STAT3 Involved in Cellular Vulnerability to Isoflurane

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Abstract

STAT3 signaling is crucial during neural spontaneous death period, a restricted developmental time window in which the neonatal brain is vulnerable to isoflurane. Here, we designed experiments to assess whether isoflurane target STAT3 to deliver its cytotoxicity. Mice at postnatal day 7 or 21, primary cortical neurons cultured for 5 or 14 days and human neuroglioma U251 cells were treated with isoflurane. A plasmid containing human wild-type STAT3, STAT3 anti-sense oligonucleotide, STAT3 specific inhibitor STA21, proteasome inhibitor MG-132 and calcineurin inhibitor FK506 were utilized to evaluate the influence of STAT3 levels on isoflurane-induced cytotoxicity. In the present study, an upregulation of STAT3 parallel with a decline in calcineurin activity as well as a decrease in the ability of isoflurane to trigger calcineurin activity and neuroapoptosis were observed in more mature neuron or brain. STAT3 survival pathway was impaired after isoflurane exposure in U251 cells and exerted a prominent effect. STAT3 disruption exaggerated isoflurane-induced oxidative injury and apoptosis, whereas, STAT3 overexpression exhibited notable cellular protection. The blockage of calcineurin activity ameliorated neural apoptosis, dendritic spine impairment and cognitive dysfunction induced by isoflurane. Overall, these results indicated that specific regulation of STAT3 was closely related with the cellular vulnerability to isoflurane.

Introduction

Accumulating evidence suggests that most commonly used clinical anesthetics causes widespread neuronal apoptosis in newborn animals [1,2], whereas the mature brain appears resistance [3,4]. Animal research further showed that anesthetics may trigger cell death in immature neurons of a specifically vulnerable age, when they require synaptic connections and neurotrophin such as NGF and BDNF for survival [5]. However, the underlying mechanism of this inherent vulnerability is largely unknown.

As a transcriptional factor and an intracellular signal transducer, signal transducer and activator of transcription-3 (STAT3) is increasingly recognized for its neuroprotective effect in various brain injuries [6]. During postnatal brain development, STAT3 is identified as a key mediator of the neurotrophin-induced survival pathway, and its protein level gradually increases as neurons pass through the developmental death period [7]. STAT3 knockdown in mature neurons induces neurotrophin dependency, whereas overexpression of STAT3 enables immature neurons to achieve resistance against neurotrophin deprivation.

Our previous study found that isoflurane impaired the STAT3-mediated survival pathway in the brain of neonatal mice. In the current study, we hypothesized that the impaired STAT3 pathway further sensitize cells to the toxicity of isoflurane. We designed gain and loss experiments to determine whether STAT3 signaling was involved in the cellular vulnerability to isoflurane.

Materials And Methods

Mice anesthesia and treatment

Animal experiments were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and were approved by the Ethics Review Board for Animal Studies of Nanjing Drum Tower Hospital. The animal treatments were similar to that in our previous study [8]. C57/BL6 mice on postnatal day 7 or 21 (from Model Animal Research Center of Nanjing University, China) were anesthetized with 1.5% isoflurane for 6 hr in 100% oxygen at 37°C. Mice in the interaction group were injected intraperitoneally with a calcineurin inhibitor FK506 (5 mg/kg, InvivoGen, USA) 30 min before isoflurane exposure.

Primary neuron culture and treatment

The primary cortical neurons were cultured in vitro for 5 days (C5) or 14 days (C14) and treated with 1.5% isoflurane in a gas mixture of 21% O_2, 5% CO_2, and balanced nitrogen for 6 hr as we described previously [9]. In the interaction experiments, neurons were pretreated with FK506 (1mM) 30 min before isoflurane exposure.

U251 cell culture and treatment

We used U251 human neuroglioma cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) in the succeeding experiments. Cells incubated in serum-free media were exposed to 2% isoflurane for 6 hr, performed similarly as our previous study [8]. The wild-type pcDNA3-6×Myc-mSTAT3 vector [10] or an antisense oligonucleotide (ASO) of STAT3 (HSS186131, Invitrogen, USA) were transfected into cells using lipofectamine^{TM} 2000 (Invitrogen, USA). An AxyPrep Endofree Plasmid Miniprep Kit (Axygen Biosciences, USA) was used to extract the plasmids from bacterial culture. After the transfection, cells were incubated at 37°C in a CO_2 incubator for 48 hr prior to isoflurane exposure. The untransfected cells without isoflurane exposure and the empty vector-transfected cells were set as the controls. In the interaction studies, cells were pretreated with a proteasome inhibitor MG-132 (30mM, Calbiochem, Germany) [11], FK506 (1mM) or a STAT3 inhibitor STA21 (30mM, Enzo Life Sciences, Switzerland) [12] 30 min before isoflurane exposure.

Mitochondria isolation

A mitochondria isolation kit for cultured cells (Thermo Scientific, USA) was used to evaluate the levels of mitochondria-located STAT3 and cytochrome C oxidase released from mitochondria into cytosol in U251 cells. After exposed to isoflurane, cells grown on 10-cm culture dishes (appropriately $1 \acute{} 10^7$ cells incubated in 9 ml of culture media per dish) were collected, centrifuged at 850×g for 2 min, and supernatant was then discarded. Subsequently, 800 μL of mitochondria isolation reagent A (with 100:1 protease inhibitors), provided in the kit, was added to each sample. The resulting mixtures were incubated on ice for exactly 2 min. Option A, which used the reagent-based method, was utilized in accordance with the manufacturer's protocol.

Real-time PCR (RT-PCR)

U251 cells (n = 4 wells) grown in six-well plates were treated with lysis buffer provided by an Rneasy Mini Kit (QIAGEN, Hilden, Germany) immediately after isoflurane treatment. The frontal cortex was harvested, frozen in liquid nitrogen, and stored at −80°C before use. The tissues (n = 4) were ground in liquid nitrogen using a small mortar and then treated with lysis buffer. Retrotranscription and PCR analysis were carried out as we described previously [9]. STAT3, cyclin D1, Mcl-1, survivin, and Bcl-xl genes (3 repetitions per sample) were amplified using specific oligonucleotide primers (GenScript, USA; see table, Supplemental Digital Content , which is a table listing sequences of all primers used in this study). Housekeeping gene, b-actin, was used as internal standard. The no template sample was used as negative control.

Western blot analysis

The brain tissues were immediately removed and stored in -80℃ after anaesthesia . The frontal cortex were homogenized with lysis buffer containing 1% protease and phosphatase inhibitor cocktail (Thermo scientific, USA) on ice for 30 min. For the *in vitro* experiments, lysis buffer was added to six-well plates seeded with U251 cells or primary neurons immediately after 6-h isoflurane exposure. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific, USA) and then each sample (25 μg) was separated by SDS-PAGE gel. Rabbit primary antibodies against STAT3 (1:1000; Epitomics, USA), Tyr705-phosphorylated STAT3 (pY705-STAT3, 1:1000; Epitomics), Bcl-xl (1:1000; Epitomics), survivin (1:1000; Epitomics), Mcl-1 (1:1000; Epitomics), cytochrome C (1:1000; Abcam, USA), MnSOD (1:1000; Abcam), activated caspase-3 (1:500; Bioworld), caspase-3 (1:500; Bioworld), Bim (1:1000; Epitomics), ubiquitin (1:1000; Thermo scientific, USA) and loading control GAPDH (1:2500; Bioworld) was used. GeneTools image analysis software was used for densitometric analysis of immunoblots.

