indicated that BDNF participated in the process of neuronal apoptosis reduction, but they did not clarify the mechanism by which BDNF might have acted in this process [11].

BDNF is a key neurotrophin in the process of synaptogenesis and also is involved in neurogenesis and neuroplasticity in the brain. BDNF is initially synthesized as proBDNF (proneurotrophin), which is cleaved to form mature BDNF. Both proBDNF and mature BDNF have signaling properties. BDNF is secreted as proBDNF from synaptic vesicles and is proteolytically cleaved by plasmin to form mature BDNF [12, 13]. Signaling by mature BDNF acts through the tropomyosin receptor kinase B (TrkB) to mediate neurite growth and survival, as well as cause cell differentiation [14]. On the other hand, proBDNF activation of the p75 neurotrophin receptor ($p75^{NTR}$) causes pruning of neuronal processes through actin cytoskeletal depolymerization and apoptosis [15]. Lemkuil et al. found that isoflurane neurotoxicity is mediated through a shift in the balance of signaling by mature BDNF to proBDNF/$p75^{NTR}$ (Lemkuil, Head, Pearn, Patel, Drummond and Patel 2011). Due to this shift, increased signaling by proBDNF triggered by isoflurane exposure could result in actin cytoskeletal depolymerization, leading to loss of nascent synapses and induction of neuronal apoptosis (Lemkuil, Head, Pearn, Patel, Drummond and Patel 2011).

Based on previous research, we hypothesized that exposure to isoflurane could induce cytoskeletal depolymerization of neurons mediated by proBDNF/p75NTR. We also hypothesized that Dex could mitigate isoflurane-induced neuronal apoptosis, and reverse down-regulation of BDNF. We assessed whether dexmedetomidine could alleviate the neurotoxic effects of isoflurane by converting increasing BDNF expression and protecting the integrity of the actin cytoskeleton. This study was conducted to test our hypotheses.

Methods

Animals

Seven day old (postnatal day 7, PND7) neonatal Swiss CD-1 mouse pups (Adult male and female mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd), weighing 8 ± 0.4g, were used in this study. The ethical committee of Qingdao Agricultural University approved all of the procedures described in this study.
All protocols used in this study were approved by the laboratory animal use ethical committee of Qingdao Agricultural University (approval number #QDAU 4322). In addition, all protocols using live animals followed internationally approved guidelines for the use of animals in research (National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC: The National Academies Press. https://doi.org/10.17226/5140).

Anesthetic Neurotoxicity Model

A total of 28 7PND mice were divided into four groups: Dex15+iso (intraperitoneal injection of 15mg/kg Dex (dexmedetomidine)), Dex20+iso (intraperitoneal injection of 20mg/kg Dex), Dex25+iso

(intraperitoneal injection of 25mg/kg Dex), and the control group (intraperitoneal injection of 20 mg/kg normal saline). After injection, the mouse pups were placed in a small box (15cm×15cm×15cm) and anesthetized using 2% isoflurane (Iso) in air for 2h. The concentration of isoflurane was monitored by a probe put in the box. The interior temperature of the experimental box was maintained at 30℃. After the 2h anesthesia period, the pups were returned to their mother.

Morris water maze

When 21-day-old mice were tested in the Morris water maze (MWM), which is used to assess spatial learning abilities in rodents. The maze requires subjects to remember spatial cues to navigate in an open swimming tank to find a submerged escape platform (Vorhees and Williams 2006). An overhead camera was used to record the swim path of the mice. The water temperature was maintained at 25℃, and a sufficient amount of nontoxic white paint was added to the water in the tank to make it opaque and render the submerged platform virtually invisible. An automated tracking system (Xinruan, Shanghai, China) was used to analyze the swim path of each mouse and calculate the escape latencies. Four positions around the perimeter of the maze, arbitrarily indicated as N, S, E, and W, were used as the initiation points where the mice were released at the beginning of each trial. The order in which the starting points was random, and each starting point was used only once during each session. Once the mouse located the platform, it was allowed to remain there for 30 s before being removed from the tank. During the training period, if a mouse failed to locate the platform in 2min, the mouse was picked up when it was in the middle portion of the swim path and placed on the platform for 15s. Each mouse was trained 7 times by releasing the mouse into the water from the same position as the first trial[16]. After training, each mouse was subjected to the probe trial.

