

# Isoflurane Increases Neuronal Cell Death Vulnerability by Downregulating miR-214

Hailiang Yan<sup>1,4</sup>, Tao Xu<sup>1,4</sup>, Hongfeng Zhao<sup>3</sup>, Kuo-Chieh Lee<sup>2</sup>, Hoau-Yan Wang<sup>2</sup>, Yan Zhang<sup>1,4\*</sup>

**1** State Key Laboratory of Biomembrane and Membrane Biotechnology, College of Life Sciences, Peking University, Beijing, China, **2** Department of Pharmacology, Physiology and Neuroscience, Sophie Davis School of Biomedical Education/City University of New York Medical School, New York, New York, United States of America, **3** Beijing No.4 High School, Beijing, China, **4** Department of Urology, Peking University People's Hospital, Beijing, China

## Abstract

Since accumulating evidence suggests the application of anesthetics may increase the risk of Alzheimer's disease (AD), we investigated the cytotoxicity of inhaled general anesthesia in neurons and its underlying mechanism. Using primary cultured rat hippocampal neurons as the study model, here we show that isoflurane increases vulnerability to intracellular or extracellular amyloid  $\beta$  with or without serum deprivation. This isoflurane-induced effect is mediated by the downregulation of miR-214 level that lead to an elevated expression of Bax, a prominent target for miR-214. We conclude that isoflurane increases cell death in the presence of amyloid  $\beta$  by increasing Bax level through downregulating miR-214. Our data provide a new insight for inhaled anesthetics toxicity and indicate a possible mechanistic link between anesthetic application and neurodegeneration in AD.

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\* E-mail: yanzhang@pku.edu.cn

These authors contributed equally to this work.

## Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by senile plaques and neurofibrillary tangles in elderly [1]. With increasing number of older people worldwide undergo surgery every year, accumulating evidence indicates the association between anesthetic application and risk for AD. Inhaled anesthetics can impair cognitive function especially in aged individuals [2] and are associated with neuronal damage and prolonged cognitive impairment [2,3,4,5,6,7,8,9,10,11,12,13]. While the age of AD onset has been linked to surgery history in which inhaled anesthetics was used [14,15,16,17], there is also evidence indicates that there is no correlation between the usage of general anesthesia and AD [18]. Hence, current clinical data are inconsistent in associating inhaled general anesthesia and risk for AD.

Despite the conflicting clinical evidence on the risk of AD after the usage of inhaled anesthetics, potential mechanisms responsible for neuronal damage following inhaled anesthesia have been suggested. In accord, some anesthetics promote protein misfolding and aggregation, one of the mechanisms associating with cell death in neurodegenerative diseases [12,19,20]. Inhaled anesthetics are small haloalkanes or haloethers which can bind to hydrophobic cavities of proteins [21,22,23] and have the ability to increase the levels of small oligomers [24] which may include amyloid  $\beta$  (A $\beta$ ) peptides aggregates, the main component in senile plaques. Isoflurane was shown to alter A $\beta$  precursor protein (APP) processing, upregulate A $\beta$  production and induce apoptosis in H4

neurogloma cells [12]. In APP-transfected H4 cells, desflurane and hypoxia can also increase A $\beta$  production and caspase activation [25]. Nuclear magnetic resonance (NMR) data indicate that halothane, isoflurane and desflurane interact with A $\beta$  that leads to its oligomerization [26,27]. Similarly, inhaled anesthesia increased hyperphosphorylated tau, the primary component of neurofibrillary tangles [28,29,30,31,32,33], although there is evidence that the hypothermia, which is induced by anesthesia, rather than anesthetics themselves, causes tau hyperphosphorylation [28]. Further, recent data show that intravenous anesthetic propofol and inhaled anesthetic isoflurane both promote tau hyperphosphorylation [34,35]. Taken together, our understanding of how anesthetics increase neuronal cell death remains very limited.