Detection of intracellular ROS production

A fluorescent probe carboxy-H2DCFDA (Invitrogen, Eugene, USA) was used to assess the levels of intracellular reactive oxygen species (ROS). U251 cells grown in six-well plates were incubated with ROS detection solution for 30 min at 37 °C in the dark (*n* = 6 wells). Each sample was then washed thrice with Hank's buffer and immediately observed under a Fluoview Fv10i confocal microscope (Olympus, Japan). A fluorescence spectrophotometer (F-7000, Hitachi, Japan) was also used to measure the cellular fluorescence of cells grown on 96-well plates (*n* = 6 wells).

Flow cytometric detection of apoptosis

A FITC Annexin V apoptosis detection kit (BD Biosciences, USA) was used to quantify the apoptotic rate in U251 cells (*n* = 6 wells). Cells grown on six-well plates were collected, washed twice with cold PBS, resuspended in 1 ´ binding buffer at a concentration of 1 ´ 10^6 cells/ml, and then incubated with 5 μL FITC Annexin V as well as 5 μL PI for 15 min in the dark. The apoptotic rate was analyzed by a flow

cytometer (BD Biosciences, USA) and expressed as the percentage of FITC Annexin V positive cells out of the total number of cells counted.

Immunofluorescence analysis

U251 cells or primary neurons grown on six-well plates were fixed in 4% PFA for 30 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 5% BSA for 1 hr (n = 3). Rabbit primary antibodies against STAT3 (1:100), Drebrin (1:100; Abcam) and Alexa Fluor 488 donkey anti-rabbit secondary antibodies (1:200; Molecular Probes, USA) were used for immunofluorescence analysis. Images (n = 3) were captured using a Fluoview Fv10i confocal microscope (Olympus, Japan) and analyzed using Image-Pro Plus analysis software (Media Cybernetics, Inc., Rockville, MD).

TUNEL staining

U251 cells or primary neurons grown on six-well plates were assayed through TUNEL staining by using the *in situ* cell death detection kit (Roche Applied Science, Germany) per the manufacturer's protocol. After fixed in 4% PFA for 1 hr, cells were permeabilized with 0.1% Triton X-100 for 2 min on ice and stained with TUNEL for 1 hr at 37 °C in the dark. Representative images were obtained at 200´ magnification by using a TCS SP2 multiphoton confocal microscope (Leica, Germany).

Calcineurin activity assays

The activity of calcineurin was determined in the frontal cortex obtained from mice that were killed immediately after anesthesia or age-matched controls (n = 6). Fresh tissues were homogenized with lysis buffer containing 1% protease inhibitor (Thermo scientific, USA). A colorimetric calcineurin cellular activity assay kit (Calbiochem, Germany) was used to perform the test as we previously described [9].

Intellicage test

Mice assigned for behavior assessment were allowed to recover in 100% oxygen for 20 min after isoflurane exposure and reunited with their mothers. Mice were housed in a temperature-controlled (21± 1 °C) room with a 12-h light/dark cycle and free access to food and water. Four weeks after isoflurane exposure, place learning was performed in the IntelliCage system (NewBehavior Inc., Zurich, Switzerland) as we described before [8]. Briefly, the test started from an adaptation period of 6 days with *ad libitum* access to obtain water in all the corners. The least preferred corner for each mouse was chosen, and for the following 4 days, mice could obtain access to water only in their assigned correct corner. A brief air puff (1 bar/2 s) was triggered as a punishment if the mice visit an incorrect corner. When the test turned to the reversal learning phase, the opposite corner to the initial correct corner was reassigned as correct corner to each mouse for the next 4 days. Place learning was assessed as a ratio of number of visits in correct corner to all corners.

Statistics

Data are expressed as mean ± SD. The Western blot results, levels of mRNA and intracellular ROS, apoptotic rate, and activity of calcineurin were analyzed using one-way ANOVA for overall differences among groups followed by Bonferroni post hoc test for multiple comparisons. Unpaired Student's t-test was used for comparisons between 2 groups. Repeated measures ANOVA followed by Bonferroni post hoc test was used to examine statistical comparisons among the four groups on correct visit ratios at different time point in Intellicage test. The sample sizes used were based on previous experience. All analyses were performed using SPSS 13.0 software (IBM Corporation, Armonk, USA). $P < 0.05$ (2-tailed) was considered as significant difference.

Results

STAT3 levels were closely related with neural vulnerability to isoflurane

In previous study we have found that isoflurane exposure increased the activity of calcineurin, which specifically promotes STAT3 degradation. To determine whether calcineurin-mediated degradation of STAT3 is also time-restricted as the neurotoxicity of isoflurane to the brain [13], mice on postnatal day 7 (P7) and day 21 (P21) were anesthetized with 1.5% isoflurane for 6 hr. Our study showed that isoflurane markedly increased the level of activated caspase-3 in the frontal cortex of P7 mice (Figure 1A; $n = 4$, 1.50 ± 0.16 vs 0.91 ± 0.09, $P < 0.001$), but not in P21 mice ($n = 4$, 1.13 ± 0.19 vs 0.99 ± 0.08, $P = 0.96$). In line with this observation, we found that the protein level of STAT3 in the frontal cortex of P21 mice were obviously elevated ($n = 4$, 1.87 ± 0.15 vs 1.05 ± 0.04, $P < 0.001$) when compared with that in P7 mice, whereas, the calcineurin activity was significantly lower (Figure 1B; $n = 6$, 0.87 ± 0.16 vs 2.10 ± 0.34, $P < 0.001$). Moreover, there was a 2-fold increase in calcineurin activity in P7 mice after isoflurane exposure ($n = 6$, 4.40 ± 0.70 vs 2.10 ± 0.34, $P < 0.001$), but the increase of calcineurin activity in P21 mice was minimal ($n = 6$, $P = 0.21$).

The *in vitro* system exhibited a similar developmental regulation. Compared with the primary cortical neurons cultured for 5 days (C5), a significant upregulation in STAT3 protein level (Figure 1C; $n = 4$, 1.70 ± 0.13 vs 0.99 ± 0.08, $P < 0.001$) was observed in C14 neurons, at the instance when isoflurane did not induce apoptosis ($P = 1.00$). Moreover, a notable decline of STAT3 protein levels ($n = 4$, 0.62 ± 0.08 vs 0.99 ± 0.08, $P = 0.002$) that coincident with an increase in calcineurin activity (Figure 1D; $n = 6$, 3.85 ± 0.63 vs 1.87 ± 0.34, $P < 0.001$) after isoflurane exposure was observed in C5 but not in more mature C14 neurons ($n = 4$, 1.47 ± 0.13 vs 1.70 ± 0.13, $P = 0.06$ for STAT3 protein levels; $n = 6$, 0.89 ± 0.25 vs 0.54 ± 0.16, $P = 0.76$ for calcineurin activity). Collectively, these findings suggested that calcineurin-mediated STAT3 degradation may involved in neural vulnerability to isoflurane.