Neuronal Cell Cultures

Neurons were isolated from anesthetized PND 4 mouse hippocampi. The mice were gently restrained and decapitated for neurons preparation as approved by the ethical committee of Qingdao Agricultural University. Both left and right hippocampi were dissected on ice and digested using 0.25% trypsin in D-Hanks medium. Neurons were isolated from a total of 10-14 neonatal 4-day-old mouse pups, combined, then cultured at 37℃ in 5% CO_2 for 3-4 days in Neurobasal-A medium, supplemented with 2% B27, 1% penicillin, and streptomycin. The cultured neurons were divided into three groups: a 1.5% Iso group, a 2% Iso group, and a control group (treated with normal saline). The isoflurane or normal saline were added to the culture medium, respectively (These results are shown in the supplemental material). After evaluating the toxic effect of the different concentrations of isoflurane, the 2% isoflurane exposure was chosen to use in the experiments that included exposure to Dex. The Dex experiments were divided into four groups, dex25+iso （25mg/ml of dex was added to the culture medium）, dex20+iso (20mg/ml of dex was added to the culture medium), dex15+iso (15mg/ml of dex was added to the culture medium), and a control group (nothing was added to the culture medium). Then the neurons were cultured at 37℃ in 5% CO_2 and 2% isoflurane for 4 days.

Protein Extraction and Western blot

Neuronal proteins were isolated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 10% acrylamide gels (Solarbio, Beijing China). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, Massachusetts, USA) using electroblotting. Then the membranes with the transferred proteins were blocked in phosphate-buffered saline with 0.1% Tween (PBST) containing 10% bovine serum albumin (BSA) at room temperature for 2h. The membranes were incubated for 2h in Tris-buffered saline with 0.1% Tween (TBST) with primary antibodies, including rabbit anti-BDNF (1:800), rabbit anti-RhoA (1:1000), rabbit anti-Caspase-3 (1:1000), and rabbit anti-GAPDH (1:1000) (Boster, Wuhan, China). After three washes for 10 min each in TBST, the membranes were incubated for 1h at 37℃ with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and diluted at 1:800 (Beyotime, Beijing, China). Then the membranes were washed three times in TBST and processed using the enhanced chemiluminescence (ECL) detection system. An antibody to GAPDH was used as the loading control. All experiments were repeated three times. The expression level of proteins was calculated as an average of the densities per band area from each group.

Cytoskeletal Depolymerization Quantification

Actin from neurons was labeled with 200mg/ml Alexa-488-phalloidin prior to observation. The neurons were fixed for 20 min with 4% paraformaldehyde at room temperature and washed three times in PBS. The neurons were treated for 20min with 0.3% Triton X-100, then washed 3 times in PBS for 5min each. Then the neurons were stained for 30min in the dark, using phalloidin labeled with rhodamine, and washed three times in PBS for 5min each. Finally, the stained neurons were incubated with DAPI (4’,6-diamidino-2-phenylindole) for 5min at room temperature and imaged under a fluorescence microscope (Nikon Eclipse 50i, Japan).

Statistical Analysis

Results from this study were given as the mean ± SD. Analysis of variance (ANOVA) was used to analyze the data. Significant differences were set at $p \leq 0.05$. Statistical analyses were carried out using SPSS23.0. GraphPad Prism 5.0 software was used to plot the graphs. Data for the latency time to find the hidden platform in the Morris water maze were analyzed using a two-way repeated-measures ANOVA. All assays were carried out independently and in triplicate.

Results

Dexmedetomidine attenuated isoflurane-induced cognitive impairment.

The results from the Morris water maze revealed that all mice were able to locate the platform. However, the escape latencies for the isoflurane-exposed and the dex15+iso groups were significantly longer compared to the dex20+iso and dex25+iso groups (Fig.1A). These results indicated that exposure to 20mg/kg and 25mg/kg dexmedetomidine (Dex) could significantly reduce the cognitive impairment that resulted from early postnatal exposure to isoflurane. Furthermore, analysis of the escape trajectories

revealed that mice in the dex20+iso and dex25+iso groups swam shorter paths to reach the platform compared to the other groups (Fig 1B), which also indicated a reduction in cognitive impairment.

