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The antioxidative effect of STAT3 involved in cellular vulnerability to isoflurane

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Abstract

Background The vulnerable period to neurotoxicity of isoflurane overlaps with a developmental stage characterized by programmed neuronal death. STAT3 has been identified as a crucial molecule involved in survival pathways during this period. We aimed to investigate the role of STAT3 in cellular vulnerability to isoflurane.

Methods C57/BL6 mice on postnatal day 7 or 21, primary neurons derived from mice embryos at gestational days 14–16 and cultured for 5 or 14 days, as well as 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. The levels of Western blot results, mRNA, intracellular ROS, apoptotic rate, and calcineurin activity were analyzed using unpaired Student's t-test or one-way ANOVA followed by Bonferroni post hoc test, as appropriate.

Results Elevated levels of STAT3, reduced activity of calcineurin, as well as a diminished response to isoflurane-induced calcineurin activation and neuroapoptosis were observed in more mature brain or neurons. Isoflurane accelerated the degradation of ubiquitin-conjugated proteins but did not facilitate ubiquitin conjugation to proteins. STAT3 was of particular importance in the all ubiquitin-conjugated proteins degraded by isoflurane. Knockdown or inhibition of STAT3 nuclear translocation exacerbated isoflurane-induced oxidative injury and apoptosis, while STAT3 overexpression mitigated these effects. Finally, this study demonstrated that FK506 pretreatment mitigated the apoptosis, ROS accumulation, and the impairment of neurite growth in primary neurons after exposed to isoflurane.

Conclusions These findings indicate that specific regulation of STAT3 was closely related with the cellular vulnerability to isoflurane via an antioxidative pathway.

Keywords Cellular vulnerability to isoflurane, STAT3, Antioxidative effect

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Background

Growing evidence suggests that the administration of commonly used clinical anesthetics induces widespread neuronal apoptosis in newborn animals [1, 2]. Conversely, the mature brain exhibited a degree of resistance to these effects [3, 4]. Animal studies have also demonstrated that anesthetics can initiate cell death in immature neurons. Nevertheless, the precise mechanism underlying this inherent vulnerability remains largely unknown.

The vulnerable period to the proapoptotic effect of anesthetics coincides with a developmental stage of programmed neuronal death, when neurons require neurotrophins for survival [5, 6]. Numerous studies have demonstrated that the apoptotic neurodegeneration triggered by isoflurane, which is a commonly used volatile anesthetic, in the developing brain is associated with suppressed brain-derived neurotrophic factor signaling [7–9]. However, the key site of survival-promoting proteins in the pathological processes of isoflurane-induced neuroapoptosis is still elusive.

Signal transducer and activator of transcription-3 (STAT3), a transcriptional factor and an intracellular signal transducer, has garnered increasing recognition for its neuroprotective effects in various brain injuries [10]. During the postnatal development of the brain, STAT3 has been identified as a crucial molecule of the survival pathway mediated by neurotrophins [11]. Knockdown of STAT3 in mature neurons has been shown to induce neurotrophin dependency, whereas overexpression of STAT3 confers resistance to neurotrophin deprivation in immature neurons [12]. In this study, we designed experiments involving gain and loss of STAT3 function to elucidate the precise mechanisms underlying the cellular vulnerability to isoflurane.

Methods

Mice anesthesia and treatment

All animal experiments conducted in this study were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the Ethics Review Board for Animal Studies of Nanjing Drum Tower Hospital, following a protocol similar to our previous research [13]. A total of 88 male C57/BL6 mice obtained from the Model Animal Research Center of Nanjing University, on postnatal day 7 or 21, were housed in a controlled environment with a 12-h light/dark cycle and a temperature maintained at 21 ± 1 °C. Mice were randomly divided into anesthesia and age-matched control group. To induce anesthesia, mice were exposed to 1.5% isoflurane in 100% oxygen at 37 °C for 6 h. 1.5% was approximately 0.6 MAC of isoflurane in C57BL6/J mice aged 7–8 days and neuronal apoptosis was significantly increased after a 6 h exposure to 1.5% isoflurane [1]. Isoflurane was delivered from an isoflurane vaporizer (MSS International Limited, UK) into a sealed plastic box with soda lime placed under the bottom partition. The mice breathed spontaneously, and the isoflurane concentration was continuously monitored using a Vamos gas monitor (Drager, Germany). To prevent excessive CO_2 accumulation, the mice were transferred to another plastic box with 100% oxygen and free of isoflurane for 10 min after every 2 h of isoflurane exposure [14]. None of the mice died during anesthesia. Both 30% [1] and 100% [14] oxygen have been reported in previous in vivo studies on isoflurane. In our study, 100% oxygen was used for anesthetizing the mice, and age-matched control mice were exposed to 100% oxygen for the same duration to exclude the possibility that the observed changes were caused by the 100% oxygen exposure.

Primary neuron culture and treatment

Primary cortical neurons were derived from C57/BL6 mice embryos at gestational days 14–16 and cultured in vitro for either 5 days (C5) or 14 days (C14). Neurons were then treated with 1.5% isoflurane in a gas mixture of 21% O_2, 5%CO_2 and balanced nitrogen for 6 h, following the methodology described in our previous study [15]. The concentration of isoflurane used in primary neurons was determined based on a previous study [8], which demonstrated that exposure to 1.4% isoflurane for 4 h increased apoptosis in primary neurons cultured in vitro for 5 days. We adjusted the concentration and exposure time of isoflurane to 1.5% for 6 h to align with the conditions used in our animal experiments. 21% oxygen was used in vitro studies of isoflurane because this concentration is conventionally used in cell culture. In the interaction experiments, neurons were pretreated with FK506 (1μM) 30 min prior to isoflurane exposure.

U251 cell culture and treatment

U251 human neuroglioma cells (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were exposed to 2% isoflurane for 6 h as we described previously [13]. The use of 2% isoflurane in human neuroglioma cells is a commonly employed model in in vitro studies of isoflurane [16–18]. We utilized U251 human neuroglioma cells because we have successfully achieved STAT3 overexpression and knockout in this cell line through transfections. An AxyPrep Endofree Plasmid Miniprep Kit (Axygen Biosciences, USA) was used to extract the plasmids from bacterial culture. The cells were transfected with either the wild-type pcDNA3-6×Myc-mSTAT3 vector [19] or an antisense oligonucleotide (ASO) targeting STAT3 (HSS186131, Invitrogen, USA) using lipofectamine™ 2000 (Invitrogen, USA) and then incubated for 48 h before isoflurane exposure. The untransfected

cells without isoflurane exposure and the empty vector-transfected cells were set as the controls. For the interaction studies, cells were pretreated with either a proteasome inhibitor, MG-132 (30μM, Calbiochem, Germany) [20], FK506 (1μM) or a STAT3 inhibitor, STA-21 (30μM, Enzo Life Sciences, Switzerland) [21], 30 min before isoflurane exposure.

Mitochondria isolation

We used a mitochondria isolation kit for cultured cells (Thermo Scientific, USA) to evaluate the levels of mitochondria-located STAT3 and cytochrome C oxidase released from mitochondria into cytosol in U251 cells. After exposure to isoflurane, cells grown on 10-cm culture dishes (appropriately 1×10^7 cells incubated in 9 ml of culture media per dish) were collected and centrifuged at 850×g for 2 min. The supernatant was discarded, and each sample was then treated with 800 μL of mitochondria isolation reagent A (containing 100:1 protease inhibitors) provided in the kit. After a 2-min incubation on ice, mitochondria isolation was performed using Option A method following the manufacturer's protocol.