Isoflurane exposure impaired STAT3 survival pathway in U251 cells

To determine whether STAT3 is crucial in cellular vulnerability to isoflurane, we tested the role of ectopic STAT3 in isoflurane-induced apoptosis. We employed U251 human neuroglioma cells, which showed caspase-3 activation and ROS accumulation after a 6-h isoflurane exposure in our previous study. The present study showed that decreased protein levels of STAT3, pY705-STAT3 and its downstream survival

targets were observed in U251 cells after isoflurane exposure (Figure 2A; $n = 4$, $P < 0.001$ for STAT3 and pY705-STAT3, $P = 0.001$ for Bcl-xl and survivin). Immunofluorescence showed that isoflurane exposure induced a 30% reduction of STAT3 staining (Figure 2B; $n = 6$, $P < 0.001$) in U251 cells, indicating that this cell model may be used for the study of STAT3 function in isoflurane-induced cytotoxicity *in vitro*.

In contrast with the reduction of STAT3 protein, the mRNA level of STAT3 in U251 cells was increased remarkably after 6-h isoflurane exposure (Figure 2C; $n = 4$, 2.34 ± 0.14 vs 1.04 ± 0.08, $P < 0.001$), indicating that isoflurane-induced STAT3 reduction may be caused by a post-transcriptional mechanism. Protein degradation is known to be achieved mostly through the ubiquitin (Ub)–proteasome pathway (UPP) [14]. To determine the role of STAT3 degradation in isoflurane, a proteasome inhibitor MG132 was introduced (Figure 2D). Interestingly, our data showed that isoflurane significantly decreased the levels of total ubiquitinated proteins in U251 cells (n = 4, $P < 0.001$). MG132 prevented this downregulation of ubiquitinated proteins ($P < 0.001$), so as STAT3 ($P = 0.012$). However, MG-132 coincubation did not lead to any further enhancement of protein ubiquitination ($P = 0.14$), indicating that isoflurane accelerated the degradation of ubiquitinated proteins, but not protein ubiquitination. Moreover, this MG132 effect enhanced the levels of Bim ($P < 0.001$), which is an essential initiator of apoptosis, with an increased caspase-3 activation ($P < 0.001$) after isoflurane treatment. In contrast, pretreatment with FK506, a calcineurin-specific inhibitor, prevented the degradation of STAT3 (Figure 2E, $n = 4$; $P < 0.001$) and cleavage in caspase-3 ($P < 0.001$), without significant influence to the decreased levels of total ubiquitinated proteins ($P = 1.0$) after isoflurane exposure. These findings highlighted the prominent importance of STAT3 in isoflurane-induced cytotoxicity.

Ectopic STAT3 protected cells from isoflurane-induced cytotoxicity *in vitro*

U251 cells were then transiently transfected with a wild-type STAT3 gene-containing vector STAT3-pcDNA3. Western blot analysis confirmed that the cells transfected with STAT3-pcDNA3 expressed high levels of STAT3 protein (Figure 3A; $n = 4$, $P < 0.001$) and its downstream anti-apoptotic factors, e.g. Mcl-1($P = 0.006$) and survivin ($P = 0.008$), at 48 hr post-transfection. Real-time PCR showed that the transcript of STAT3 target genes e.g. cyclin D1 (Figure 3B; $n = 4$, $P < 0.001$), Mcl-1 ($P < 0.001$), survivin ($P = 0.014$) and Bcl-xl ($P = 0.003$) were remarkably upregulated, when compared to those of controls.

Next, we found that STAT3 overexpression was able to restore isoflurane-induced decline of STAT3 and its downstream anti-apoptotic proteins (Figure 3C; $n = 4$, $P = 0.011$ for Mcl-1, $P = 0.006$ for survivin) as well as mitochondria-located STAT3 (Figure 3D; $n = 4$, $P < 0.001$), which has been previously confirmed to play a major role in modulating mitochondrial respiration and antioxidative stress [15]. Simultaneously, cytochrome c that released from mitochondria into cytoplasm after isoflurane exposure was also prevented (Figure 3D; $n = 4$, $P < 0.001$).

The protective effects of STAT3 involved an antioxidative stress mechanism

ROS accumulation was reported to be a critical event in triggering the cytotoxicity of isoflurane [16]. To determine whether the cellular protective function of STAT3 involved an antioxidative stress mechanism,

we used an antisense oligonucleotide (ASO) to knockdown STAT3 and a specific inhibitor STA21 to hinder STAT3 dimerization, together with the STAT3 overexpression assay. Western blot and immunofluorescence analyses showed that STAT3-ASO efficiently knocked down STAT3 expression in U251 cells (Figure 3A; $n = 3$, $P < 0.001$), meanwhile 6-h STA-21 treatment markedly inhibited the nuclear translocation of STAT3, without significant influence on its total protein level. STAT3 knockdown or its nuclear-translocation disruption resulted in a more than 10-fold increase of ROS in U251 cells after isoflurane exposure, when compared with that of controls (Figure 3B; $n = 6$, $P < 0.001$). The apoptotic rates after isoflurane exposure (Figure 3C & D; $n = 6$, 12.42 ± 2.23%) were obviously augmented in cells with STAT3 knockdown (24.78 ± 4.65%, $P < 0.001$) or STA21 treatment (19.10 ± 3.31%, $P < 0.001$). By contrast, STAT3 overexpression mitigated isoflurane-induced ROS accumulation (Figure 3B; $n = 6$, $P < 0.001$) and apoptosis (Figure 3C & D; $n = 6$, 7.51 ± 1.33%, $P = 0.002$). These protective effects of STAT3 were also confirmed by TUNEL staining (Figure 3E; 3 wells per group, 6 images per well) and Western blot analysis of cleaved caspase-3 (Figure 3F & G; $n = 4$). Notably, STAT3 disruption or overexpression did not affect the levels of ROS and apoptosis in isoflurane-untreated cells.

Mechanistically, the antioxidative effect of STAT3 was linked to its canonical activity as a nuclear transcription factor. We found the protein level of manganese-containing superoxide dismutase (MnSOD or SOD2), a critical cellular antioxidant enzyme and a direct target of STAT3 [17,18], was decreased (Figure 3F & G; $n = 4$, $P < 0.001$) after isoflurane exposure. The decline was further aggravated in cells with STAT3 disruption, but was restored by STAT3 overexpression ($P < 0.001$), suggesting that isoflurane-induced ROS accumulation is at least partially ascribed to impaired ROS scavenging.

Calcineurin inhibition alleviated the neurotoxicity of isoflurane

Since STAT3 levels were closely related with cellular vulnerability to isoflurane, we next examined whether calcineurin-specific inhibitor FK506 have a long-term protective effect against the neurotoxicity of isoflurane. STAT3 degradation (Figure 4A; $n = 4$, $P < 0.001$) and apoptosis (Figure 4B; 4 wells per group, 6 images per well) induced by isoflurane were prevented by FK506 pretreatment in C5 neurons. After exposed to isoflurane, C5 neurons were maintained in culture for 5 days and then dendritic spines were stained using a neuronal F-actin marker, drebrin. Some recent studies on neurological disorders accompanied by cognitive deficits suggested that the loss of drebrin from dendritic spines is a common pathognomonic feature of synaptic dysfunction [19]. In the present study, we observed that neurons in the control group exhibited extensive and overlapping neurites. A significant reduction in the number of dendritic spines was detected in neurons exposed to isoflurane. Pretreatment with FK506 attenuated the isoflurane-induced loss of dendritic spines (Figure 4C; 4 wells per group, 6 images per well).