Dexmedetomidine alleviated isoflurane-induced neuronal apoptosis in neonatal mice

The presence of apoptosis, as indicated by the level of expressed activated caspase-3 protein in neurons (Fig.2), was highest when neonatal mice were exposed to 2% isoflurane for 2h. Neuronal apoptosis was reduced when the mouse pups were injected with Dex prior to exposure to isoflurane. Specifically, apoptosis in neurons from the dex20+iso and dex25+iso groups was significantly lower compared to the dex15+iso and the isoflurane-only treated neurons. Furthermore, the expression of activated caspase-3 in all Dex treated groups was significantly lower than that observed in the isoflurane-only treated mice (Fig.2).

Dexmedetomidine attenuated isoflurane-induced actin cytoskeletal depolymerization

Published research reported that isoflurane-induced neurotoxicity was due to depolymerization of the actin cytoskeleton [14]. Therefore, we assessed whether Dex could attenuate the actin cytoskeletal depolymerization induced by isoflurane. Two experimental groups (unexposed neurons and neurons exposed to 2% isoflurane) were evaluated to determine the neurotoxic effects of isoflurane on the actin cytoskeleton. The results revealed that exposure to 2% isoflurane significantly induced actin cytoskeletal depolymerization compared to control neurons exposed to normal saline (supplemental material, Fig.supplyment).

Dex, at three different concentrations, 15 mg/ml、 20 mg/ml, and 25 mg/ml) was added to the cell culture medium before adding 2% isoflurane. The phalloidin staining revealed that Dex diminished the actin cytoskeletal depolymerization induced by isoflurane. All Dex experimental groups (15mg/ml, 20 mg/ml, and 25mg/ml) the integrity of the neuron cytoskeletal structure was significantly protected (Fig.3).

Dexmedetomidine attenuated isoflurane-induced actin cytoskeletal depolymerization via the BDNF-RhoA signal pathway

Schubert et al. and others demonstrated that RhoA regulates actin dynamics in neurons and causes growth cone collapse [17, 18]. RhoA is an important regulator of the actin cytoskeleton, and isoflurane exposure leads to increased RhoA activation, actin depolymerization, and neuronal apoptosis [14]. Therefore, we examined the expression of RhoA. Our results showed that isoflurane exposure significantly increased RhoA expression, and Dex decreased the expression of RhoA. Specifically, the expression of RhoA in hippocampal neurons in the dex25+iso and dex20+iso groups were significantly lower compared to expression in the dex15+iso and isoflurane only groups (Fig. 4). We also examined the expression of BDNF in this study. Exposure to isoflurane alone caused BDNF expression in cultured hippocampal neurons to decrease, but this effect was reversed when cells were exposed to Dex plus isoflurane (Fig.5). We also observed that Dex attenuated the adverse effects of isoflurane in a dose-dependent manner in the Dex25 + iso and Dex20+iso groups.

Discussion

Isoflurane exposure has been found to induce cognitive impairment, increase inflammatory cytokines, and cause cell injury in the hippocampus of rodents [8]. The neurotoxicity of isoflurane in the neonatal rat hippocampus due to $p75^{NTR}$-RhoA activation and actin depolymerization has been reported previously [14]. Dex is a highly efficient and α_2 adrenoceptor agonist. In recent years, Dex has been found to exhibit prominent neuroprotective effects. Dex was the first drug discovered to exert a protective effect on isoflurane-induced neurocognitive dysfunction [10]. However, the mechanism of Dex neuroprotection was still unclear.

We demonstrated in this study that neonatal exposure to isoflurane-induced cognitive impairment in mice, and that Dex pretreatment could provide neuroprotection against isoflurane-induced cognitive dysfunction.

The Morris water maze task was developed to test spatial learning and memory in rodents [19]. The task relies on the ability of rodents to use distant visual cues to navigate from positions around the perimeter of a swim tank and locate a submerged platform that allows escape from the water[19]. Previous studies found that rats exposed to isoflurane were slower to find the escape platform in the Morris water maze task when compared with control rats [20]. In our study, we found that the higher concentrations of Dex that were used reduced the severity of the neurocognitive dysfunction induced by exposure to isoflurane. The escape latencies of the experimental mice that received 20μg/kg and 25 μg/kg Dex were significantly lower compared to the mice exposed to isoflurane alone or the 15μg/kg Dex + iso group. Therefore, we concluded that 20μg/kg and 25μg/kg Dex have a neuroprotective effect in mice exposed to isoflurane.

