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Original Article

Halothane increases neuronal cell death vulnerability by downregulating miR-214 and upregulating Bax

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Abstract: Inhalational general anesthesia is widely used in clinical practice, but there have been plenty of evidence shows that application of inhalational anesthetics may increase the risk of Alzheimer's disease (AD). Halothane is a common inhalational anesthetics. We cultured rat primary neurons as study model to investigate cytotoxicity of halothane in neurons. We found that halothane could enhance the cytotoxicity induced by intracellular or extracellular amyloid β (Aβ) with or without serum deprivation. In addition, halothane induced the enhanced cytotoxicity through downregulation of miR-214 level which can lead to increasing expression of Bax. Therefore our data suggest that halothane increases cell death induced by Aβ through increasing Bax level by downregulating miR-214. Our study indicates a probable connection between anesthetic application and AD, while provides a new perspective for the studies of inhalational anesthetics cytotoxicity.

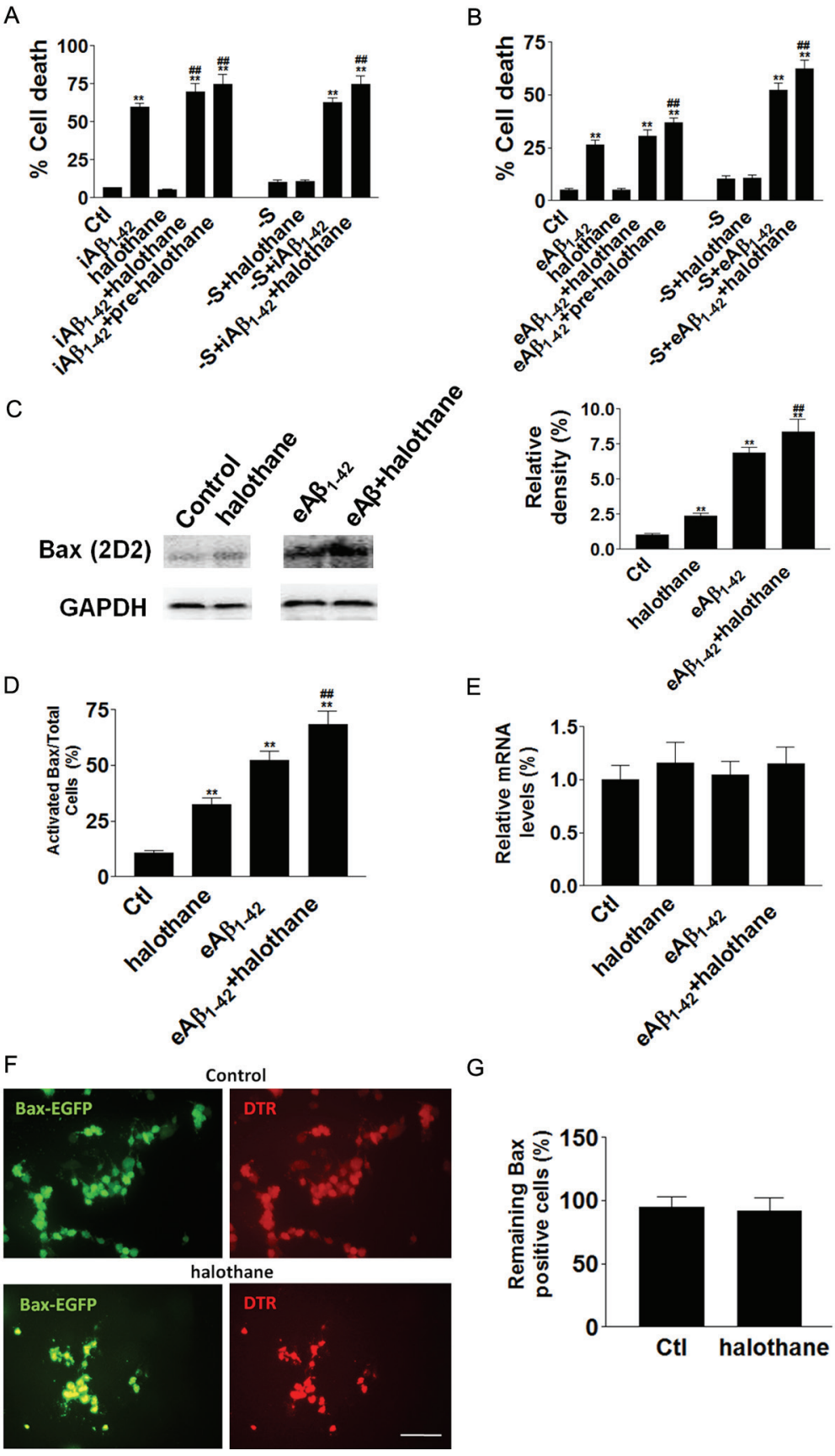
Keywords: Halothane, miR-214, Bax, cell death, Alzheimer's disease