Real-time PCR (RT-PCR)

U251 cells 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 until further use. The frozen tissue was ground in liquid nitrogen using a small mortar and subsequently treated with lysis buffer. Retrotranscription and PCR analysis were performed following our previously described methods [15]. Amplification of the STAT3, cyclin D1, Mcl-1, survivin, and Bcl-xl genes (3 repetitions per sample) were carried out using specific oligonucleotide primers (GenScript, USA). The sequences of all primers used in this study can be found in the supplemental material. The housekeeping gene, β-actin, served as the internal standard. The no template sample was used as negative control.

Western blot analysis

Mice in the anesthesia group were sacrificed by cervical dislocation after isoflurane exposure. Age-matched control mice were sacrificed using the same method after a brief period of 1.5% isoflurane anesthesia to induce unconsciousness. The brain tissues were immediately removed and stored in -80℃. The frontal cortex was homogenized with ice-cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Thermo scientific, USA). For the in vitro experiments, lysis buffer was added to six-well plates seeded with U251 cells or primary neurons immediately after isoflurane exposure. A BCA protein assay kit (Thermo Scientific, USA) was used to determine the protein concentration 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.

Detection of intracellular ROS production

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

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 according to the manufacturer's protocol. The apoptotic rate was then analyzed using 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 with 4% PFA for 30 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 5% BSA for 1 h. Immunofluorescence staining was performed using rabbit primary antibodies against STAT3 (1:100), Doublecortin (1:100) and Drebrin (1:100; Abcam), followed by incubation with Alexa Fluor 488 donkey anti-rabbit secondary antibodies (1:200; Molecular Probes, USA). Drebrin, a protein whose loss from dendritic spines is a characteristic feature of synaptic dysfunction in neurological disorders [22], was investigated in experiments on neurite growth. Following exposure to isoflurane, primary neurons were maintained in culture for 5 days and then stained with Drebrin antibodies. Images were captured using a Fluoview Fv10i confocal microscope (Olympus, Japan) and analyzed using Image J software (NIH, Bethesda, MD, USA).

TUNEL staining

U251 cells or primary neurons grown on six-well plates were subjected to TUNEL staining using an in situ cell death detection kit (Roche Applied Science, Germany). After fixed in 4% PFA for 1 h, the cells were permeabilized with 0.1% Triton X-100 for 2 min on ice and then stained with TUNEL for 1 h at 37 °C in the dark. Images were obtained at 200× magnification using a TCS SP2 multiphoton confocal microscope (Leica, Germany).

Calcineurin activity assays

The activity of calcineurin in the frontal cortex was assessed in mice immediately after anesthesia or in age-matched controls. Fresh tissues were homogenized with lysis buffer containing 1% protease inhibitor (Thermo scientific, USA). A calcineurin activity assay kit (Calbiochem, Germany) was used to perform the test as we described previously [15].

Statistics

Statistical analysis was performed using SPSS 13.0 software (IBM Corporation, Armonk, USA). Data were assessed for normal distribution using the Shapiro-Wilk test and expressed as mean ± SD. The levels of Western blot results, mRNA, intracellular ROS, apoptotic rate, and calcineurin activity were analyzed using unpaired Student's t-test or one-way ANOVA followed by Bonferroni post hoc test, as appropriate. $P < 0.05$ (2-tailed) was regarded as statistically significant.

Results

The developmental regulation of STAT3 mediated by calcineurin was closely related with neural vulnerability to isoflurane

Protein and mRNA levels of STAT3 in the frontal cortex were assessed in mice exposed to isoflurane on postnatal day 7 (P7) for varying durations. The protein levels of STAT3 were significantly increased after 30 min of isoflurane exposure (Fig. 1A; $n=4$, $P<0.001$), but significantly decreased after isoflurane exposure for 4 h ($n=4$, $P<0.001$). In contrast, the mRNA levels of STAT3 showed a remarkable increase after 1 h of isoflurane exposure (Fig. 1B; $n=4$, $P<0.001$) and remained elevated until isoflurane exposure for 6 h.

To investigate the relationship between STAT3 regulation and time-restricted neurotoxicity of isoflurane, mice on postnatal day 7 (P7) and day 21 (P21) were exposed to isoflurane for 6 h. Our study demonstrated that isoflurane significantly increased the level of activated caspase-3 in the frontal cortex of P7 mice (Fig. 1C; $n=4$, 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, $P=0.96$). Furthermore, the protein levels of STAT3 in the frontal cortex of P21 mice were significantly higher when compared with that in P7 mice ($n=4$, 1.87 ± 0.15 vs. 1.05 ± 0.04, $P<0.001$). The activity of calcineurin, a protein phosphatase specifically promotes STAT3 degradation, was significantly lower in the frontal cortex of P21 mice (Fig. 1D; $n=6$, 0.87 ± 0.16 vs. 2.10 ± 0.34, $P<0.001$). Interestingly, 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, $P<0.001$), while the increase in calcineurin activity in P21 mice was minimal ($n=6$, $P=0.21$).

In the in vitro system, similar developmental regulation was observed. Compared with primary cortical neurons cultured for 5 days (C5), a significant upregulation in STAT3 protein level (Fig. 1E; $n=4$, 1.70 ± 0.13 vs. 0.99 ± 0.08, $P<0.001$) was observed in neurons cultured for 14 days (C14), at the instance when isoflurane did not induce apoptosis ($P=1.00$). Additionally, a notable reduction in STAT3 protein levels ($n=4$, 0.62 ± 0.08 vs. 0.99 ± 0.08, $P=0.002$) accompanied by an increase in calcineurin activity (Fig. 1F; $n=6$, 3.85 ± 0.63 vs. 1.87 ± 0.34, $P<0.001$) was observed in C5 neurons after isoflurane exposure, whereas no significant changes were observed 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).

STAT3 was the key element in the all ubiquitinated proteins degraded by isoflurane in U251 cells

To investigate the role of STAT3 in cellular vulnerability to isoflurane, we examined the effects of ectopic STAT3 expression on isoflurane-induced cytotoxicity in U251 cells. Decreased protein levels of STAT3, pY705-STAT3 and its downstream survival targets were observed in U251 cells following isoflurane exposure (Fig. 2A; $n=4$, $P<0.001$ for STAT3 and pY705-STAT3, $P=0.001$ for Bcl-xl and survivin). Immunofluorescence staining revealed a 30% reduction of STAT3 in U251 cells after isoflurane exposure (Fig. 2B; $n=6$, $P<0.001$).