To explore the mechanism of neuroprotection by Dex, we assessed apoptosis in neonatal mouse hippocampal neurons. Previous studies reported that isoflurane exposure triggered apoptotic cell death in neonatal brains in different experimental animals, such as macaques and rodents [21–23]. To confirm whether Dex could protect against neuronal apoptosis, we assessed the expression of activated caspase-3 in neonatal mouse hippocampal cells. The results showed that exposure to 2% isoflurane resulted in significant neonatal neuronal apoptosis in the mouse hippocampus (supplemental material, Fig. supplyment). In our study, we found that exposure to even a low concentration of Dex (15ug/kg) could reduce neuronal apoptosis that was caused by exposure to isoflurane. Therefore, we propose that Dex is able to protect neurocognitive function in neonatal mice exposed to isoflurane by reducing the extent of apoptosis that occurs in hippocampal neurons. However, the mechanism by which Dex reduces the neuronal apoptosis in the hippocampus is still unclear.

Lemkuil et al. have demonstrated that isoflurane could activate RhoA in neuronal and brain tissues, and that activated RhoA depolymerizes actin, resulting in cytoskeletal depolymerization and apoptosis in mice [14]. In this study, we determined whether Dex could protect neurons through inhibition of RhoA. First, we determined whether Dex could reduce cytoskeletal depolymerization by protecting actin. Our results showed that when mouse neurons were pre-treated with 15μg/kg Dex, the cytoskeletal

depolymerization caused by isoflurane exposure was reduced significantly. This result indicated that Dex might protect neurons by preventing cytoskeletal depolymerization.

Furthermore, to confirm whether RhoA mediated this effect, we assessed the expression of RhoA in hippocampal neurons. We found that Dex reduced the expression of RhoA in a dose-dependent manner compared to the group that was exposed to isoflurane only. This indicated that Dex affected the same signal pathway as isoflurane. Adverse effects on actin assembly have been linked to cognitive and behavioral changes [24, 25]. Thus, Dex may have a significant role in preventing cytoskeletal depolymerization that leads to cognitive dysfunction.

BDNF can be released from dendritic spines during the induction of long-term synaptic plasticity [26, 27]. This autocrine role of BDNF is important in protein synthesis, and BDNF can induce the local synthesis of several molecules, including Arc,LIMK1, and CaMKII, which are crucial in synaptic plasticity [28, 29]. RhoA regulates actin dynamics in neurons, and BDNF controls cytoskeletal depolymerization via RhoA. Therefore, we determined whether Dex could prevent cytoskeletal depolymerization by altering BDNF expression. We evaluated the differences in BDNF protein expression after exposure to different concentrations of Dex and isoflurane. The results showed that Dex could influence the expression of BDNF in hippocampal neurons. In recent years, numerous reports have indicated that exposure to Dex increased BDNF expression [30, 31]. We also found that 15 μg/ml Dex increased the expression of BDNF, and with exposure to increased concentrations of Dex, the BDNF protein expression in the mouse hippocampus also increased.

Several recent reports have documented that anesthetics are neurotoxic in neonatal animals during the critical period of synaptogenesis[32–34]. Fortunately, Dex has been found to have neuroprotective effects during synaptogenesis. Dex exposure could alleviate the neurotoxicity caused by isoflurane through inhibition of RhoA activation by increasing BDNF expression. This would result in a reduction of cytoskeletal depolymerization and neuronal apoptosis. Therefore, the results reported here provide a mechanistic framework upon which novel therapeutic approaches for the prevention of anesthetic neurotoxicity might be developed.

Conclusions

Dex could reverse decreased spatial learning and memory abilities caused by isoflurane exposure in neonatal mice, and this process might be mediated through the BDNF/RhoA signal pathway to prevent actin depolymerization in developing neurons.

Abbreviations

Dex: dexmedetomidine; PND: postnatal day; RhoA: a small guanosine triphosphatase that can depolymerize actin; BDNF: brain-derived neurotrophic factor; TrkB: tropomyosin receptor kinase B; $P75^{NTR}$:

p75 neurotrophin receptor; Iso: isoflurane; PVDF: polyvinylidene difluoride; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Declarations

Ethics approval and consent to participate

This study was approved by the ethical committee of Qingdao Agricultural University (#2020-06-22). And there are no patient participate in this study.