Introduction

Inhalational general anesthesia is widely used in clinical practice, but there is accumulating evidence showing that inhalational anesthetics can induce cognitive impairment and neuronal damage [1-7]. And there are also studies showing that inhalational general anesthesia damages cognitive function especially in aged individuals [1]. Alzheimer's disease (AD) is a kind of neurodegenerative disease which is prevalent in aged people [8]. The typical symptoms of AD are always memory loss and cognitive impairment [9]. There have been some studies indicated that surgery history in which inhalational general anesthesia was used linked to the Alzheimer's disease [10-13]. The clinical evidence indicates that there may exist connection between inhalational general anesthesia and AD.

Except for the clinical evidence, there are correspondingly mechanism studies showing that some anesthetics induce proteins misfolding and aggregating [7, 14], which are associated with cell death in AD. Inhalational anesthetics can likewise increase hyperphosphorylated tau, which can further form neurofibrillary tangles (NFT) in AD patients' brains [15-18]. There are also data showing inhalational anesthetics can alter Aβ precursor protein (APP) processing, increase Aβ level and lead H4 neuroglioma cells apoptosis [7]. However, at the same time, there is another study shows tha it is probable that the anesthesia is not an independent risk factor for AD [19]. Similarly, there is evidence showing that it is the hypothermia which induced by the anesthesics instead of the anesthetics themselves causes tau hyperphosphorylation [18], indicating that anesthetics may just be an indirect risk factor for AD. Thus so far, the connection between inhalational general anesthesia and AD is still controversial.

Halothane is a kind of inhalational general anesthetics widely be used in surgery. Halothane can enhance plaque deposition [1] and Aβ oligomerization [14], suggesting that halothane may be related to the AD. In our research, we found that halothane could enhance Aβ cytotoxicity in rat primary neurons. And at the same time, halothane enhanced Bax


A
% Cell death
Ctl
iAβ1-42
halothane
iAβ1-42+halothane
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-S
-S+halothane
-S+iAβ1-42
-S+iAβ1-42+halothane
B
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Ctl
eAβ1-42
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-S
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C
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halothane
eAβ1-42
eAβ+halothane
Bax (2D2)
GAPDH
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Ctl
halothane
eAβ1-42
eAβ1-42+halothane
F
Control
Bax-EGFP
DTR
halothane
Bax-EGFP
DTR
G
Remaining Bax positive cells (%)
Ctl
halothane

Figure 1. Halothane enhanced Aβ cytotoxicity and Bax level. A. Halothane enhanced intracellular $Aβ_{1-42}$ (iAβ) toxicity with or without serum serum deprivation (-S). Cell death were measured by TUNEL staining. B. Pre-treatment of halothane for 3 hours enhanced extracellular $Aβ_{1-42}$ (eAβ) toxicity. Halothane enhanced extracellular $Aβ_{1-42}$ (eAβ) toxicity with serum serum deprivation (-S). C. Left panel: Western blots show that total Bax (indicated by 2D2 antibody) levels were increased by halothane in the absence or presence of $eAβ_{1-42}$. Right Panel: Quantitative data showed that total Bax level were increased significantly by halothane. Data represent Means ± SE (n = 3). D. Quantitative data showed that activated Bax (indicated by 6A7 antibody) levels were increased by halothane. E. Quantitative RT-PCR results showed that halothane treatment did not alter Bax mRNA levels significantly. F. Representative images showed that neurons were microinjected(indicated by DTR) with Bax-EGFP plasmid in control group or halothane treatment group. Scale bar: 50 μm. G. Quantitative data showed that halothane did not alter Bax levels at 12 hours after halothane treatment. Data represent Means ± SE (n = 200). $^{**}p < 0.01$ compared with the control. $^{\#\#}p < 0.01$ compared with the Aβ group.

level while decreased miR-214 level. So we conclude that halothane enhances Bax level through downregulating miR-214 and thus increases Aβ-induced neuronal cell death.