In our present study, we examined the underlying mechanism responsible for isoflurane-induced neuronal cell death when combined with A $\beta$  or serum deprivation insults in cultured primary rat neurons. We found that isoflurane decreased the level of miR-214, which inhibited Bax activation. Therefore, isoflurane enhanced A $\beta$  cytotoxicity through downregulating miR-214.

## Results

### Isoflurane Increased A $\beta$ and Serum Deprivation Cytotoxicity

To further investigate the mechanisms of general anesthesia induced cytotoxicity in cultured primary rat neurons, we treated the cells in a closed chamber with incubation of 0.5% isoflurane

for 3 hours. Simultaneously, the neurons were insulted with intracellular  $A\beta_{1-42}$  (iA $\beta$ ), extracellular  $A\beta_{1-42}$  (eA $\beta$ ), or in combination with serum deprivation (-S). In the un-insulted neurons, isoflurane did not induce significant cell death 24 hours after the treatment (Fig. 1A). In contrast, isoflurane markedly increased cell death in neurons that were microinjected with  $A\beta_{1-42}$  peptide (iA $\beta_{1-42}$ ) when compared with neurons with iA $\beta_{1-42}$  alone (Fig. 1A). However, when neurons were treated with  $A\beta_{1-42}$  peptide in culture medium (eA $\beta_{1-42}$ ), isoflurane did not enhance cell death compared with eA $\beta_{1-42}$  alone (Fig. 1B). In contrast to iA $\beta_{1-42}$  or eA $\beta_{1-42}$ , iA $\beta_{1-40}$  and eA $\beta_{1-40}$  or the control reversed peptides iA $\beta_{42-1}$  and eA $\beta_{42-1}$  did not alter the cell viability (Fig. 1A and 1B). Application of isoflurane did not change the cell death in the presence of iA $\beta_{1-40}$ , eA $\beta_{1-40}$ , iA $\beta_{42-1}$  and eA $\beta_{42-1}$  (Fig. 1A and 1B). When neurons were pre-incubated with isoflurane for 3 hours and then insulted with either iA $\beta_{1-42}$  or eA $\beta_{1-42}$  peptide, isoflurane pre-treatments significantly increased the cell death (Fig. 1C and 1D). We also examined the cytotoxicity of isoflurane in the presence of serum deprivation. Our results showed that with serum deprivation for 3 days, application of isoflurane for 3 hours did not alter cell death levels (Fig. 1E). Isoflurane enhanced cell death significantly only when iA $\beta_{1-42}$  or eA $\beta_{1-42}$  peptides were combined with serum deprivation (Fig. 1E and 1F).

### Bax Levels were Enhanced with Isoflurane Treatment

Pre-incubation of isoflurane potentiated the toxicity induced by both iA $\beta_{1-42}$  or eA $\beta_{1-42}$  peptides in neurons (Fig. 1C and 1D). To investigate the underlying mechanism responsible for the elevated cytotoxicity of isoflurane treatment, we examined the protein levels of Bax in the absence or presence of eA $\beta_{1-42}$  peptide application. Our Western blot data show that isoflurane markedly increased total Bax levels in the absence and presence of eA $\beta_{1-42}$  peptide (Fig. 2A). Using an antibody specifically detects the activated Bax (6A7), our results showed that isoflurane also increased activated Bax in the absence and presence of eA $\beta_{1-42}$  (Fig. 2B). When neurons were microinjected iA $\beta_{1-42}$  peptide (Fig. 2C), the level of activated Bax was increased as indicated by increased 6A7 staining in more neurons (Fig. 2D). The increased activated Bax level is not mediated by the elevated Bax mRNA since quantitative RT-PCR data indicated that there was no significant difference in Bax mRNA levels in these treatment groups (Fig. 2E). These data suggest that Bax protein levels might be modified by anesthetics at post-transcriptional levels. To rule out the possibility that isoflurane might decrease Bax degradation to enhance Bax level, we microinjected Bax-EGFP fusion protein expressing construct to primary neurons. A successful injection was indicated by the presence of Dextran Texas Red (DTR) staining. The cells were then treated with isoflurane for 3 hours immediately after Bax-EGFP injection. At 12 hours after anesthetics treatment, the numbers of EGFP positive cells versus DTR positive cells were counted. Our results showed that there was no significant difference between the treatment groups compared with the control group (Fig. 2F), suggesting that isoflurane did not affect Bax degradation.