The post-transcriptional mechanism was also involved in isoflurane-induced STAT3 downregulation in the brain of P7 mice. The protein levels of STAT3 were not significantly decreased until isoflurane exposure for 4 hr (Figure 4D; $n = 4$, $P < 0.001$). In contrast, the mRNA level of STAT3 was increased remarkably after 1-h isoflurane exposure (Figure 4E; $n = 4$, $P < 0.001$). Intriguingly, the protein level of STAT3 was elevated initially, and the underlying mechanism remained to be determined.

Finally, we used the Intellicage system to assess the long-term effect of FK506 on isoflurane-induced cognitive dysfunction. As we described before, the cognitive impaired induced by isoflurane exposure was displayed in a more demanding task. During the first three days of learning phase, the isoflurane-treated mice did not show a significant disability in recognizing the correct corner. When test turned to the reversal learning phase, as compared to mice in the control group, the ratio for making a correct visit was approximately 20% lower in the isoflurane-treated mice (Figure 4F; $n = 8$, $P < 0.01$ at day 4-8), reflecting that they had not learned to drink successfully in their new assigned corner. Mice pretreated with FK506 showed significant improvement in spatial memory as compared to that in the isoflurane group (Figure 4F; $n = 8$, $P < 0.01$ at day 4-7, $P < 0.05$ at day 8).

Discussion

In the present study, we propose the hypothesis that STAT3 signaling is involved in the cellular vulnerability to isoflurane. Our results showed that calcineurin-mediated degradation of STAT3 is closely related with the cytotoxicity of isoflurane in the brain of mice and cultured primary cortical neurons. Subsequently, we used the U251 cell line to determine the underlying mechanism and found that STAT3 is of particular importance in isoflurane-induced neurotoxicity. STAT3 disruption exaggerated, while STAT3 overexpression mitigated isoflurane-induced oxidative injury and apoptosis.

Calcineurin is a calcium and calmodulin dependent serine/threonine protein phosphatase that is highly expressed in brain [20]. Recently, it has also been shown that calcineurin promotes the degradation of STAT3 and controls the critical developmental death of neurons in newborn brain [21]. The present study found that the isoflurane exposure resulted in a 2-fold increase of calcineurin activity in the frontal cortex of P7 mice, but not in P21 mice. Moreover, the calcineurin activity of P21 mice was significantly lower than that of P7 mice, accompanied by a marked elevation in STAT3 protein levels in the frontal cortex. A similar developmental regulation of STAT3 was exhibited in primary cortical neurons cultured *in vitro*. Compared with C14 neurons, an upregulation of STAT3 parallel with a decline in calcineurin activity as well as with a decrease in the ability of isoflurane to trigger calcineurin activity were observed in more mature C14 neurons. This finding revealed that calcineurin-mediated STAT3 degradation may be closely related with developmental stage-related vulnerability of the brain to isoflurane stress. Given previous research showing that isoflurane induce neurodegeneration by activating inositol 1,4,5-trisphosphate (IP3) receptors [22,23], it is possible that the upstream trigger of calcineurin activity could be the excessive release of calcium from the endoplasmic reticulum.

The ubiquitin-proteasome pathway is the major intracellular non-lysozymal mechanism for degrading proteins and is involved in the regulation of a number of key signaling pathways. Proteins destined for degradation are conjugated by an ubiquitin chain and the ubiquitinated proteins are subsequently targeted for degradation by proteasome [24]. Previous studies have shown that ubiquitin metabolism affects growth inhibition of isoflurane in yeast [25,26]. A recent research revealed that isoflurane preconditioning decreased ubiquitin-conjugated protein aggregation and prevented free ubiquitin depletion in the CA1 region of hippocampal after global cerebral ischemia in mice [27]. It is noteworthy

that we found isoflurane exposure triggered a 40% decrease in total ubiquitin-conjugated proteins levels in U251 cells, while when co-incubation with a proteasome inhibitor MG132, the ubiquitin-conjugated proteins levels were not increased, indicating that isoflurane accelerated the degradation of ubiquitin-conjugated proteins, but not promote ubiquitin conjugate to protein. In addition, our study found that MG132 result in aggravated apoptosis after isoflurane exposure, hence it is essential to alternative techniques to specifically regulate ubiquitination and proteasomal degradation of the key protein.

As a transcription factor, STAT3 is suggested to play an instructive role in brain development. STAT3-mediated cytokine signaling regulates gliogenesis as well as neurogenesis during brain development [28]. Neurons in newborn brain are under selection through a process of developmental death, depend on neurotrophin for survival. Overexpression of STAT3 completely attenuated neurotrophin dependency in this state, suggesting STAT3 is a key mediator of survival pathway that neurons acquire in this vulnerable period [29]. Moreover, it has been reported that calcineurin mediate STAT3 degradation via directing STAT3 to the E3 ubiquitin ligase complex [21]. To identify whether STAT3 is a key mediator of the cellular vulnerability to isoflurane, we performed gain- and loss-of-function studies in U251 cells. We confirmed that the levels of STAT3, p-STAT3 and its downstream anti-apoptotic proteins were significantly reduced after isoflurane exposure in U251 cells. In comparison with global blockade the degradation of ubiquitinated proteins by MG132 aggravated apoptosis after isoflurane exposure, specifically blocking the degradation of STAT3 by calcineurin-inhibitor FK506 prevented caspase-3 activation after isoflurane exposure, revealing the particular importance of STAT3 in isoflurane-induced cytotoxicity.

This notion was further confirmed by the result that STAT3 overexpression mitigated isoflurane-induced ROS accumulation, caspase-3 activation and apoptosis in U251 cells, whereas cells with STAT3 knockdown exhibited more sensitive to the toxicity of isoflurane, mimicking the cellular phenomenon of immature neurons with relatively lower levels of STAT3. Moreover, STAT3 knockdown or overexpression did not show significant influence on the basal levels of ROS and apoptotic rate in isoflurane-untreated cells, suggesting that STAT3 was functioning in a stress-related protective mechanism.

Oxidative injury was regarded as a key event in isoflurane-induced cell apoptosis [30,31]. Previous studies have reported both enhanced ROS production [32] and impaired ROS scavenging were involved in the ROS accumulation after isoflurane exposure [33]. It has been demonstrated that isoflurane impaired SOD but not catalase activity, while it remains unclear whether the impaired ROS scavenging capability is an initial event or a result of excess ROS accumulation. ROS is generated when electrons released from the mitochondrial electron transport chain (ETC) incompletely reduce O2, mainly form complexes I and III. STAT3 has recently been identified to colocalize with complex I and shown essential for optimal function of the electron transfer chain [15,34,35]. Mitochondrial STAT3 appeared to exert protection against certain diseases [18,36,37] by inhibiting oxidative stress or mitochondrial permeability transition pore (mPTP) opening [38,39]. Notably, the present study showed that mitochondria-located STAT3 was also decreased after isoflurane exposure and was able to be restored by STAT3 overexpression. Meanwhile, we found that inhibiting the nuclear translocation of STAT3 by STA21 greatly aggravated isoflurane-induced ROS accumulation and apoptosis as well as STAT3 knockdown. Thus, STAT3 overexpression may contribute

to cytoprotection not only through its transcriptional activity such as upregulating anti-oxidative and anti-apoptotic proteins, but also through its mitochondrial function that reducing ROS generation.