Consent for publish

There are no attached video and participant patients in this study.

Availability of data and material

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

Competing interests

The authors declare that all authors do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted

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Authors' Contributions

All ahthors have read and approved the manuscript. The detailed contribution of each author were listed below:

Liang Chi prepare this paper and collect the data.; Xiaoyang Yao performed the experiments of western blotting. Caixia Gao cultured the cells; Huansheng Dong performed the anesthesia part experiment and analyze data. Huanqi Liu designed the whole experiment .

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None

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Figures

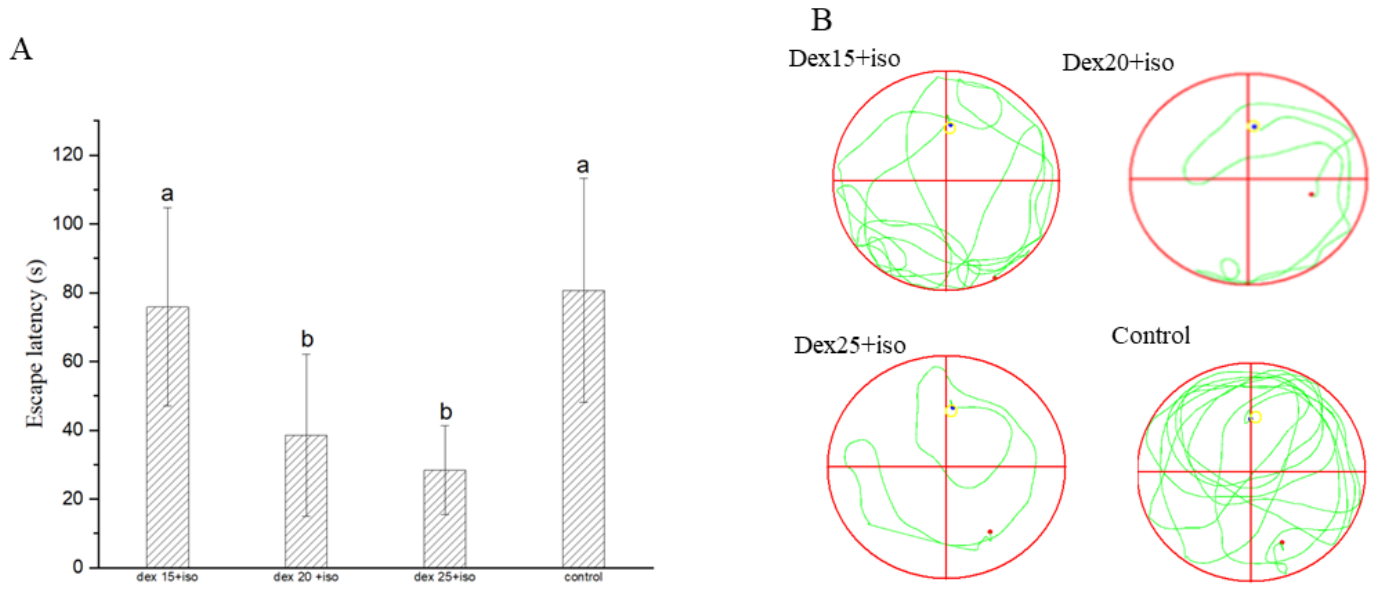

Figure 1

The Morris water maze task performed by mice treated with isoflurane and Dex. A: The escape latency times exhibited by the different experimental groups. B: The swimming trajectory of mice in the Morris water maze. Data are shown as mean ± SD. A one-way ANOVA was performed to determine the

significant differences between the means. Columns labeled with different letters indicate significant differences between experimental groups ($p < 0.05$).