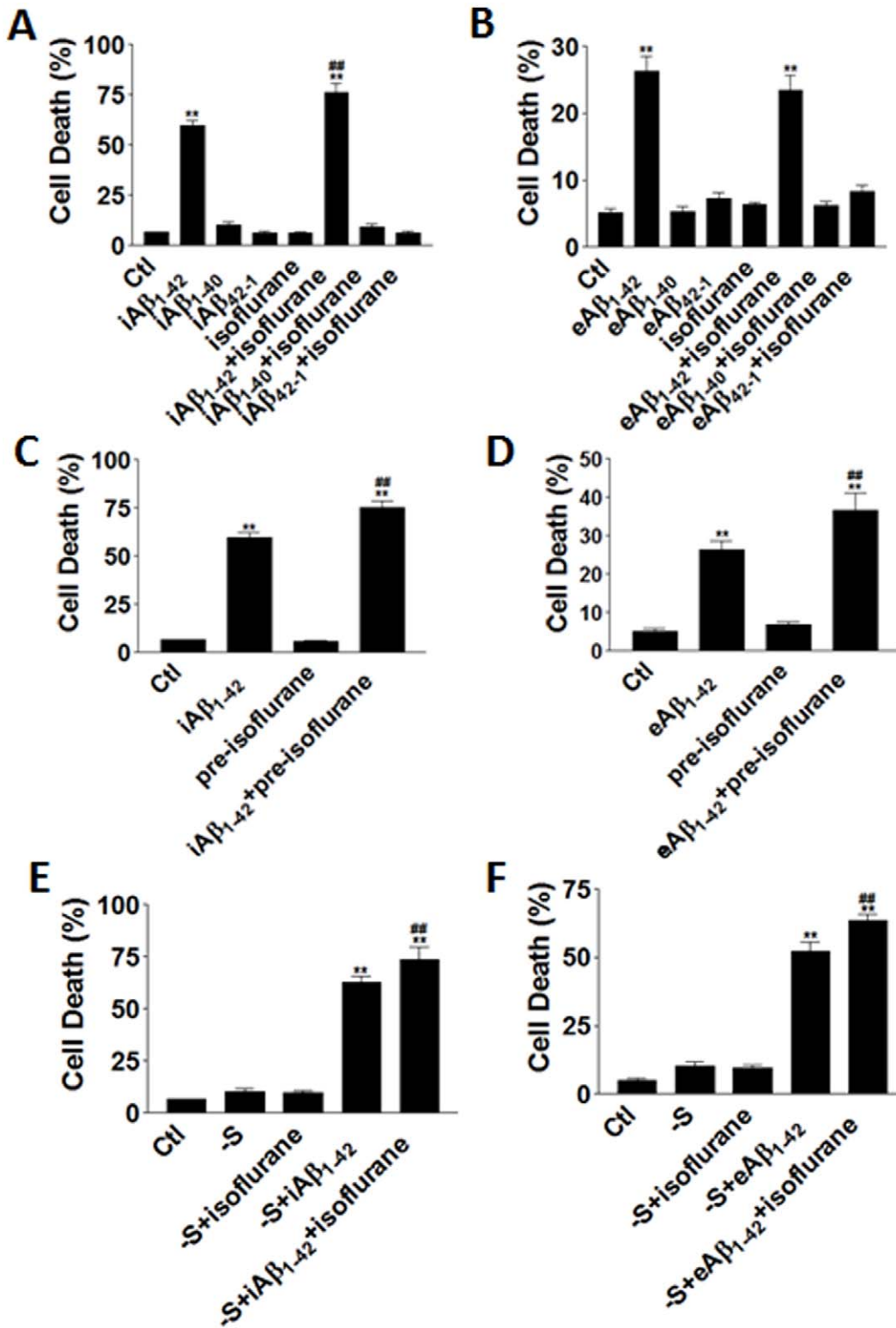
### Isoflurane Increased Bax Level through Downregulating miR-214