Results

Halothane enhanced intracellular $Aβ_{1-42}$ (iAβ) and extracellular $Aβ_{1-42}$ (eAβ) cytotoxicity

As Aβ is widely believed to be an important peptide in the pathological process of AD, we investigated if halothane could influence the cytotoxicity induced by Aβ. We treated the neurons with 0.5% halothane for 3 hours with or before the neurons were insulted with iAβ, eAβ or serum deprivation (-S). We found that halothane did not induce remarkable cell death in the neurons that were not insulted or insulted by serum deprivation (**Figure 1A** and **1B**), but halothane treatment or pre-treatment significantly increased cell death in the neurons insulted by iAβ (**Figure 1A**), and pre-treatment of halothane for 3 hours enhanced eAβ toxicity significantly (**Figure 1B**). Similarly, in the neurons insulted by serum deprivation for 3 days, halothane can also increased cell death significantly when the neurons were insulted by iAβ or eAβ (**Figure 1A** and **1B**).

Halothane treatment enhanced Bax level in neurons

To further investigate the mechanism of the enhanced cytotoxicity induced by the treatment of halothane, we checked the protein levels of Bax in the neurons treated with or without halothane. Our data showed that the total Bax was upregulated when the neurons were treated with halothane (**Figure 1C**), and when the neurons insulted by eAβ, halothane treatment could also increase the protein level of Bax (**Figure 1C**). At the same time we examined the activated Bax by confirmationally specific antibody 6A7, and found that halothane increased the protein level of activated Bax in the neurons that were insulted by the eAβ (**Figure 1D**). Nonetheless, the Bax mRNA level did not change significantly when the neurons treated with halothane (**Figure 1E**). These results suggested that halothane might alter the levels of Bax in the post-thanscription process. The mRNA level of Bax was then examined by quantitative RT-PCR and our data suggested that no difference of Bax mRNA level was detected in halothane treated neurons. To examine if halothane increased the Bax level through preventing the degeneration of Bax, we microinjected Bax-EGFP plasmid to the rat neurons and then treated the neurons with halothane for 3 hours. Dextran Texas Red (DTR) was used to show successful injection. Twelve hours after the halothane treatment, the numbers of the Bax positive cells and the DTR positive cells were counted to calculate the percentage of the Bax positive cells in the DTR positive cells. We found there was no noticeable difference between the control group and the halothane treatment group (**Figure 1F** and **1G**). This result showed that halothane had no effect on the Bax degeneration.

Halothane raised Bax level through miR-214

To further investigate how halothane raised Bax level, we utilized microRNA array to check the levels of microRNAs in the neurons treated with or without halothane. Among the microRNAs checked, miR-214 was significantly downregulated in the neurons treated with halothane compared to the control (**Figure 2A**). Furthermore, we examined the change of miR-214 level *in vivo*. We treated rats with inhaled 1% halothane for 3 hours, and then we tested the miR-214 levels in the rats' whole brain tissues and cerebrospinal fluid (CSF). The data