In contrast to the decrease in STAT3 protein levels, the mRNA levels of STAT3 in U251 cells were significantly increased after 6 h of isoflurane exposure (Fig. 2C; $n=4$, 2.34 ± 0.14 vs. 1.04 ± 0.08, $P<0.001$). To assess the role of STAT3 degradation in isoflurane-induced cytotoxicity, a proteasome inhibitor MG132 was introduced initially (Fig. 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 the reduction of total ubiquitinated proteins ($P<0.001$) as well as STAT3 ($P=0.012$) after isoflurane exposure. However, MG132 coincubation did not lead to further enhancement of protein ubiquitination ($P=0.14$). Additionally, MG132 enhanced the levels of Bim, an essential initiator of apoptosis ($P<0.001$), and increased caspase-3 activation ($P<0.001$) after isoflurane treatment. In contrast, pretreatment with FK506, a specific calcineurin

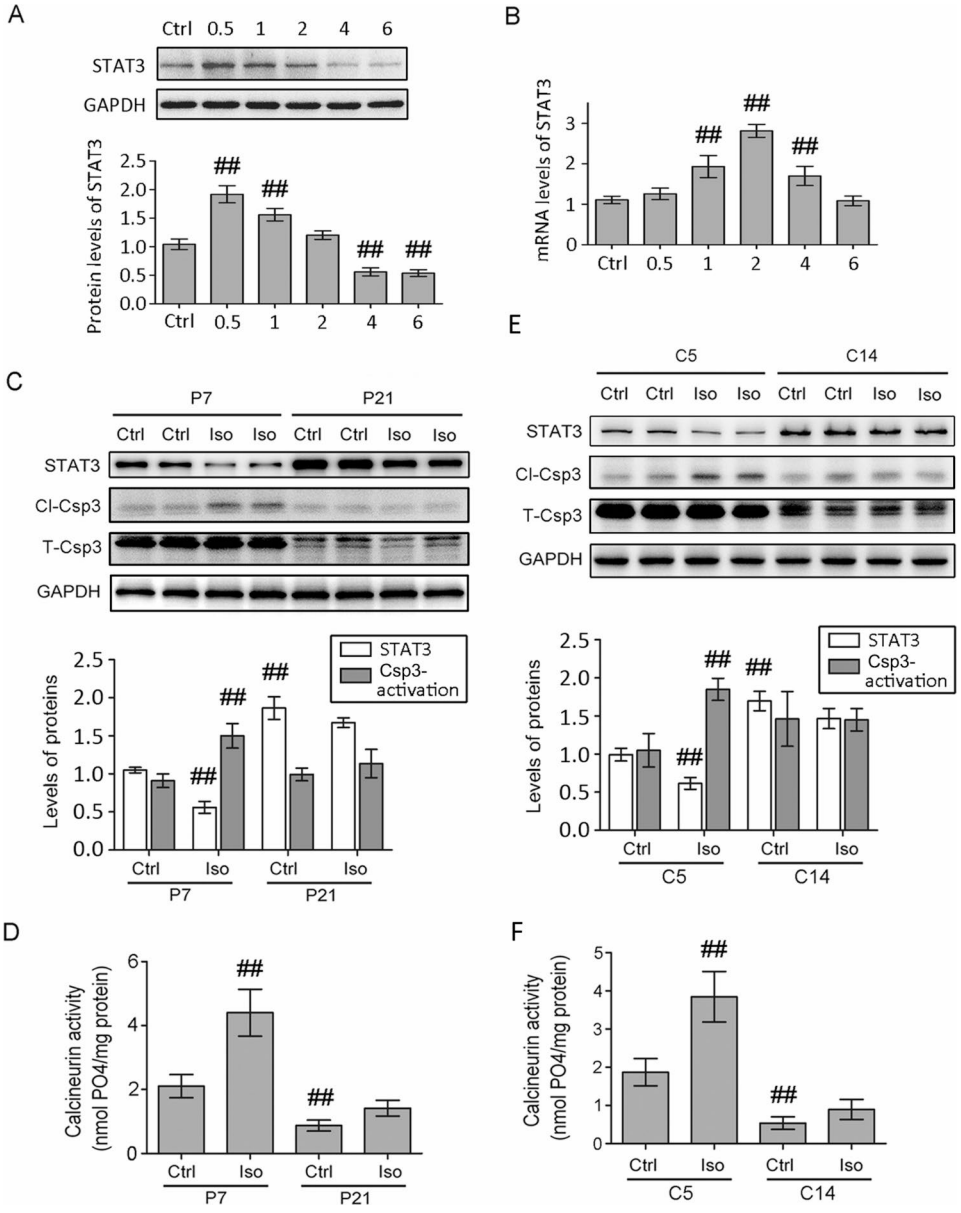

Fig. 1 STAT3 degradation mediated by calcineurin was closely related with the neural vulnerability to isoflurane. **(A)** The protein levels of STAT3 in the frontal cortex of mice on postnatal day 7 were assessed after isoflurane exposure for varying durations ($n=4$). GAPDH was used as an internal control. ## $P < 0.01$ *versus* control. The polyvinylidene difluoride membranes with transferred proteins were cropped according to the protein ladders and then incubated with different antibodies. The contrast and brightness of the blot images were adjusted uniformly. **(B)** The mRNA levels of STAT3 in the frontal cortex of mice on postnatal day 7 were assessed after isoflurane exposure for varying durations by Real-Time PCR ($n=4$). ## $P < 0.01$ *versus* control. **(C)** Isoflurane significantly increased the level of activated caspase-3 ($n=4$, $P<0.001$) in the frontal cortex of mice on postnatal day 7 (P7), but not in mice on postnatal day 21 (P21) ($n=4$, $P=0.96$). The protein levels of STAT3 were significantly higher in P21 mice compared to P7 mice ($P<0.001$). 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* P7 control mice. (**D)** The activity of calcineurin in the frontal cortex of P21 mice was significantly lower than that in P7 mice ($n=6$, $P<0.001$). Isoflurane increased calcineurin activity ($n=6$, $P<0.001$) in the frontal cortex of P7 mice, while the increase in P21 mice was minimal ($n=6$, $P=0.21$). The activity of calcineurin was expressed in nanomolar PO_4 per milligram of protein. ## $P < 0.01$ *versus* control in P7. (**E)** The primary cortical neurons were cultured in vitro for 5 days (C5) or 14 days (C14) prior to experiments. A similar stage-dependent proapoptotic effect of isoflurane accompanied by a developmental regulation of STAT3 was also exhibited in neurons ($n=4$). ## $P < 0.01$ *versus* C5 control neurons. (**F)** The activity of calcineurin in C14 neurons was significantly lower than that in C5 neurons ($n=6$, $P<0.001$). No significant changes were observed in C14 neurons after isoflurane exposure ($n=6$, $P=0.76$). ## $P < 0.01$ *versus* C5 control neurons. Ctrl, control; Iso, isoflurane; T-Csp3, full-length caspase-3; Cl-Csp3, activated caspase-3