To further investigate the mechanism responsible for isoflurane-induced increase in Bax level, we performed microRNA array assay in the neuronal extracts from control and isoflurane-treated primary neurons. Among the microRNAs altered by isoflurane application, miR-214 was significantly downregulated (Fig. 3B). In accord with the notion that miR-214 level may be related to Bax abundance, Bax 3'UTR was predicted to be a target of miR-214

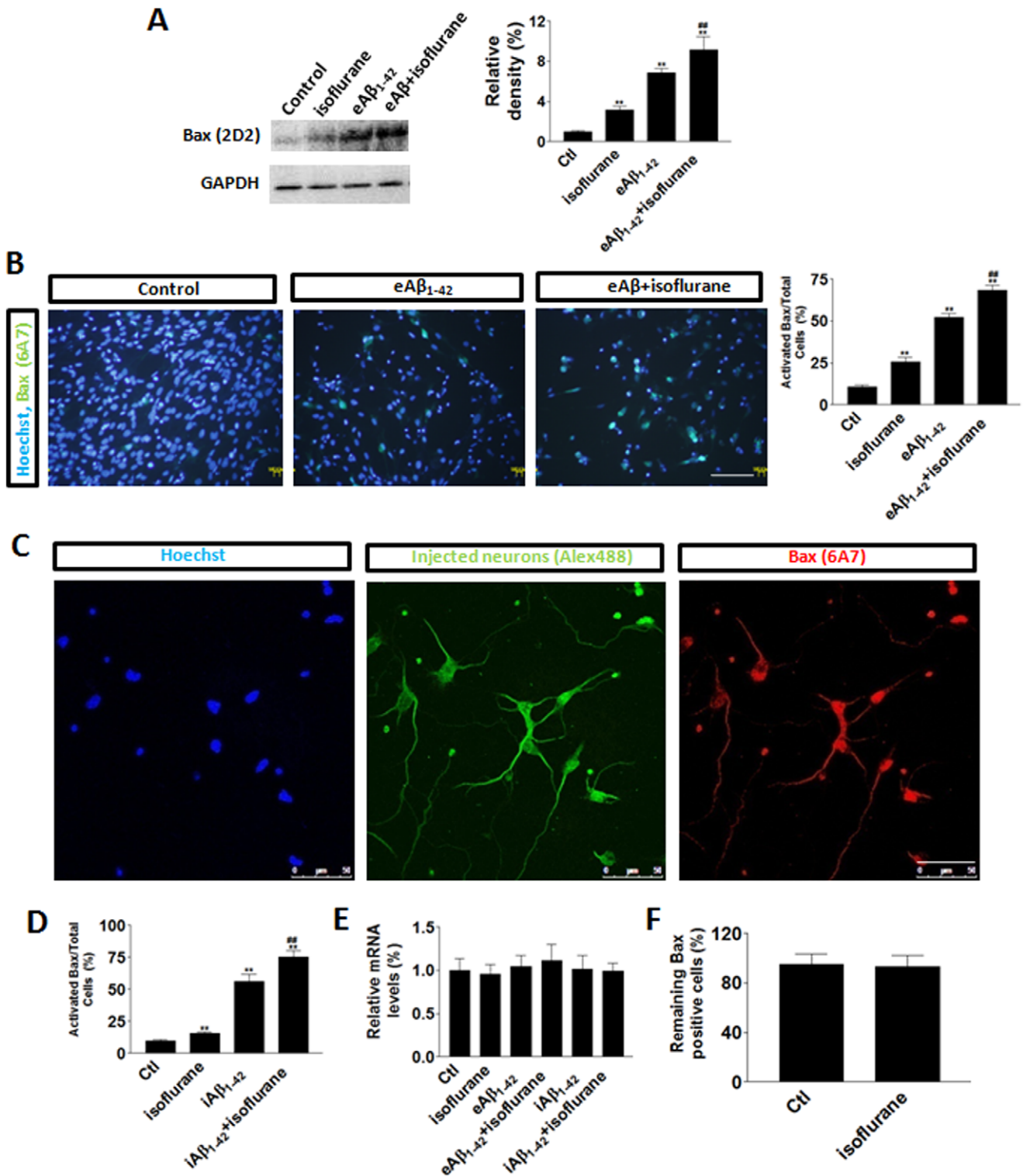
(Fig. 3A). To this end, we transfected the neuroblastoma SH-Sy5y cells with human Bax 3'UTR regulated EGFP construct and co-transfected with control, scramble microRNA, miR-214, mimic miR-214, miR-214 inhibitor or mutant miR-214, respectively. The transfection efficiency was indicated by RFP (Fig. 3C). Our data showed that the relative number of EGFP positive cells in RFP positive cells was decreased in the miR-214 and mimic miR-214 transfected groups, but not in the scramble, miR-214 inhibitor or mutant miR-214 groups (Fig. 3D), suggesting that miR-214 downregulated Bax expression. Similarly, Bax 3'UTR regulated luciferase assay showed miR-214 from 20 to 100 nM efficiently decreased Bax expression (Fig. 3E). Time course of miR-214 indicated that maximal inhibition happened at 12–24 hours after transfection, although the effect might last for at least 48 hours (Fig. 3F).