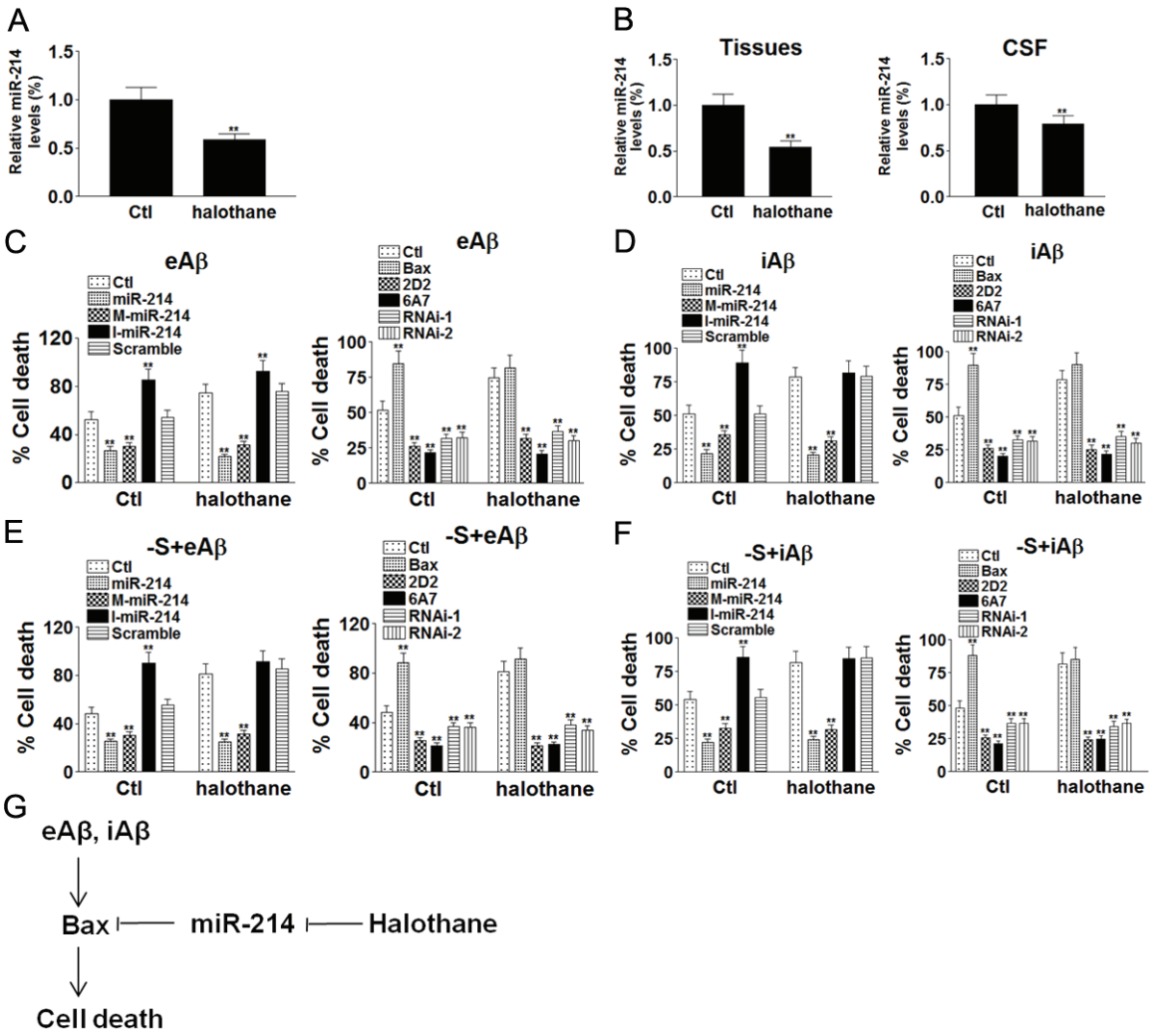

Figure 2. Halothane regulated Aβ-induced cytotoxicity through miR-214. A. Levels of relative miR-214 was downregulated in halothane treatment group. B. miR-214 levels were significantly decreased in halothane treated rats' brain tissues and CSF. C. Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) decresed cell death induced by halothane in the presence of eAβ. Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax si-RNAs (RNAi-1 and RNAi-2) reversed cell death induced by halothane in the presence of eAβ. D. Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) decresed cell death induced by halothane in the presence of iAβ. Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax si-RNAs (RNAi-1 and RNAi-2) reversed cell death induced by halothane in the presence of iAβ. E. Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) decresed cell death induced by halothane in the presence of eAβ and serum deprivation (-S). Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax si-RNAs (RNAi-1 and RNAi-2) reversed cell death induced by halothane in the presence of eAβ and serum deprivation (-S). F. Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) decresed cell death induced by halothane in the presence of iAβ and serum deprivation (-S). Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax si-RNAs (RNAi-1 and RNAi-2) reversed cell death induced by halothane in the presence of iAβ and serum deprivation (-S). G. Schematic drawing for halothane cytotoxicity mechanism. $^{**}p < 0.01$ compared with the control.

showed that miR-214 level significantly decreased in the brain tissues and CSF following the halothane treatment (**Figure 2B**).