Taken together, the present study demonstrated that STAT3-mediated survival pathway as well as its anti-oxidative effect were impaired after isoflurane exposure and contributed to the cellular vulnerability to isoflurane. The connections between calcineurin-mediated STAT3 degradation and neuronal vulnerability to isoflurane reported here point out novel therapeutic target for the prevention and treatment of isoflurane-induced neurotoxicity, not only to the immature brain, but also the aged brain, in which a marked decrease of STAT3 was also reported [40]. In addition to STAT3, a marked decrease in the amount of ubiquitin-conjugated proteins protein was found after isoflurane exposure. Continued analysis of additional proteins relevant to isoflurane-response degradation is imperative in future studies.

Declarations

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

The software application and custom code are available from the corresponding author upon reasonable request.

Authors’ contributions

All authors contributed and critically reviewed and approved the manuscript. Q.G., Z.M., X.G., H.W. and Y.Y. conceived and designed the experiments. Y.Y., S.S., Y.L., W.Z., J.Z., Y.S., and J.H. performed the experiments. Y.Y., S.S., and X.Y. analysed the data. Z.M., Q.G., X.G., H.W. and Y.Y. prepared the manuscript.

Ethics approval

Animal experiments were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Review Board for Animal Studies of Nanjing Drum Tower Hospital.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

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Figures

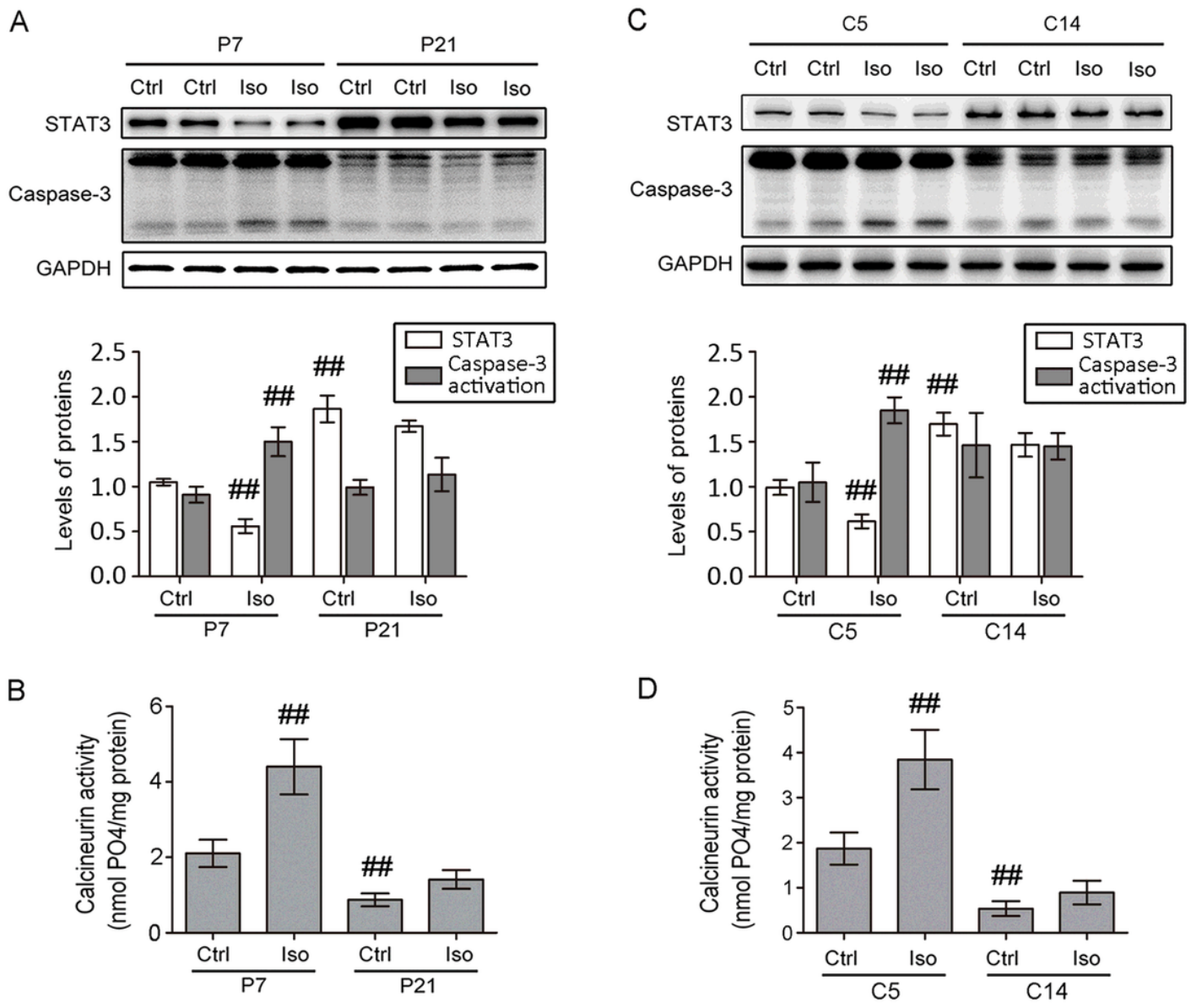

Figure 1

STAT3 degradation mediated by calcineurin was closely related with the neural vulnerability to isoflurane. (A) Isoflurane markedly increased caspase-3 activation (n = 4, P < 0.001) in the frontal cortex of mice on postnatal day 7 (P7), but not in P21 mice (n = 4, P = 0.96). A significant upregulation of STAT3 protein level (P < 0.001) was observed in P21 mice, when compared with that in P7 mice. The protein levels of STAT3 were normalized to GAPDH. The levels of caspase-3 activation were assessed by quantifying the ratio of cleaved and full-length caspase-3 first and then compared with that of control conditions. ## P <0.01 versus control on P7. (B) The developmental upregulation of STAT3 was parallel with a decrease in calcineurin activity at later postnatal stage (n = 6, P < 0.001). Isoflurane increased calcineurin activity (n = 6, P < 0.001) in the frontal cortex of P7 mice, but not in P21 mice (n = 6, P = 0.21). The activity of calcineurin was expressed in nanomolar PO4 per milligram of protein. ## P < 0.01 versus control on P7.

(C) The primary cortical neurons were cultured in vitro for 5 days (C5) or 14 days (C14) prior to experiments. A similar stage-dependent pro-apoptotic effect of isoflurane accompanied by a developmental upregulation of STAT3 in more mature C14 neurons was also exhibited in vitro system (n = 4). ## P < 0.01 versus control on C5. (D) A decrease in calcineurin activity (n = 6, P < 0.001) as well as with a decrease in the ability of isoflurane to trigger calcineurin (n = 6, P = 0.76) activity was observed in more mature C14 neurons. ## P < 0.01 versus control on C5. Ctrl = Control, Iso = Isoflurane.