To further confirm the role of miR-214 in regulating Bax level, mutant Bax 3'UTR was made by altering 2 nucleotides at WT Bax 3'UTR region. When mutant Bax 3'UTR was co-transfected into SH-Sy5y cells with either scramble or miR-214 sequence, luciferase reporter assay showed no significant difference between scramble and miR-214 transfected groups (Fig. 4A), suggesting that miR-214 targeted Bax 3'UTR specifically. To further proof that miR-214 regulate Bax 3'UTR selectively, microtubule-associated protein 2 (MAP2) 3'UTR was co-transfected with scramble or miR-214 sequence. Our data prove no noticeable difference between scramble and miR-214 groups (Fig. 4A). Since microRNAs bind to mRNAs at specific regions (binding domain, BD), we made Bax 3'UTR with double BD (2BD) and triple BD (3BD). Our data showed that miR-214 inhibits Bax expression increased proportionally with 2BD or 3BD construct (Fig. 4A), thus confirming that miR-214 targeted Bax and downregulated its expression. Western blot data showed that in SH-Sy5y cells, miR-214 and mimic miR-214 decreased Bax level (Fig. 4B), while Bax mRNA levels were not altered by miR-214 (Fig. 4C). These data together indicate that miR-214 regulated Bax mRNA translation. The relevance of isoflurane *in vivo* was tested in the rats treated with inhaled 1% isoflurane for 3 hours and the levels of miR-214 were then measured in the whole brain tissues and CSF. Our data indicated that following anesthetic treatment, miR-214 level was decreased significantly in both brain tissues and CSF (Fig. 4D). To assess whether AD affects the level of miR-214, we measured miR-214 expression in the postmortem tissues from AD patients or age matched controls. The RT-PCR results of hippocampal tissues from 8 matched control-AD pairs with miR-214 showed that there was no discernible difference detected between two diagnostic groups ( $p = 0.38281$ , two-tailed Student's *t*-test). There are no significant correlation between miR-214 expression and age ( $R^2 = -0.69457$ ) or postmortem intervals ( $R^2 = -0.39699$ ) (Fig. 4E).