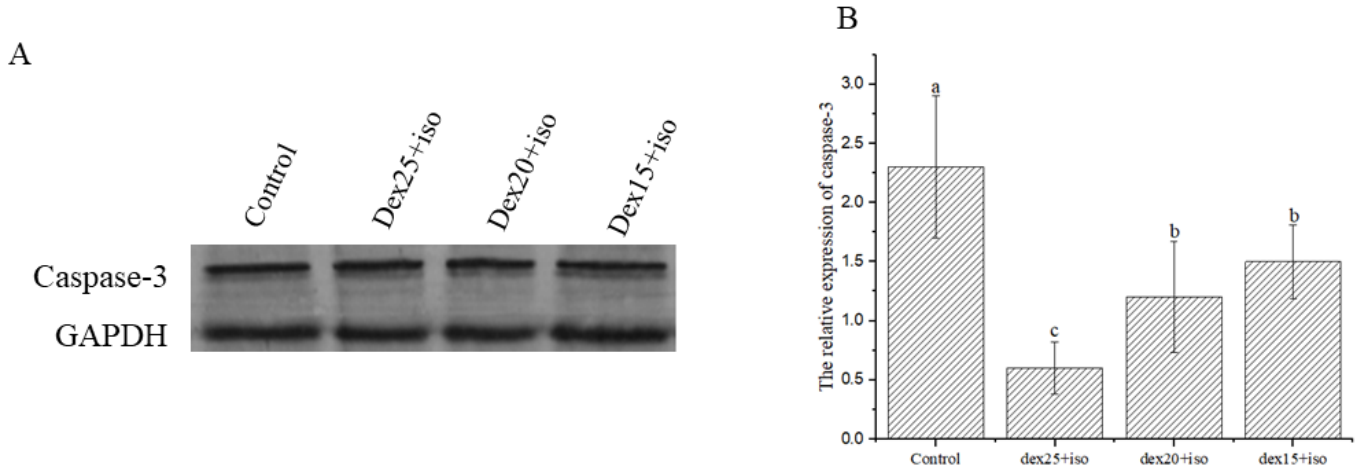

Figure 2

The expression levels of activated caspase-3 protein in hippocampal neurons in different experimental groups. A: Immunoblot analysis shows a decrease in the apoptosis marker, activated caspase-3, with Dex treatment. B: Quantitative data are shown in the graph. Data are shown as means ± SD. A one-way ANOVA was performed to determine the significant differences between the means. Columns labeled with different letters indicate significant differences between experimental groups ($p < 0.05$).