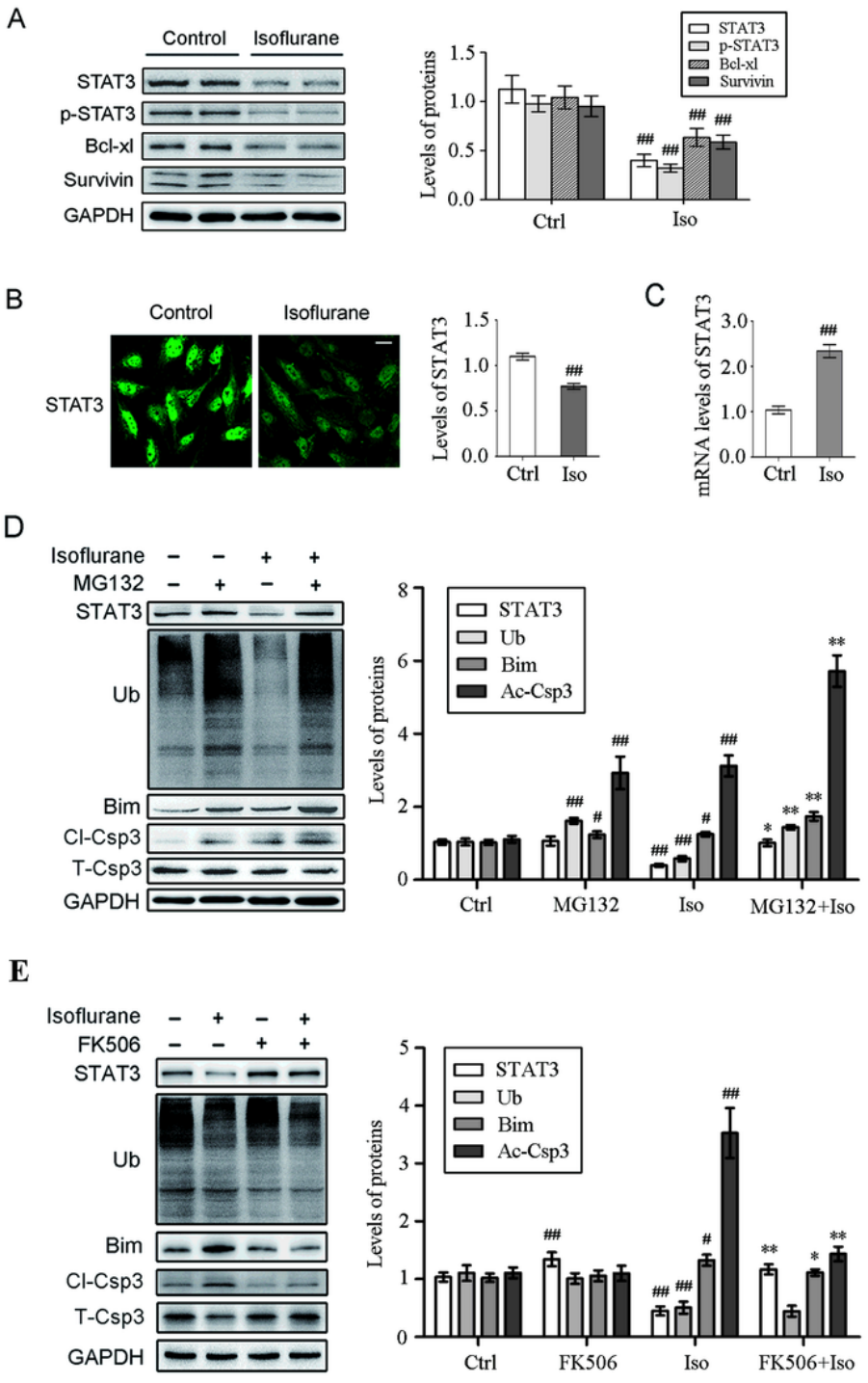

Figure 2

Isoflurane exposure impaired STAT3 survival pathway and ubiquitinated protein degradation in U251 cells. (A) The protein levels of STAT3, pY705-STAT3 and its downstream Bcl-xl and survivin were significantly declined ($P < 0.001$ for STAT3 and pY705-STAT3, $P = 0.001$ for Bcl-xl and survivin, $n = 4$) in U251 cells after a 2% isoflurane exposure for 6hs. The levels of protein were expressed as optical density normalized to housekeeping gene GAPDH. (B) Immunofluorescence analysis detected a notable reduction of STAT3 in U251 cells, especially in the nucleus ($n = 6$, $P < 0.001$). Scale bar, 30 μm. (C) The mRNA level of STAT3 in U251 cells was increased obviously ($n = 4$, $P < 0.001$) after 6h isoflurane exposure. Housekeeping gene β-actin was used as internal standard. (D) MG132 attenuated the isoflurane-induced decline of STAT3 ($P = 0.012$) and total ubiquitinated protein ($P < 0.001$), but aggravated cleavages in caspase-3 ($P < 0.001$) in U251 cells ($n = 4$). (E) Application of FK506 attenuated isoflurane-induced degradation of STAT3 ($P < 0.001$) and cleavage in caspase-3 ($P < 0.001$), with no significant influence to the reduction of total ubiquitinated proteins ($P = 1.0$) in U251 cells ($n = 4$). p-STAT3 = STAT3 phosphorylated at tyrosine 705. Ub = ubiquitinated total proteins, T-Csp3 = full-length caspase-3, Cl-Csp3 = activated caspase-3. # $P < 0.05$, ## $P < 0.01$ versus control. * $P < 0.05$, ** $P < 0.01$ versus isoflurane.