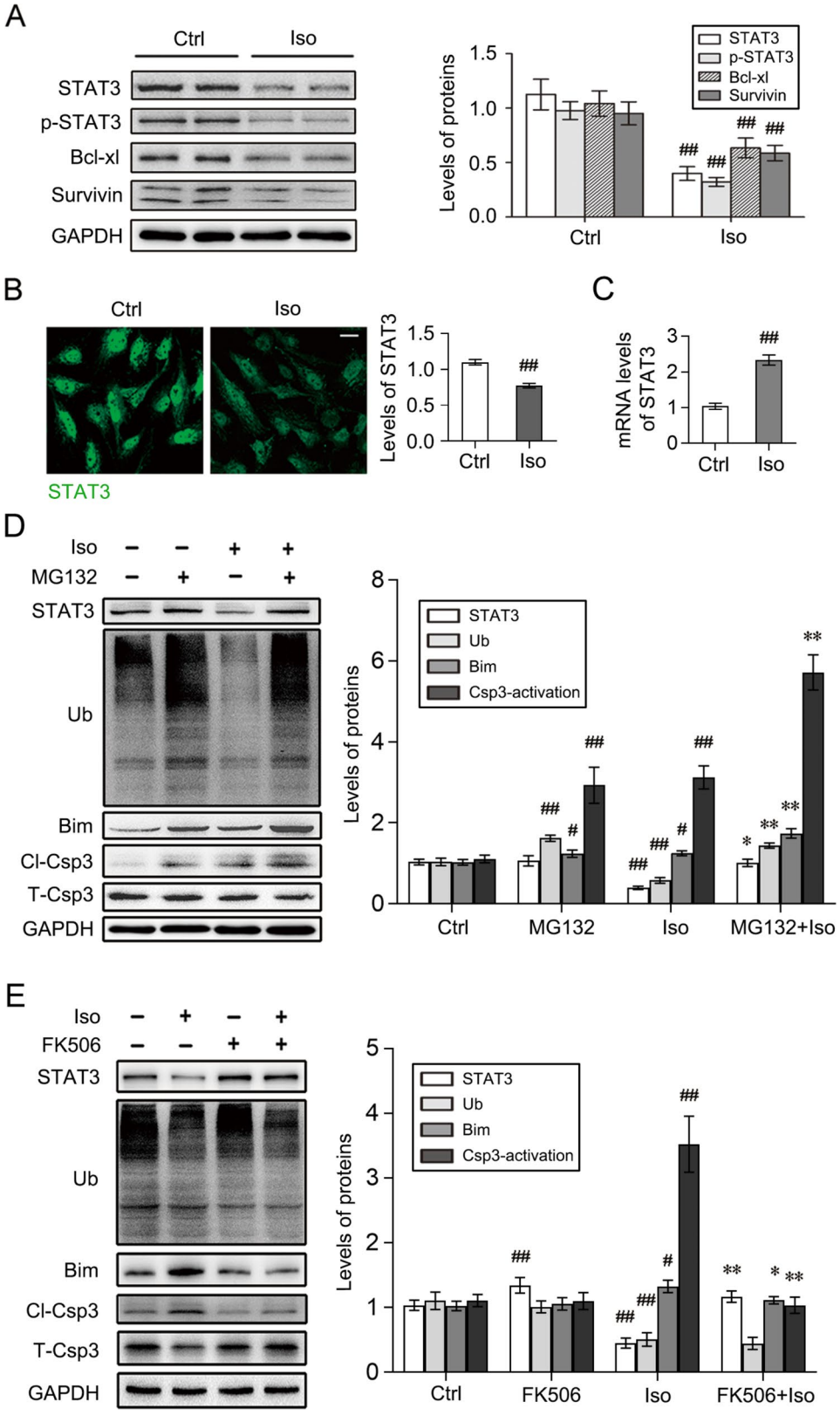

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Fig. 2 STAT3 was the key element in the all ubiquitinated proteins degraded by isoflurane in U251 cells. (**A)** The protein levels of STAT3, pY705-STAT3 and its downstream survival targets were significantly decreased ($P<0.001$ for STAT3 and pY705-STAT3, $P=0.001$ for Bcl-xl and survivin, $n=4$) in U251 cells after isoflurane exposure for 6 h. GAPDH was used as an internal control. The blots were cropped according to the ladders and then incubated with different antibodies. The contrast and brightness of the blot images were adjusted uniformly. (**B)** Immunofluorescence staining revealed 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 significantly increased ($n=4$, $P<0.001$) after 6 h of isoflurane exposure. Housekeeping gene β-actin was used as internal standard. (**D)** MG132 prevented the reduction of both STAT3 ($P=0.012$) and total ubiquitinated protein ($P<0.001$), but enhanced caspase-3 activation ($P<0.001$) following isoflurane exposure in U251 cells ($n=4$). **(E)** Pretreatment with FK506 prevented STAT3 degradation ($P<0.001$) and caspase-3 activation ($P<0.001$) without affecting the levels of total ubiquitinated proteins ($P=1.0$) following isoflurane exposure 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 only

inhibitor, prevented the degradation of STAT3 (Fig. 2E, $n=4$; $P<0.001$) and cleavage of caspase-3 ($P<0.001$) without affecting the levels of total ubiquitinated proteins ($P=1.0$) after isoflurane exposure.

Ectopic STAT3 protected cells from isoflurane-induced cytotoxicity in U251 cells

To investigate the protective role of STAT3 against isoflurane-induced cytotoxicity, U251 cells were 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 (Fig. 3A; $n=4$, $P<0.001$) as well as its downstream anti-apoptotic factors, such as Mcl-1 ($P=0.006$) and survivin ($P=0.008$), after 48 h of post-transfection. Real-time PCR showed that the transcript levels of STAT3 target genes, including cyclin D1 (Fig. 3B; $n=4$, $P<0.001$), Mcl-1 ($P<0.001$), survivin ($P=0.014$), and Bcl-xl ($P=0.003$), were significantly upregulated when compared to those of controls.

Next, we observed that STAT3 overexpression was able to restore the decline of STAT3 and its downstream anti-apoptotic proteins after isoflurane exposure (Fig. 3C; $n=4$, $P<0.001$ for STAT3 and pY705-STAT3, $P=0.011$ for Mcl-1, $P=0.006$ for survivin), as well as mitochondria-located STAT3 (Fig. 3C; $n=4$, $P<0.001$). Additionally, the release of cytochrome C from mitochondria into the cytoplasm after isoflurane exposure was also prevented by STAT3 overexpression (Fig. 3C; $n=4$, $P<0.001$).

The protective effects of STAT3 involved an antioxidative stress mechanism in U251 cells

To investigate whether the cellular protective function of STAT3 involves an antioxidative stress mechanism, we utilized an antisense oligonucleotide (ASO) to knockdown STAT3 and a specific inhibitor STA-21 to inhibit STAT3 dimerization, in addition to the STAT3 overexpression assay.

Western blot analysis and immunofluorescence staining confirmed that STAT3-ASO efficiently reduced STAT3 expression in U251 cells (Fig. 4A; $n=3$, $P<0.001$). Treatment with STA-21 for 6 h significantly inhibited the nuclear translocation of STAT3 without affecting its total protein levels. STAT3 knockdown or its nuclear-translocation disruption resulted in a more than 10-fold increase of ROS accumulation in U251 cells after isoflurane exposure, when compared with that of controls (Fig. 4B; $n=6$, $P<0.001$). The apoptotic rates after isoflurane exposure (Fig. 4C; $n=6$, $12.42 \pm 2.23\%$) were obviously augmented in cells with STAT3 knockdown ($24.78 \pm 4.65\%$, $P<0.001$) or STA-21 pretreatment ($19.10 \pm 3.31\%$, $P<0.001$). Conversely, STAT3 overexpression mitigated isoflurane-induced ROS accumulation (Fig. 4B; $n=6$, $P<0.001$) and apoptosis (Fig. 4C; $n=6$, $7.51 \pm 1.33\%$, $P=0.002$). These protective effects of STAT3 were confirmed by TUNEL staining (Fig. 4D; $n=3$) and Western blot analysis of cleaved caspase-3 (Fig. 4E; $n=4$). Notably, STAT3 disruption or overexpression did not affect the levels of ROS and apoptosis in cells without isoflurane exposure. Moreover, we observed a decrease in the protein level of manganese-containing superoxide dismutase (MnSOD) after isoflurane exposure (Fig. 4E; $n=4$, $P<0.001$). The decline in MnSOD was further exacerbated in cells with STAT3 disruption but was restored by STAT3 overexpression ($P<0.001$).