To test the effect of miR-214 on cell death, We first treated the neurons with halothane for 3 hours and then microinjected the rat primary neurons with miR-214, mimic miR-214 (M-miR-214), miR-214 inhibitor (I-miR-214) or scramble sequence and meanwhile the neurons were insulted with iAβ or eAβ. After 24 hours treatment, the percentages of cell death showed that miR-214 or M-miR-214 injection inhibited cell death significantly while I-miR-214 injection

enhanced cell death (**Figure 2C** and **2D**). In addition, we microinjected the rat primary neurons with Bax neutralizing antibodies 2D2 and 6A7 [20] and siRNAs to Bax and at the same time, the neurons were insulted with iAβ or eAβ following 3 hours halothane treatment (**Figure 2C** and **2D**). The data indicated that miR-214 can decrease cell death induced by halothane in the presence of iAβ or eAβ while Bax suppression can also decrease cell death induced by halothane in the presence of iAβ or eAβ (**Figure 2C** and **2D**). And similarly, when the neurons insulted by serum deprivation, miR-214 and Bax suppression could also decrease cell death induced by halothane in the presence of iAβ or eAβ (**Figure 2E** and **2F**).

Discussion

In the present study, we found that halothane treatment alone did not induce noticeable neuronal cell death, but significantly enhanced cell death induced by iAβ or eAβ, so halothane maybe just a kind of catalytic agent in the process of cell death induced by Aβ, or halothane make the neurons more sensitive to the Aβ cytotoxicity. Aβ is generally believed to be a key pathogenic factor in AD [21-23]. Previous studies have showed that iAβ or eAβ induce neurons death through activating Bax [20, 24] and Bax 3'UTR is the target of miR-214 to downregulate the expression of Bax [25]. Therefore, our data suggest that halothane can increase cell death vulnerability through miR-214 and Bax. Since iAβ or eAβ induces cell death through Bax [20], it indicates that halothane enhances Bax level through miR-214 and thus increases cell death induced by iAβ or eAβ (**Figure 2G**). Therefor we believe that it is very probable halothane is related to the risk of AD. There are also other studies showing similar discoveries that some other inhalational general anesthetics like sevoflurane, desflurane with hypoxia, isoflurane, sevoflurane with nitrous oxide can induce cell apoptosis and enhance Aβ formation [26-30]. To our knowledge, our study is the first to report that halothane enhances Aβ induced cell death through miR-214, and this is a new insight for the studies of halothane or other anesthetics toxicity and application.

Although we have found that halothane enhanced Aβ induced cell death through miR-214, but there may be some other pathways taking part in this process. The inhalational anesthetics are small hydrophobic molecules that bind mainly in internal protein cavities [31, 32]. Nuclear magnetic resonance (NMR) data have showed that halothane could specifically interacts with $A\beta_{40}$ and $A\beta_{42}$ and induced structural alternation of Aβ [33]. Halothane is able to enhance the levels of small oligomers [34], like Aβ oligomerization rates [14]. So it is probable that halothane can also influence Aβ cytotoxicity through regulating Aβ directly.

As our further investigation, we find that miR-214 takes part in the process that halothane increasing cell death induced by Aβ. Presenelin-1 (PS1), another key protein in AD pathological process, was showed to be a potential target for miR-214 [35]. MiR-214 may play a more complex role in AD and there may be more complicated underlying mechanisms in the relationship between halothane and AD. Our research also indicates that miR-214 may be a potential target for the treatment of patients who suffer from the nervous system related side-effect of halothane, especially those patients suffer from AD at the same time. Moreover, our study also suggests that the risk of AD is an important factor which should be considered when the halothane or other similar inhalational anesthetics be used in clinical practice.