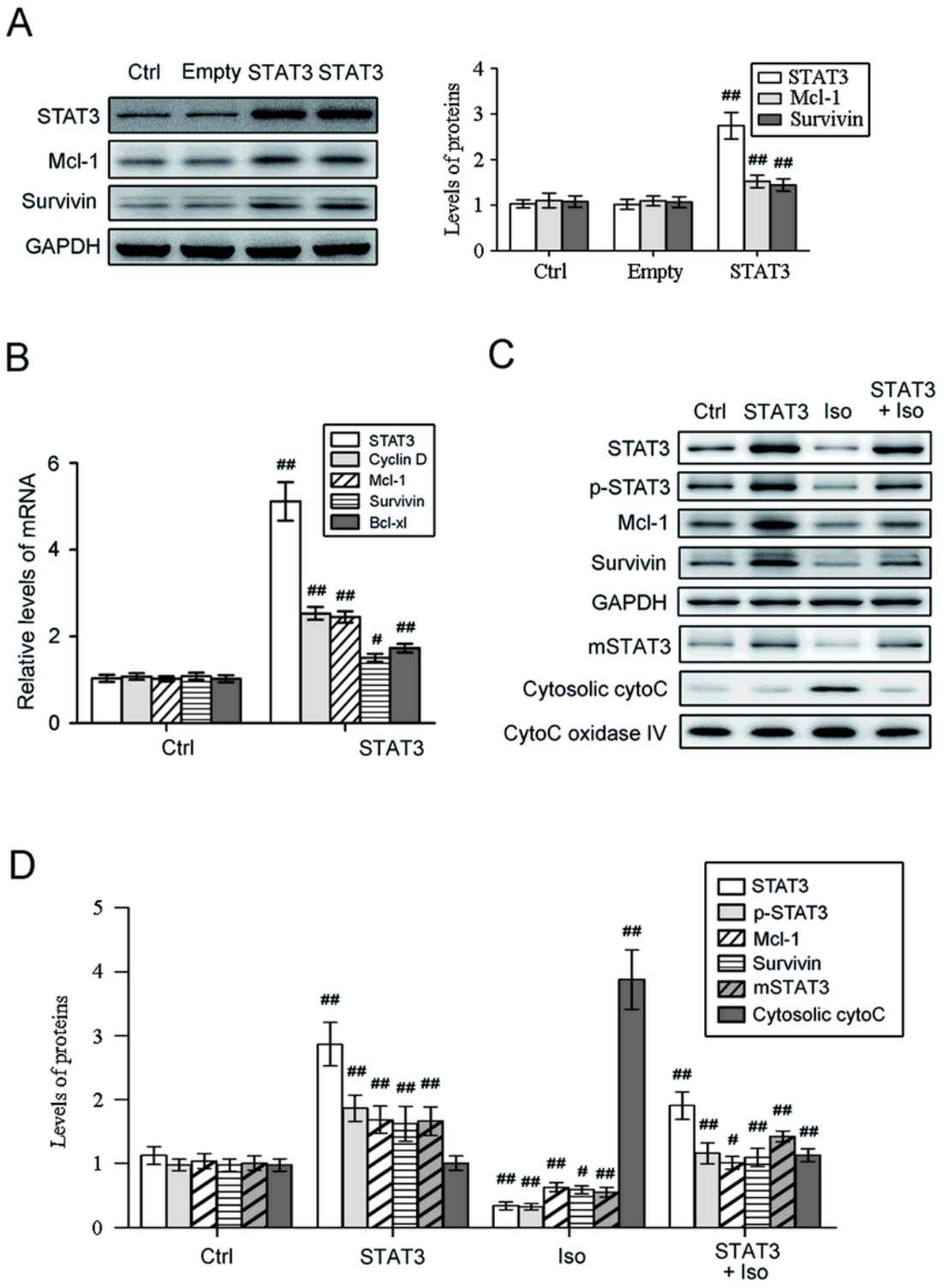

Figure 3

Overexpression of STAT3 is protective against isoflurane-induced cytotoxicity in U251 cells. Western (A) and RT-PCR (B) analysis showed that the transcription and translation of STAT3 as well as its target genes (n = 4) were remarkably upregulated after the transfection of STAT3-pcDNA3 into U251 cells (STAT3). The untransfected cells (Ctrl), and the empty vector-transfected cells (Empty) were set as controls. Representative immunoblotting images (C) and band density analyses (D) revealed that

overexpression of STAT3 (n = 4) prevented the isoflurane-induced decrease of STAT3 ($P < 0.001$), pY705-STAT3 ($P < 0.001$) and its downstream anti-apoptotic proteins ($P = 0.011$ for Mcl-1, $P = 0.006$ for survivin). The mitochondria-located STAT3 was decreased after isoflurane exposure (n = 4, $P = 0.003$). STAT3 overexpression restored this reduction ($P < 0.001$) and protected cytochrome c from releasing to cytoplasm ($P < 0.001$) that induced by isoflurane. GAPDH was used as loading controls for whole cell as well as cytosolic fraction, and cytochrome c oxidase IV was used as loading controls for mitochondrial fraction. # $P < 0.05$, ## $P < 0.01$ versus control. * $P < 0.05$, ** $P < 0.01$ versus isoflurane. mSTAT3 = mitochondria-located STAT3, cytoC = cytochrome c.

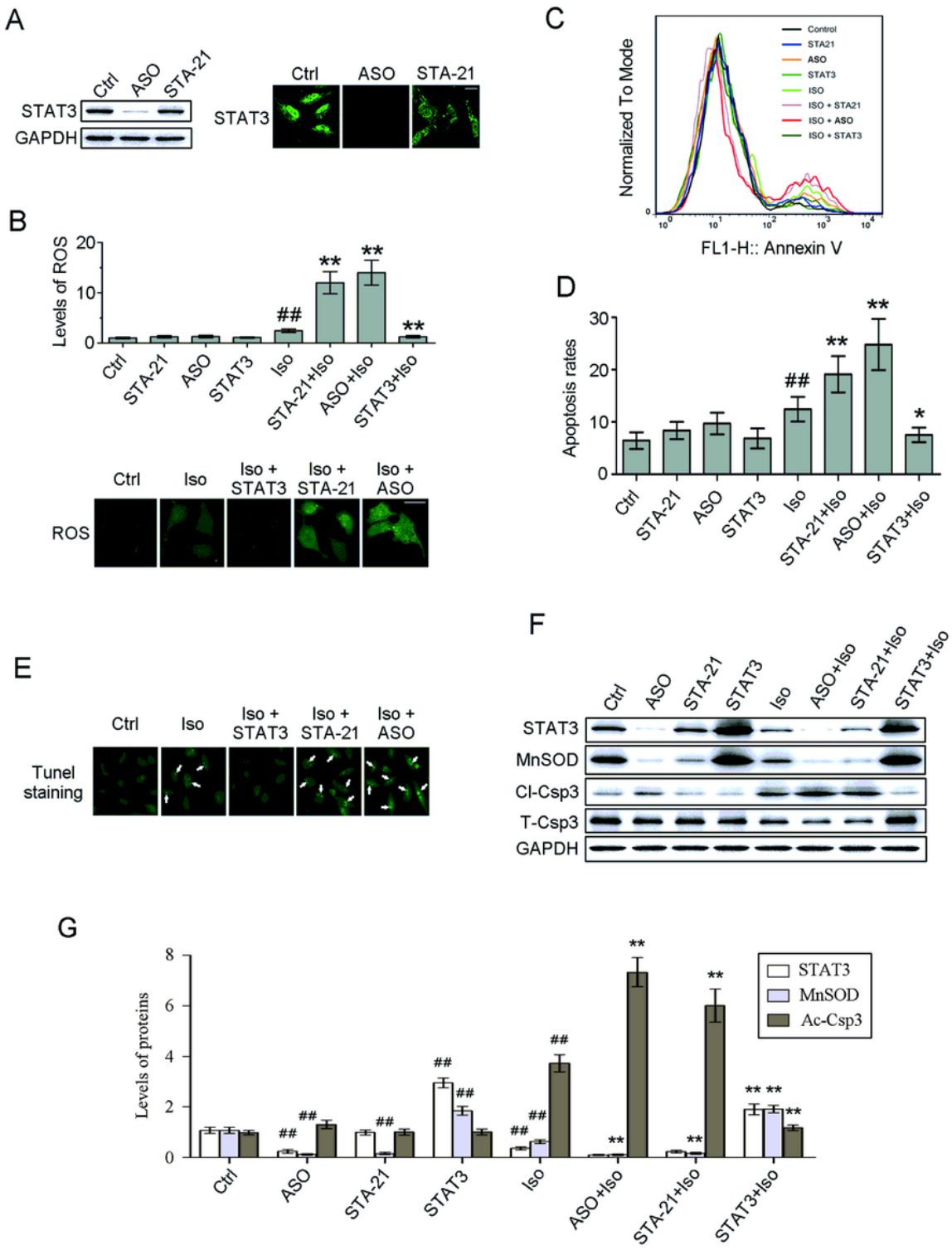

Figure 4

The protection of STAT3 against isoflurane-induced cytotoxicity was related to its anti-oxidative function. (A) Western and immunofluorescence analysis of STAT3 in U251 cells after treatment with STAT3-ASO or STA21 (n = 3). STAT3-ASO transfection resulted in a significant decline in STAT3 protein ($P < 0.001$). In contrast, STA21 treatment reduced the nuclear-located STAT3, with no obvious impact on the total protein level of STAT3. Scale bar, 30 μm. (B) The levels of intracellular ROS were detected using carboxy-