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. In primary neurons cultured for 5 days, STAT3 degradation (Fig. 5A; $n=4$, $P<0.001$), neural apoptosis (Fig. 5B; $n=4$, $P<0.001$), and ROS accumulation (Fig. 5C; $n=4$, $P<0.001$) after isoflurane exposure were prevented by FK506. Neurons in the control group exhibited extensive and overlapping neurites. Five days post isoflurane exposure, primary neurons showed a significant reduction in the number of neurite branching compared to the control group (Fig. 5D; $n=4$, $P<0.001$). FK506 pretreatment mitigated the impairment of neurite branching after isoflurane exposure ($n=4$, $P<0.001$).

Discussion

In the present study, we found that the degradation of STAT3 mediated by calcineurin plays a significant role in the susceptibility to the proapoptotic effect of isoflurane

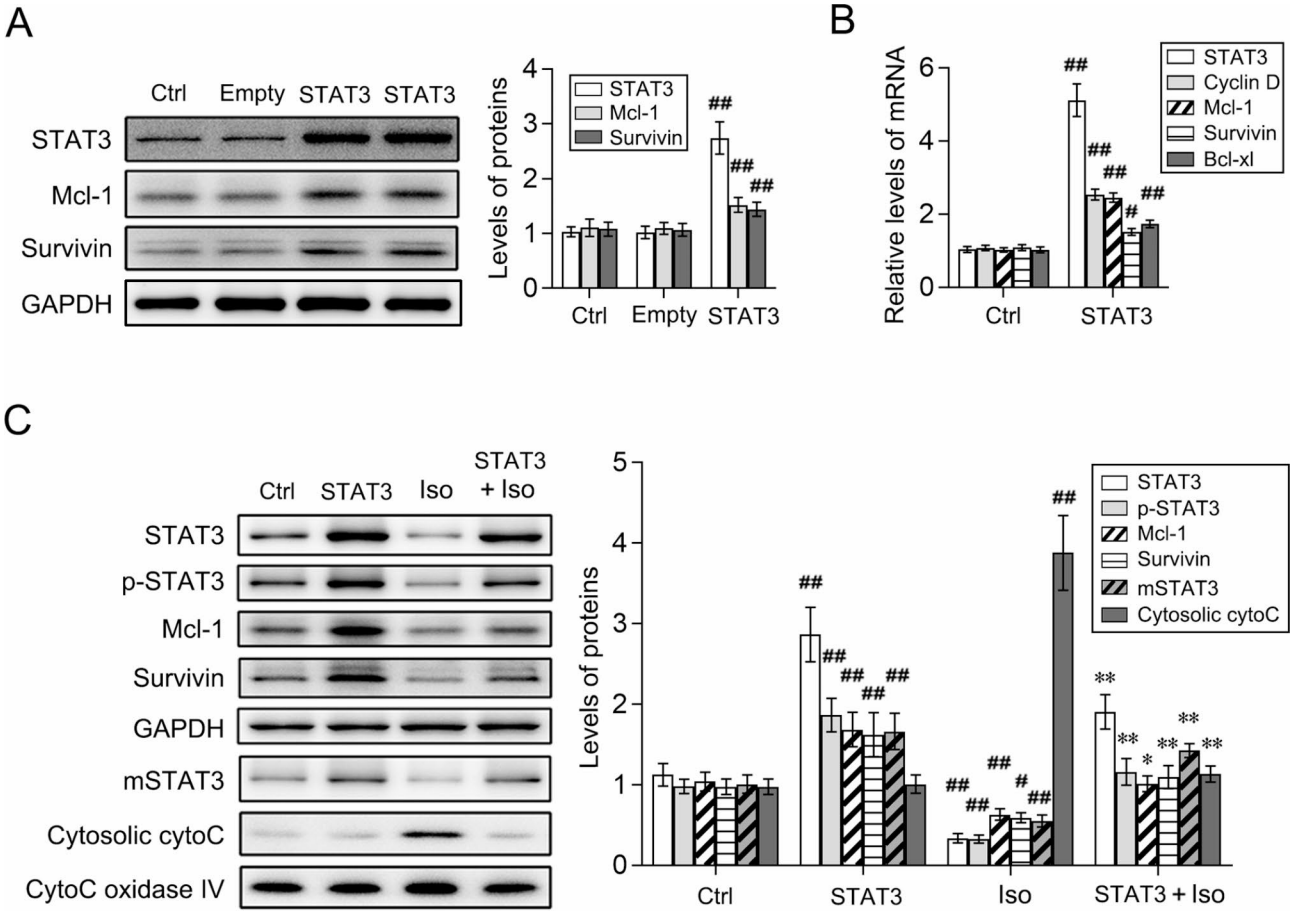

Fig. 3 STAT3 overexpression is protective against isoflurane-induced cytotoxicity in U251 cells. Western blot (**A)** and RT-PCR (**B)** analysis showed that the transcription and translation of STAT3 as well as its target genes ($n=4$) were remarkably upregulated after the transfection of STAT3-pcDNA3 into U251 cells (STAT3). The untransfected cells (Ctrl) were set as controls. Empty, empty vector-transfected cells. (**C)** Western blot analysis were performed after isoflurane exposure in U251 cells with or without STAT3 overexpression ($n=4$). STAT3 overexpression was able to restore the decline 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) induced by isoflurane. The mitochondria-located STAT3 (mSTAT3) was also decreased after isoflurane exposure ($n=4$, $P=0.003$). STAT3 overexpression prevented this reduction ($P<0.001$) and the release of cytochrome C (cytoC) from mitochondria into the cytoplasm after isoflurane exposure ($P<0.001$). GAPDH was used as loading controls for whole cell as well as cytosolic fraction. Cytochrome C oxidase IV was used as loading controls for mitochondrial fraction. The blots were cropped according to the ladders and then incubated with different antibodies. The contrast and brightness of the blot images were adjusted uniformly. # $P < 0.05$, ## $P < 0.01$ *versus* control. * $P < 0.05$, ** $P < 0.01$ *versus* isoflurane only

in the brain of mice and cultured primary cortical neurons. Subsequently, we used the U251 cell line to investigate the underlying mechanism and demonstrated that STAT3 is particularly important in the degradation of all ubiquitin-conjugated proteins induced by isoflurane. Moreover, the disruption of STAT3 exacerbated the oxidative injury and apoptosis induced by isoflurane, while the overexpression of STAT3 mitigated these effects. These findings highlight the critical role of STAT3 in the cellular response to isoflurane.