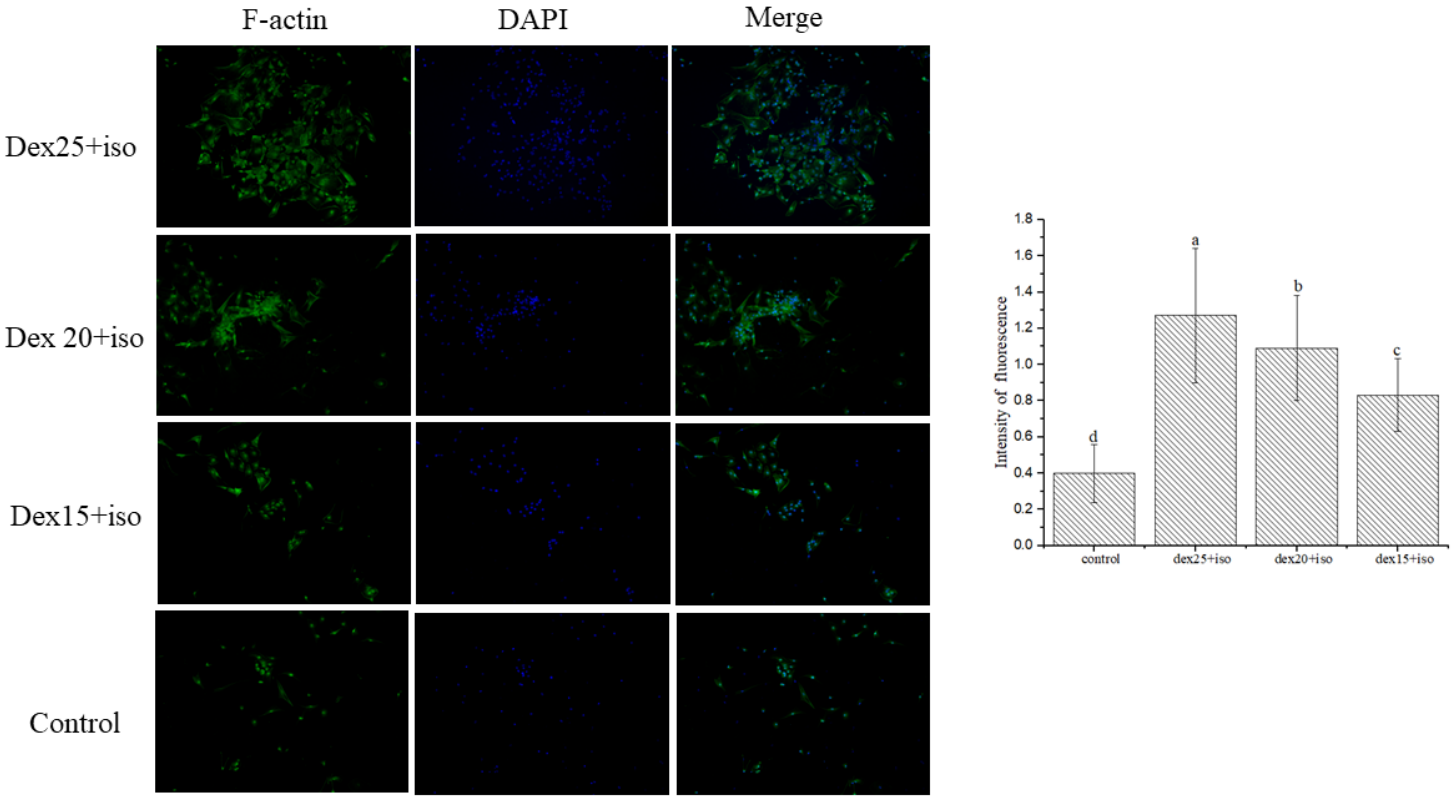

Figure 3

The actin cytoskeleton of cultured primary neurons is seen in the different experimental groups. F-actin was stained using FITC-phalloidin. The number of cultured primary neurons and the actin cytoskeleton labeling increased with exposure to Dex. Data are shown as mean ± SD. A one-way ANOVA was performed to determine the significant differences between the means. Columns labeled with different letters indicate significant differences between experimental groups ($p < 0.05$).