Materials and methods

Cell culture

Rat primary neurons were cultured from new born Sprague Dawley rat hippocampus, and the protocol following the regulations of Peking University Institutional. Animal Care and Use Committee (IACUC). In brief, the fresh rat hippocampal tissues were striped and dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone, Logan, UT). The mixture was triturated through pipette to make a homogenous mixture. Then centrifuge the flow-through after filtering the mixture through 70 μm sterilized filters. Wash the pellet once by phosphate buffered saline (PBS, 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH7.2) and once by DMEM in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, 1x antibiotic Pen-Strep (all from Invitrogen, Carlsbad, CA) with 5%

FBS. Then cells were plated on poly-L-lysine (Sigma, St. Louis, MO) coated plates or glass coverslips at the density of 5 x 10^4 cells/ml. Neurons were incubated at 37°C in DMEM with 5% FBS without phenol red and with 5% circulating CO_2. To inhibit dividing cell growth cytarabine was added to culture media 24 hours after plating at 10 mM. Medium was changed every 48 hours.

Cell and animal treatments

Aβ peptides (Bachem, King of Prussia, PA) were dissolved in sterile distilled water at 25 μM and immediately frozen at -20°C. IAβ peptides were delivered into neurons by microinjection at 10 nM at the cytosolic area while eAβ peptides were added into the medium at 1 μM. For anesthetics treatment on cultured neurons, 0.5% halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) (Guoyao Group, Beijing, China) in humidified air with 5% circulating CO_2 was applied to a closed incubator 37°C for 3 hours. As for anesthetics treatment on animals, SD rats (male, around 250 g) were anesthetized with humidified 1% halothane in 30% O_2 balanced by N_2 for 3 hours in a closed chamber. The animals were returned to their cages after exposure. For experiments described in **Figure 2**, rat miR-214, scramble microRNA control, mimic miR-214 and miR-214 inhibitor were all purchased from Qiagen (Hilden, Germany). MicroRNA levels were measured by CapitalBio (Beijing, China) microarray service with 3 repeats by using GeneChip microRNA 2.0 (Affymetrix). MiR-214 in the brain tissues and CSF was measured by TaqMan MicroRNA assay kit for rno-miR-214 according to the manufacturer's instruction (Life Technologies Corp. Shanghai, China). All Bax siRNAs (Qiagen, Hilden, Germany) were diluted into 5 nM before injection described by the manufacturer. The silencing efficiency and off-target effects of all siRNAs were verified by Qiagen.

Microinjection: Thin-walled Borosilicate glass capillaries (outer diameter=1.0 mm, inner diameter=0.5 mm) with microfilament (MTW100F-4, World Precision Instrument, Sarasota, FL) were pulled with a Flaming/Brown Micropipette Puller (P-97, Sutter, Novato, CA) to get injection needles with a tip diameter of ~0.5 μm. Microinjections were performed in the cytosol of every cell using the Eppendorf Microinjector FemtoJet and Eppendorf Micromanipulator (Eppendorf, Hamburg, Germany). Neurons were injected at the soma areas with 25 fl/shot at an injection pressure of 100 hPa, a compensation pressure of 50 hPa, and an injection time of 0.1 seconds. The solutions were injected at the indicated concentrations with 100 μg/ml dextran Texas Red (DTR, MW: 3000, Molecular Probes, Eugene, OR) or Alex488 (Molecular Probes, Eugene, OR) as fluorescent markers to recognize the successfully injected cells. Approximately 90% neurons survive the injections for at least 16 days [15].

Measurement of neuronal cell death

Cells were fixed in fresh 4% paraformaldehyde and 4% sucrose in PBS for 20 minutes at room temperature and then permeablized in 0.1% Triton X-100, 0.1% sodium citrate in PBS for 2 minutes on ice. Terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining was performed using the in situ cell death detection kit I (Roche, Quebec, Canada). Then the coverslips were washed once by distilled water for 5 minutes and mounted on glass slides to be observed by fluorescence microscope. For the non-injected cells, the percentage of cell death was determined by the ratio of the number of TUNEL-positive cells over the total of 100 cells in one count, and the average of 5 counts was calculated as the percentage of neuronal cell death in one treatment. For the cells injected, the percentage of neuronal cell death was determined by the ratio of the number of DTR-TUNEL-double-positive cells over the number of DTR-positive cells.