Isoflurane exhibits dual effects (neuroprotective or neurotoxic) in a concentration- and duration-dependent manner, both in vivo and in vitro [23–25]. Therefore, we used different concentrations, based on a literature review, to treat mice, primary neurons, and human neuroglioma cells. While prolonged exposure to isoflurane can directly induce neurotoxicity due to its inherent properties, a duration of 6 h is commonly used to study its neurotoxic effects [2, 18, 26, 27]. In the present study, the protein levels of STAT3 in the frontal cortex of mice on postnatal day 7 were initially increased but significantly decreased after 4 h of isoflurane exposure. In contrast, the mRNA level of STAT3 showed a remarkable increase after 1 h of isoflurane exposure and remained elevated throughout the observation period, suggesting the involvement of post-transcriptional mechanism in the final downregulation of STAT3 protein induced by isoflurane.

Calcineurin is a protein phosphatase that plays a crucial role in various cellular processes and is particularly abundant in the brain [28]. It is involved in the degradation of STAT3 and regulates the developmental death of

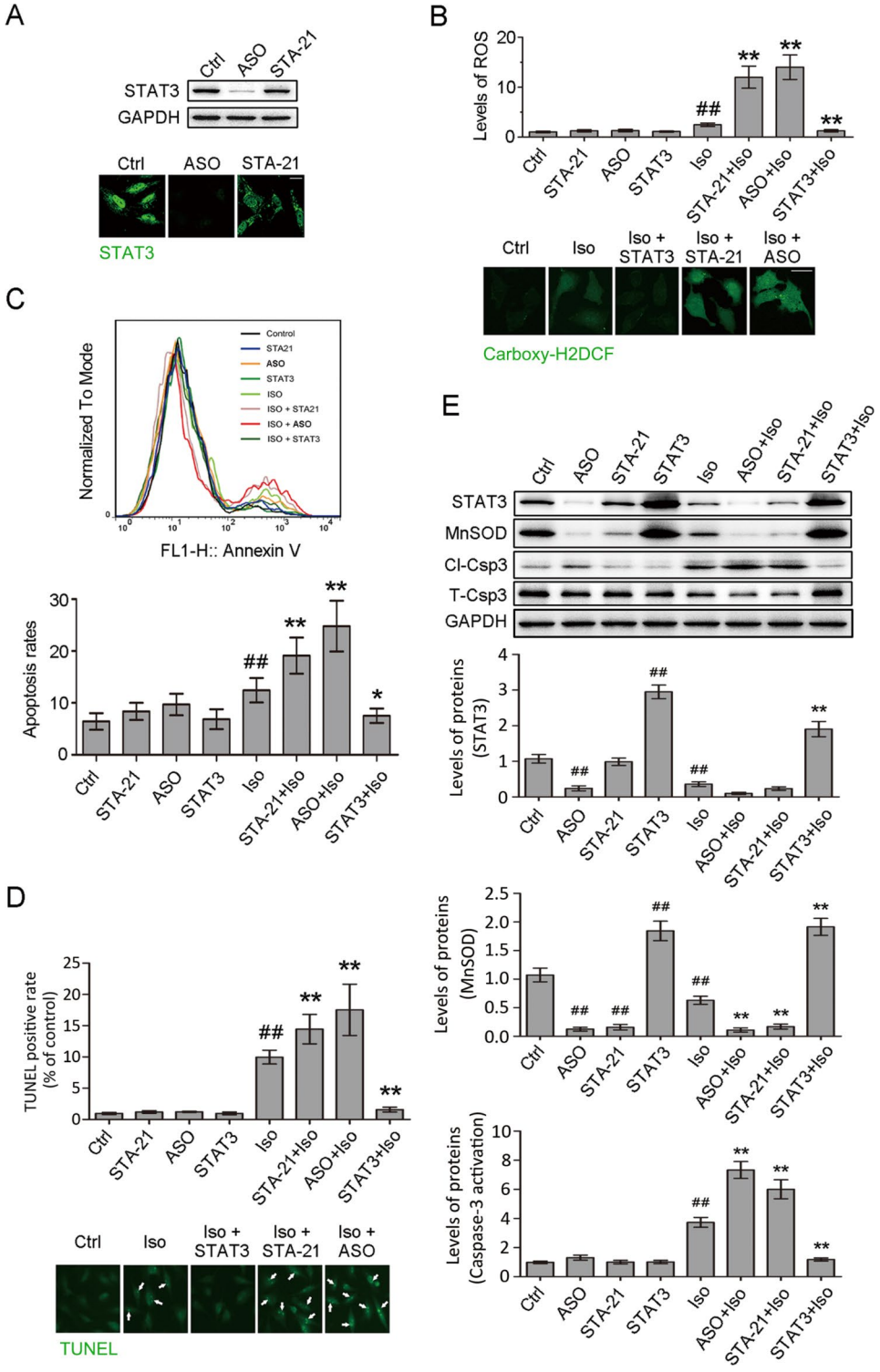

Fig. 4 (See legend on next page.)

neurons in the newborn brain [29]. In the present study, it was observed that calcineurin activity in the frontal cortex of mice was significantly lower on postnatal day 21 compared to postnatal day 7, accompanied by a notable increase in STAT3 protein levels in mice on postnatal day 21. Interestingly, isoflurane exposure resulted in a 2-fold increase in calcineurin activity in the frontal cortex of mice on postnatal day 7, but not in mice on postnatal day 21. Similar developmental regulation of STAT3 was observed in primary cortical neurons cultured in

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Fig. 4 The protection of STAT3 against the cytotoxicity of isoflurane was related to its antioxidative effect. **(A)** Western blot analysis and immunofluorescence staining of STAT3 in U251 cells treated with STAT3-ASO or STA-21 ($n=3$). STAT3-ASO efficiently reduced STAT3 expression ($P<0.001$). STA-21 treatment for 6 h significantly inhibited the nuclear translocation of STAT3 without affecting its total protein levels. Scale bar, 30 μm. ASO, antisense oligonucleotide. The blots were cropped according to the ladders and then incubated with different antibodies. The contrast and brightness of the blot images were adjusted uniformly. **(B)** The levels of intracellular ROS were detected using carboxy-H2DCFDA in U251 cells 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 images of carboxy-H2DCFDA staining in U251 cells are shown in the bottom panel. Scale bar, 30 μm. **(C)** The percentage of Annexin V positive cells was analyzed with flow cytometry ($n=6$). The apoptosis induced by isoflurane was obviously augmented in cells with STAT3 knockdown ($P<0.001$) or STA-21 pretreatment ($P=0.001$), but was mitigated in cells with STAT3 overexpression ($P=0.002$). **(D)** TUNEL staining (green) confirmed the protective effects of STAT3 overexpression. Magnification is 200 ×. Arrows indicate TUNEL-positive U251 cells (3 wells per group, 6 images per well). **(E)** Western blot analysis revealed that isoflurane downregulated the protein level of MnSOD in a STAT3-dependent manner ($n=4$). STAT3 overexpression restored the decline in MnSOD and cleavage of caspase-3 after isoflurane exposure in U251 cells ($P<0.001$). ## $P < 0.01$ *versus* control. * $P < 0.05$, ** $P < 0.01$ *versus* isoflurane only

vitro. Neurons cultured for 14 days exhibited elevated STAT3 levels, reduced calcineurin activity, and a diminished response to isoflurane-induced calcineurin activation compared to neurons cultured for 5 days. Moreover, the study revealed that the application of the calcineurin inhibitor FK506 in neurons cultured for 5 days alleviated the inhibitory effect of isoflurane on neurite growth. These findings collectively suggest that STAT3 degradation mediated by calcineurin may be closely associated with the developmental stage-related vulnerability of the brain to the effects of isoflurane.