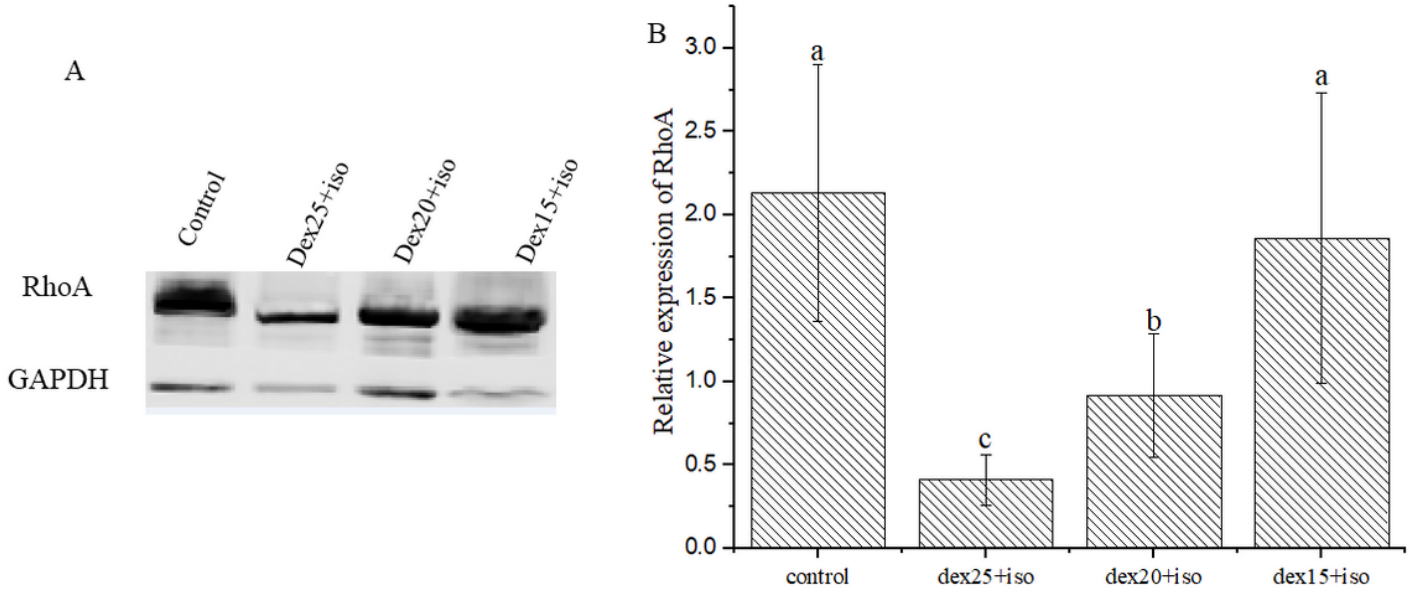

Figure 4

The expression levels of RhoA protein in hippocampal neurons in the different experimental groups. A: Immunoblot analysis shows a decrease in RhoA expression with increasing concentrations of Dex. B: Quantitative data are shown in the graph. Data are shown as mean ± SD. A one-way ANOVA was performed to determine the significant differences between the means. Columns labeled with different letters indicate significant differences between experimental groups ($p < 0.05$).

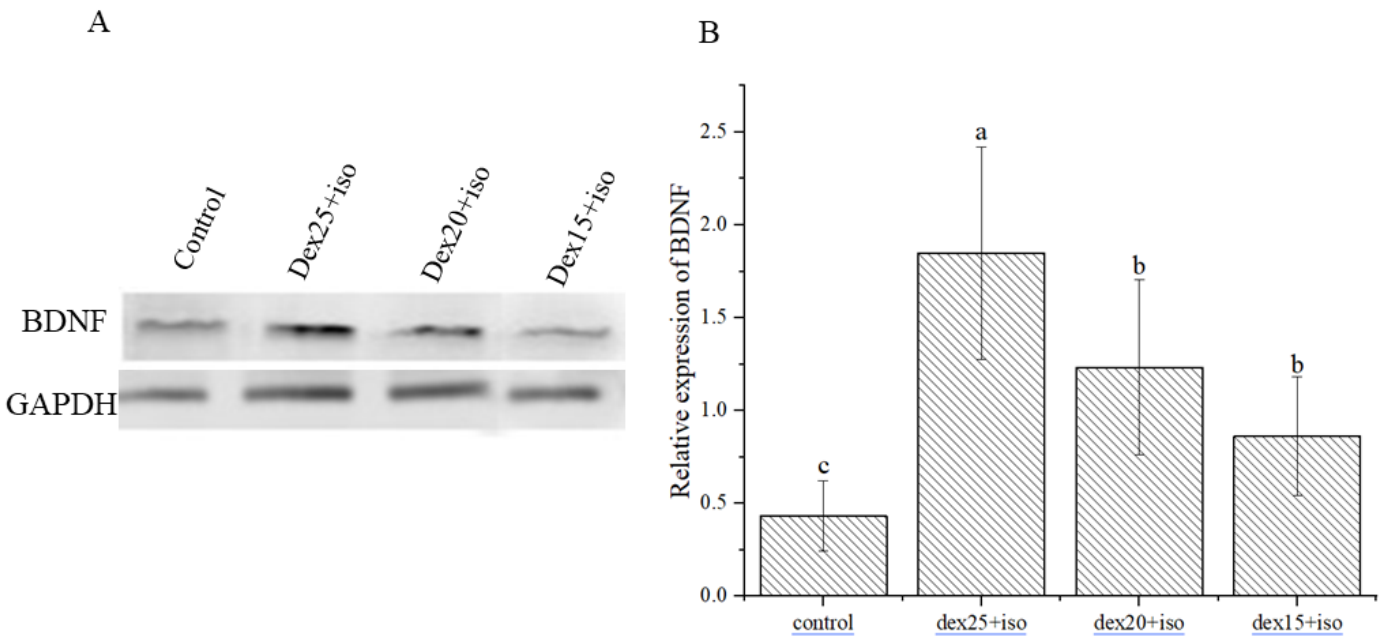

Figure 5

The expression levels of BDNF protein in hippocampal neurons in the different experimental groups. A: Immunoblot analysis shows increased BDNF expression with increasing concentrations of Dex. B: Quantitative data are shown in the graph. Data are shown as mean ± SD. A one-way ANOVA was performed to determine the significant differences between the means. Columns labeled with different letters indicate significant differences between experimental groups ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AuthorChecklistFull.pdf
- figure.rar