Immunostaining

Cells were permeablized in PBS-Triton at 4°C and blocked by 10% donkey serum at room temperature, followed by incubation with anti-activated Bax antibody (6A7, R&D, Minneapolis, MN, 1:200) at 4°C for 24 hours. Cy2 or Cy3-conjugated donkey anti-rabbit antibody was applied as secondary antibody. The nuclei were staining by Hoechst 33258 (1 μg/ml, Sigma, St. Louis, MI) for 10 minutes in dark. The coverslips were mounted with ImmunonTM mounting medium (Shandon, Pittsburgh, PA) onto glass slides and the results were analyzed by using fluorescence microscope (Olympus BH2-RFCA, Olympus, Tokyo, Japan) and digital camera

(Olympus DP70 Digital Microscope Camera, Olympus, Tokyo, Japan).

Tissue samples and extraction

Frozen hippocampal formation was lysed in QIAzol lysis reagent (Qiagen, Hilden, Germany) and homogenized with Kontes pestle. RNA was extracted by using miRNeasy kit (cat# 217004, Qiagen, Hilden, Germany). In brief, for 700 l of QIAzol, 140 l of chloroform was added and mixed for 15 seconds. After 3 minutes of incubation at room temperature, the mixture was centrifuged at 12,000 g (4°C) for 15 min. The upper aqueous layer which contains RNA was transferred to another microcentrifuge tube. Total RNA was solubilized in nuclease free water (cat# AM9932, Ambion) purified using RNeasy Mini columns (Qiagen, Hilden, Germany) according to manufacturer instructions. Nucleic acid concentrations were determined by Qubit fluorometer (cat# Q32857, Invitrogen, Carlsbad, CA) and samples were stored at 80°C.

Real time PCR (RT-PCR)

RT-PCR was performed using the QIAgen MicroRNA Reverse Transcription kit (cat# 218161, Miscript II RT) for brain tissues. cDNA concentrations were determined by Qubit fluorometer. QIAgen Miscript SYBR Green PCR kit (cat# 218073) was used to detect miR-214 expression. The QIAgen small nucleolus RNA primer (RNU 6 cat# MS00031605) was used to detect reference gene. All real time PCR reactions were performed on iCycler Real Time PCR System (BioRad, Hercules, CA) according to manufacturer's recommendation. MicroRNA quantity was calculated by the ΔCt method.

For cellular extracts, cells were harvested and total RNA was isolated with TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China). Total RNA (2 μg) was reversely transcribed using TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). Real-time PCRs were done by utilizing TransStart Green q PCR SuperMix UDGTM (Beijing TransGen Biotech Co., Ltd., Beijing, China). Primers' sequences for Bax used were as following: forward: (5'-GCAGAGGATTGCTGATG-3'); and reverse: (5'-CTCAGCCCATATTCTTCCAG-3'). Real-time PCR quantifications were run in triplicate for each sample then the average was determined. The amplification efficiency of target and housekeeping gene must be approximately equal for the sake of using the comparative Ct method for relative quantification. Expression levels for the target gene was normalized to the GAPDH of each sample [$2^{-\Delta Ct}=2^{-(Ct(target\ gene)-Ct(GAPDH))}$]. Amplification was done for 45 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, 95°C for 1 min, 59°C for 30 s and 95°C for 30 s.

Western blots: Neuronal proteins were extracted by using the cell lysis buffer (50 mM Tris, pH8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS) and the protein concentrations were measured by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Protein extracts were denatured at 100°C for 5 minutes and separated on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 80 volts for about 2 hour. Proteins were transferred to Immobilon-PTM polyvynilidene fluoride (PVDF) membrane (Millipore, Billerica, MA) at 110 volts for 2 hours. The membrane was blocked with 5% non-fat milk in Tris buffered saline with 0.1% tween 20 (TBST) at room temperature for 1 h. Anti-Bax (2D2, R&D, Minneapolis, MN) and GAPDH (Sigma, St. Louis, MO) antibodies were diluted at 1:1000 as primary antibodies. After 3 times washing of 10 minutes each with TBST, goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was added in a dilution of 1:2500 as the secondary antibody. The secondary HRP was detected by enhanced chemiluminescence. Optical density was analyzed by BioRad ChemiDox (BioRad, Hercules, CA). The relative density was determined by the aggregate absolute density of Bax/GAPDH.

Statistical evaluation: Statistical significance was assessed by one-way analysis of variances (ANOVA). The Sheffé's test was applied as a post hoc for the significant difference showed by ANOVAs. Statistical significance was indicated by a p value of less than 0.05.

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