The ubiquitin-proteasome pathway is a crucial cellular mechanism for protein degradation and is involved in the regulation of various signaling pathways. Proteins targeted for degradation are conjugated with ubiquitin molecules, and the ubiquitinated proteins are subsequently recognized and degraded by the proteasome [30]. Previous studies have demonstrated the impact of ubiquitin metabolism on growth inhibition of isoflurane in yeast [31, 32]. Additionally, recent research has revealed that isoflurane preconditioning reduces the aggregation of ubiquitin-conjugated proteins and prevents the depletion of free ubiquitin in the CA1 region of the hippocampus following global cerebral ischemia in mice [33]. In the present study, it was observed that isoflurane exposure led to a 40% decrease in the levels of total ubiquitin-conjugated proteins in U251 cells. Interestingly, co-incubation with the proteasome inhibitor MG132 did not further increase the levels of ubiquitin-conjugated proteins, indicating that isoflurane accelerated the degradation of ubiquitin-conjugated proteins but did not promote ubiquitin conjugation to proteins. Furthermore, the study found that MG132 treatment resulted in exacerbated apoptosis after isoflurane exposure, highlighting the importance of specifically regulating the ubiquitination and proteasomal degradation of key proteins in the cellular response to isoflurane-induced cytotoxicity.

STAT3, as a transcription factor, is known to play a critical role in brain development [34]. During the developmental stage of the brain, neurons undergo a process of developmental death and rely on neurotrophins for survival. Overexpression of STAT3 has been shown to eliminate the dependency of neurons on neurotrophins during this vulnerable period, suggesting that STAT3 acts as a key mediator of the survival pathway acquired by neurons [12]. Furthermore, it has been reported that calcineurin mediates the degradation of STAT3 by directing it to the E3 ubiquitin ligase complex [29]. In order to investigate whether STAT3 is a crucial mediator of cellular vulnerability to isoflurane, gain- and loss-of-function studies were conducted in U251 cells. The study confirmed that the levels of STAT3, p-STAT3, and its downstream anti-apoptotic proteins were significantly reduced after exposure to isoflurane in U251 cells. Interestingly, while global blocking of the degradation of all ubiquitinated proteins by MG132 aggravated apoptosis after isoflurane exposure, specifically blocking the degradation of STAT3 using the calcineurin inhibitor FK506 prevented caspase-3 activation following isoflurane exposure, revealing the particular importance of STAT3 in the cytotoxicity induced by isoflurane.

This notion was further supported by the result that STAT3 overexpression in U251 cells mitigated ROS accumulation, caspase-3 activation and apoptosis induced by isoflurane, whereas cells with STAT3 knockdown exhibited increased sensitivity to isoflurane toxicity, resembling the cellular phenotype of immature neurons with lower levels of STAT3. Moreover, STAT3 knockdown or overexpression did not affect the basal levels of ROS and apoptotic rate in cells not exposed to isoflurane, indicating that STAT3 functions specifically in a stress-related protective mechanism.

Oxidative injury is regarded as a key event in isoflurane-induced cell apoptosis [17, 35]. Previous studies have shown that both enhanced ROS production [36] and impaired ROS scavenging are involved in the ROS accumulation after isoflurane exposure [37]. The present study found that inhibiting the nuclear translocation of STAT3 using STA-21 significantly aggravated isoflurane-induced ROS accumulation and apoptosis, similar to the effect observed with STAT3 knockdown. This indicates that the prominent effect of STAT3 is linked to its canonical activity as a nuclear transcription factor. The protein level of MnSOD, a direct target of STAT3, was

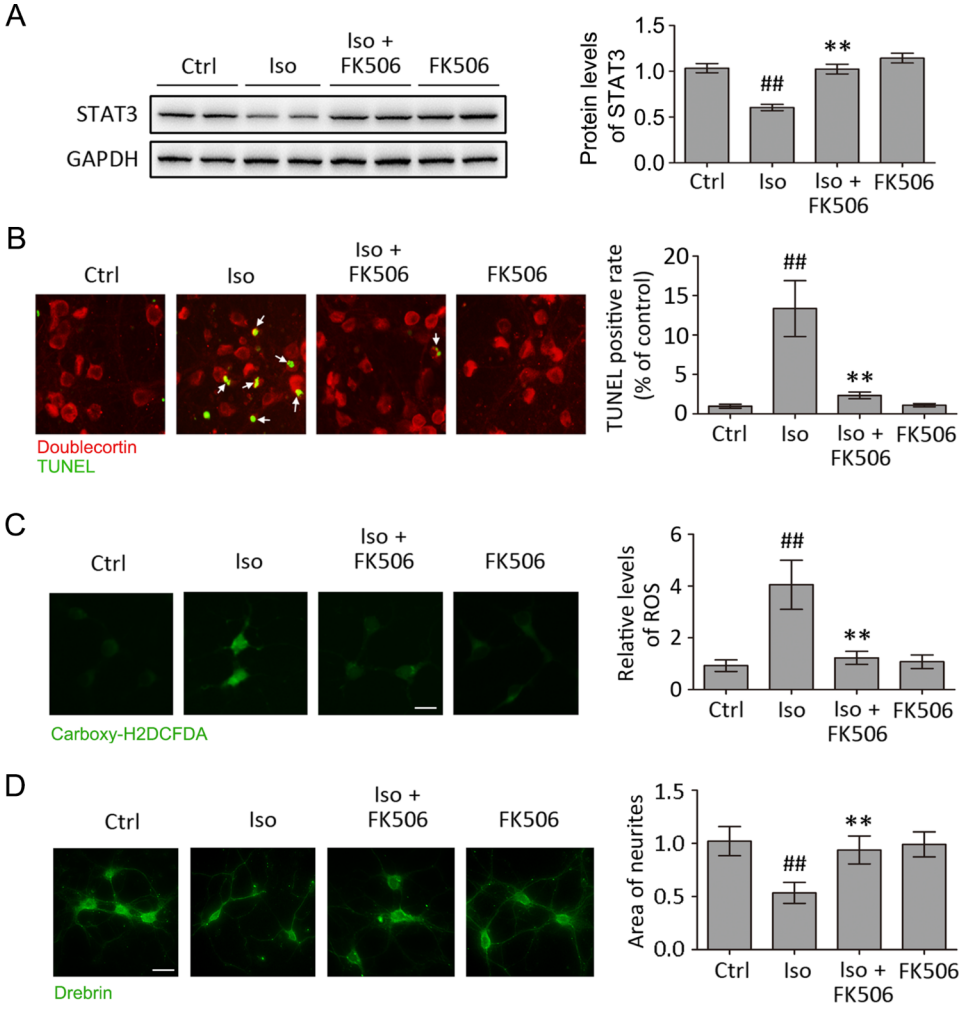

Fig. 5 Calcineurin inhibition exhibited protection against the neurotoxicity of isoflurane. **(A)** Application of FK506 prevented STAT3 degradation induced by isoflurane in primary cortical neurons cultured for 5 days ($n = 4$, $P < 0.001$). The blots were cropped according to the ladders and then incubated with different antibodies. The contrast and brightness of the blot images were adjusted uniformly. **(B)** Representative images of double staining with TUNEL staining (green) and Doublecortin (red, a neuron marker) in neurons. Statistical analysis results of the percentage of TUNEL positive neurons normalized by the control group is shown in the right panel (4 wells per group, 6 images per well). FK506 pretreatment significantly reduced the number of apoptotic neurons after isoflurane exposure. Arrows indicated TUNEL-positive neurons. **(C)** The representative images of primary neurons stained with carboxy-H2DCFDA after isoflurane exposure are shown. FK506 attenuated the ROS accumulation after isoflurane exposure in primary neurons. Graph presenting quantification of the ROS level normalized by the control group is represented in the right panel (4 wells per group, 6 images per well). Scale bar, 20 μm. **(D)** Neurons were maintained in culture for 5 days after isoflurane exposure and then stained with drebrin (green). FK506 mitigated the impairment of neurite growth induced by isoflurane (4 wells per group, 6 images per well). Scale bar, 20 μm. ## $P < 0.01$ *versus* control, ** $P < 0.01$ *versus* isoflurane only