H2DCFDA and presented as a percentage of that in controls (upper, n = 6). STAT3 disruption aggravated ($P < 0.001$), but STAT3 overexpression mitigated ($P < 0.001$) ROS accumulation induced by isoflurane. Representative immunofluorescent images of carboxy-H2DCFDA staining that exhibiting ROS generation in U251 cells were shown (bottom). Scale bar, 30 μm. (C) The percentage of Annexin V positive cells in each sample was analyzed by flow cytometry (n = 6). (D) Statistical results of flow cytometry analysis indicated that the apoptosis induced by isoflurane was obviously augmented in cells with STAT3 knockdown ($P < 0.001$) or STA21 treatment ($P = 0.001$), but attenuated by STAT3 overexpression ($P = 0.002$). (E) Similar results were observed in TUNEL staining (green). Magnification is 200 ×. Arrows indicate TUNEL-positive apoptotic U251cells (3 wells per group, 6 images per well). Representative immunoblotting images (F) and band density analyses (G) revealed that isoflurane downregulated the protein levels of MnSOD in a STAT3-dependent manner (n = 4). STAT3 overexpression restored the isoflurane-induced MnSOD decline and caspase-3 cleavage ($P < 0.001$). ## $P < 0.01$ versus control. * $P < 0.05$, ** $P < 0.01$ versus isoflurane. ASO = antisense oligonucleotide.

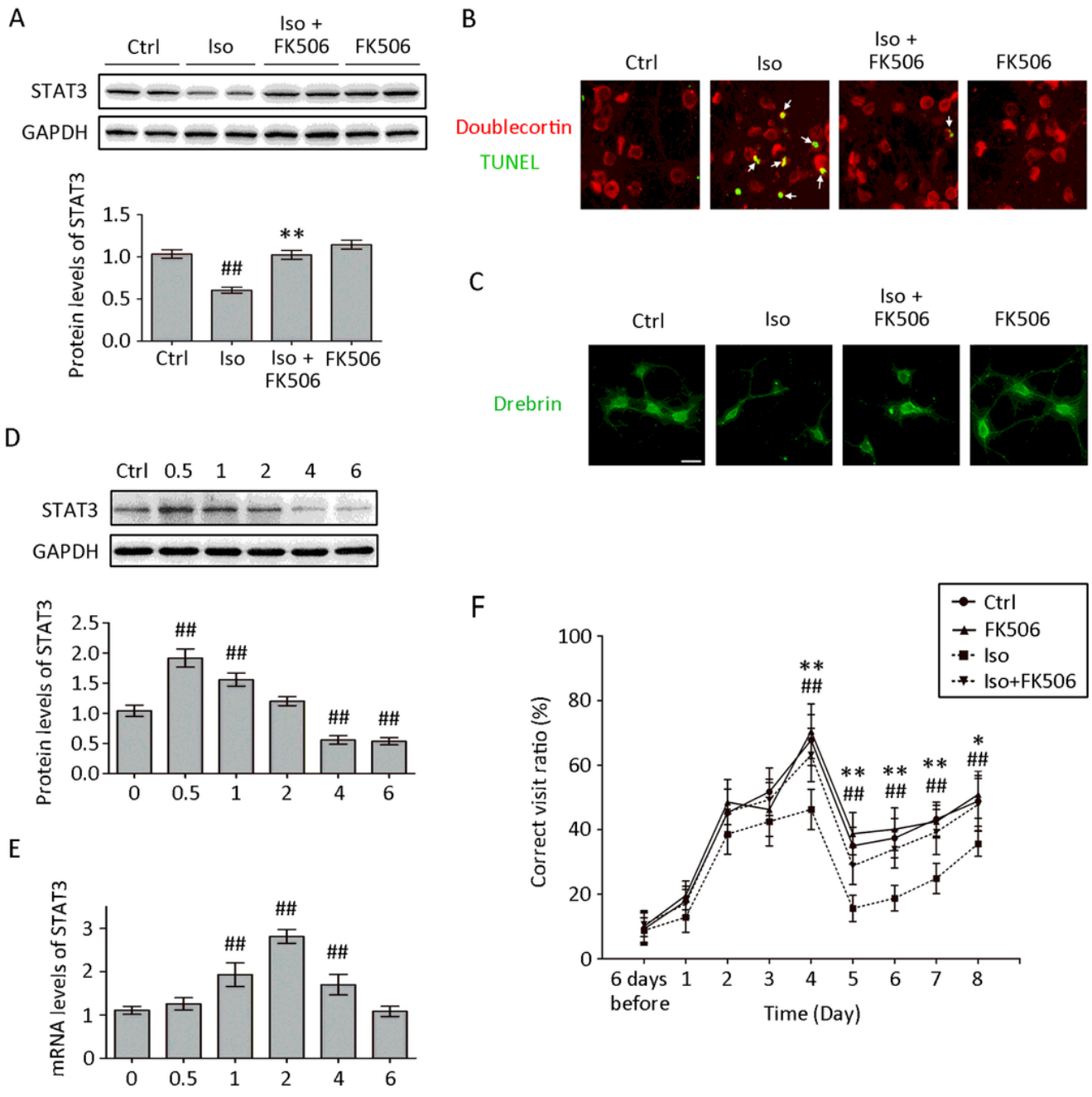

Figure 5

Calcineurin inhibition exhibited long-term protection against the neurotoxicity of isoflurane. (A) Application of FK506 attenuated STAT3 degradation induced by isoflurane in C5 neurons (n = 4, P < 0.001). (B) Double staining with TUNEL staining (green) and Doublecortin (red) was performed in C5 neurons and representative images were shown (4 wells per group, 6 images per well). FK506 pretreatment significantly reduced the apoptotic neurons as compared to that in isoflurane group. Arrows indicated TUNEL-positive neurons. (C) After exposed to isoflurane, C5 neurons were maintained in culture for 5 days and then stained with drebrin (green). FK506 mitigated isoflurane-mediated inhibition of neurite growth and reduction in dendritic spines (n = 4). Scale bar, 20 μm. (D) The protein levels of STAT3

in the frontal cortex of testing mice were measured during 6-h isoflurane exposure (n = 4). (E) Real-time PCR data showed the mRNA levels of STAT3 at the corresponding time (n = 4). Both the protein and mRNA levels of STAT3 were increased initially and then declined persistently. By 4 hrs after isoflurane exposure, STAT3 protein was far below the basal level ($P < 0.001$), in contrast, STAT3 mRNA level was higher as compared with controls ($P = 0.003$). (F) The learning behavior of mice was evaluated in the IntelliCage system four weeks after isoflurane exposure. Correct visit ratios (%) of the four groups at each time point were recorded (n = 8). Although no statistically significant was detected in the initial three days, the isoflurane-treated mice displayed a much lower ability to recognize the correct corner during the following test ($P < 0.01$ at day 4-8). FK506 pretreatment alleviated isoflurane-induced impairment of spatial memory ($P < 0.01$ at day 4-7, $P < 0.05$ at day 8). ## $P < 0.01$ versus control, * $P < 0.05$ versus isoflurane, ** $P < 0.01$ versus isoflurane.

Supplementary Files

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