decreased after isoflurane exposure. Furthermore, this decrease was exacerbated in cells with STAT3 disruption but restored by STAT3 overexpression. These findings suggest that isoflurane-induced ROS accumulation is partially attributed to impaired ROS scavenging due to MnSOD deficiency. ROS is generated during incomplete reduction of oxygen by electrons released from the mitochondrial electron transport chain (ETC), primarily from complexes I and III. STAT3 has been shown to colocalize with complex I and play an essential role in the optimal function of the electron transfer chain [38, 39]. Mitochondrial-located STAT3 has been implicated in protecting against various diseases [40, 41] by inhibiting oxidative stress or mitochondrial permeability transition pore (mPTP) opening [42, 43]. Notably, the present study also demonstrates that mitochondria-located STAT3 was decreased after isoflurane exposure but could be restored by STAT3 overexpression. Therefore, STAT3 overexpression may contribute to neuroprotection not only through its transcriptional activity, which upregulates anti-oxidative and anti-apoptotic proteins, but also through its mitochondrial function, reducing ROS generation (Fig. 6).

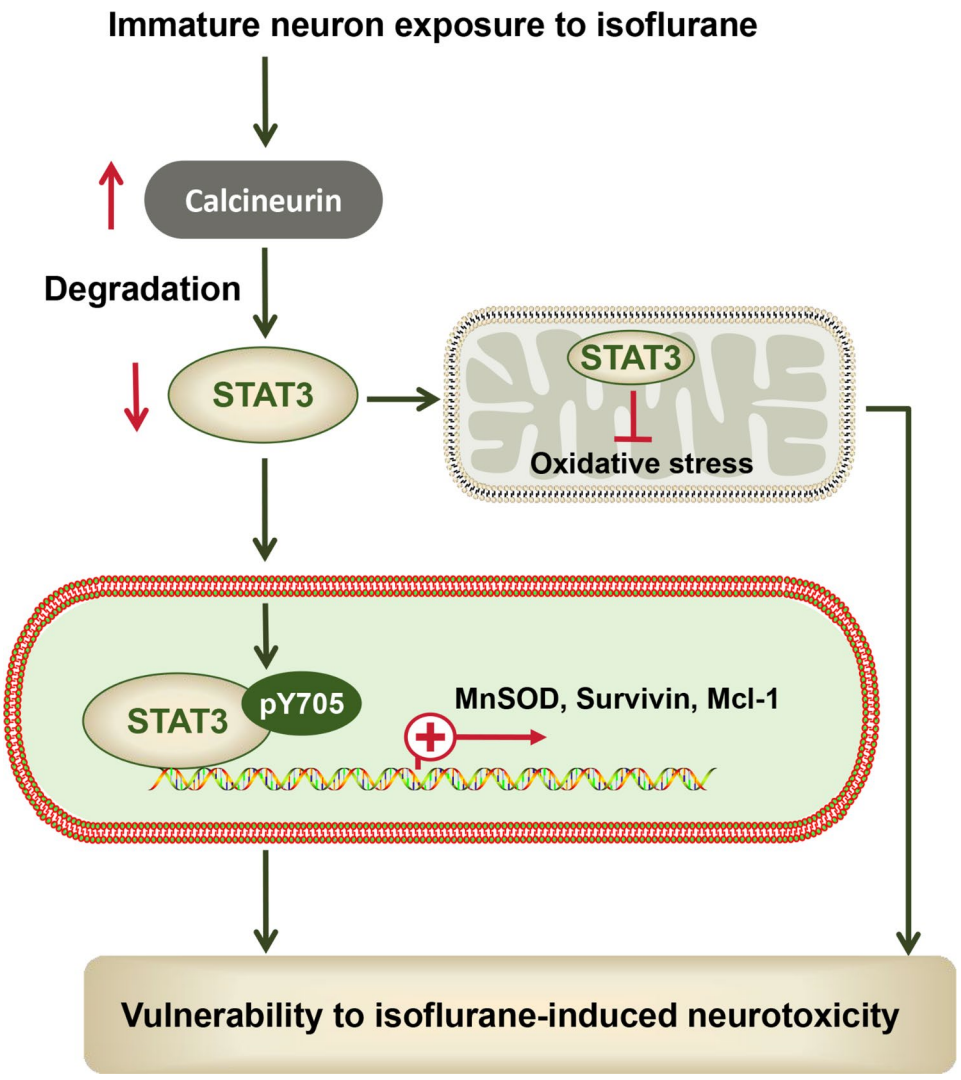

Fig. 6 Graphical summary of the protective effects of STAT3 in susceptibility to isoflurane-induced neurotoxicity. Isoflurane exposure increased calcineurin activity, a protein phosphatase that specifically promotes STAT3 degradation, in immature brain or neuron. The neuroprotective effects of STAT3 are mediated not only through its transcriptional activity, which upregulates anti-oxidative and anti-apoptotic proteins, but also through its mitochondrial function, which reduces ROS generation

Conclusions

The present study highlights the importance of specific regulation of STAT3 in determining the cellular vulnerability to isoflurane-induced toxicity, particularly through its antioxidative pathway. The findings suggest that targeting STAT3-mediated survival pathways could provide a novel therapeutic approach for the prevention and treatment of isoflurane-induced neurotoxicity, not only in the immature brain but also in the aged brain where a remarkable downregulation of STAT3 has been reported [44]. Furthermore, the study also revealed a significant decrease in the levels of total ubiquitin-conjugated proteins after isoflurane exposure, indicating the involvement of additional proteins in isoflurane-induced degradation. Further investigations focusing on these proteins will be crucial for a comprehensive understanding of the mechanisms underlying the cellular response to isoflurane and its associated neurotoxic effects.

Supplementary Information

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Supplementary Material 1

Acknowledgements
Not applicable.

Author contributions
Conceived and designed the experiments: QG, ZLM, HWW, and YY. Performed the experiments: YY and SYS. Analyzed the data: YY and SYS. Wrote the paper: YY, QG, ZLM, and HWW. All authors read and approved the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All animal experiments conducted in this study were performed following the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the Ethics Review Board for Animal Studies of Nanjing Drum Tower Hospital. The experiments conducted in this study also comply with the ARRIVE guidelines 2.0 (https://arriveguidelines.org/).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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