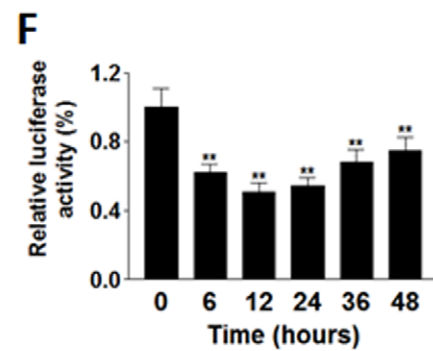
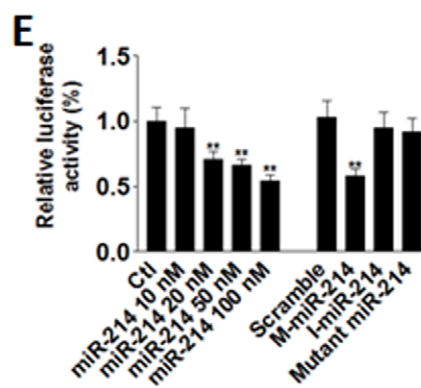
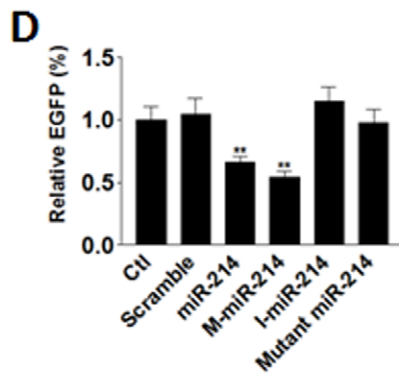
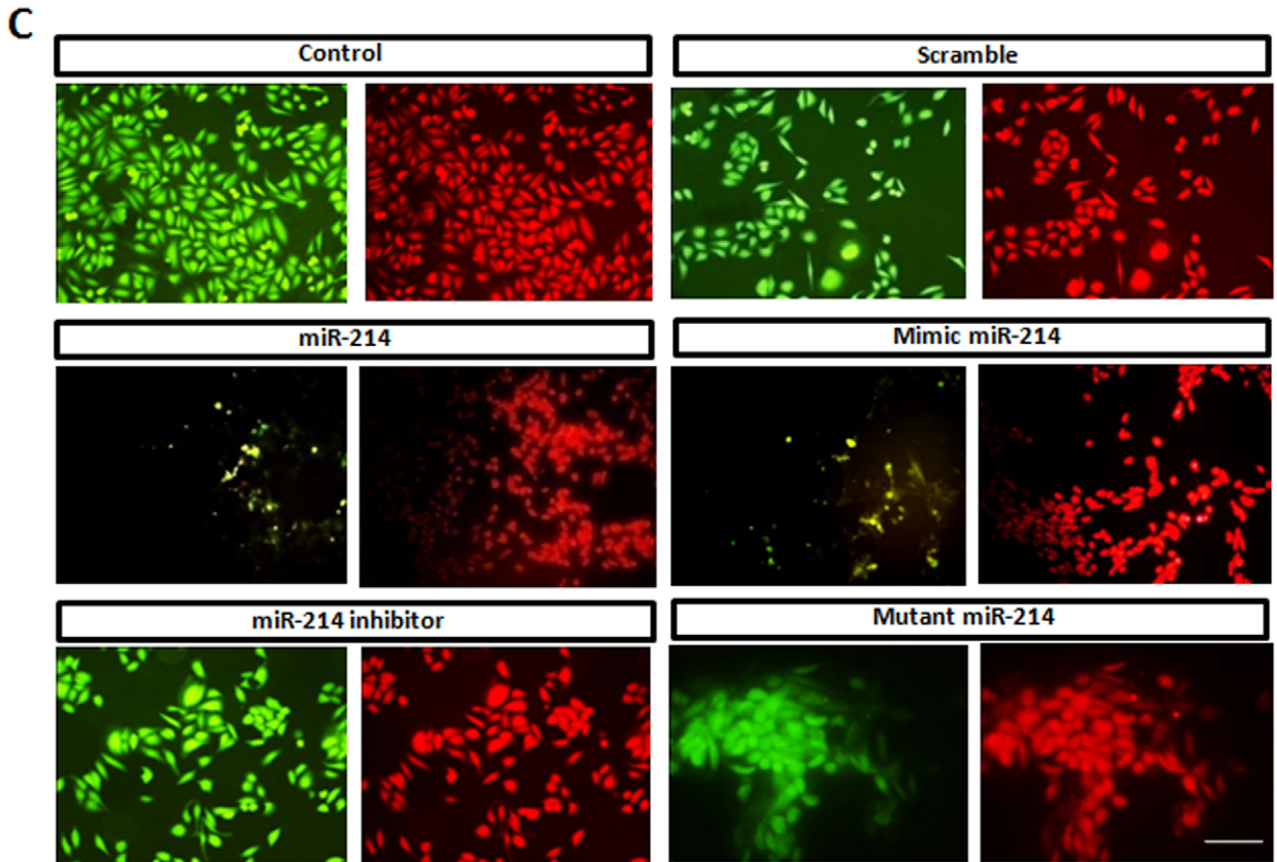
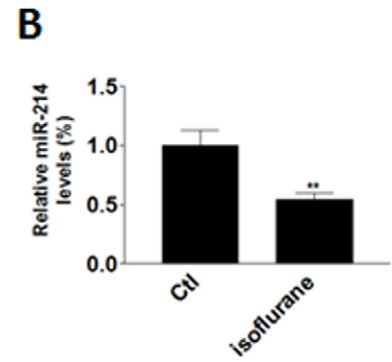
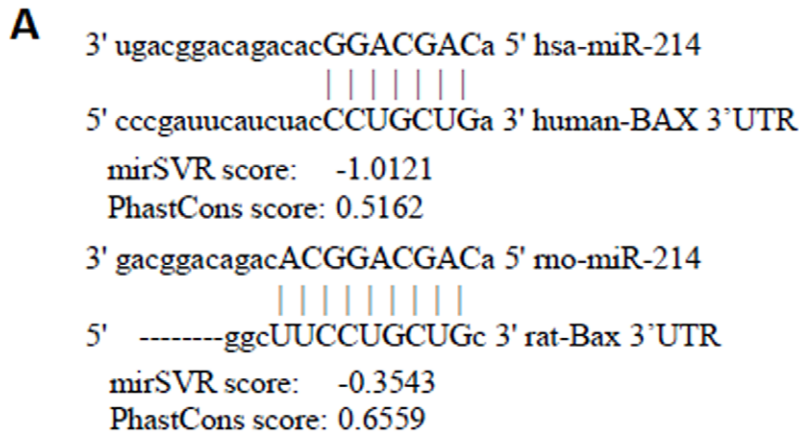
We then verified the effect of miR-214 on cell death in rat primary neurons. Neurons were microinjected with miR-214, mimic miR-214, miR-214 inhibitor or scramble sequence and insulted with eA $\beta_{1-42}$ . The cells were treated with isoflurane for 3 hours and after 24 hours of treatment, the percentages of cell death were measured. Our data showed that cell death was significantly inhibited by miR-214 or mimic miR-214 injection, and enhanced by miR-214 inhibitor injection (Fig. 4F). In parallel, we microinjected Bax construct, Bax neutralizing antibodies 2D2 and 6A7 [36] and siRNAs to Bax. Our results indicated that while Bax increased cell death significantly, its neutralizing antibodies and siRNAs decreased cell death (Fig. 4F). Our data indicate that miR-214 can reverse isoflurane-induced cell death in the presence of eA $\beta_{1-42}$ , which can be mimicked by Bax suppression. Similarly, the cell death induced by isoflurane with iA $\beta_{1-42}$  and the



**Figure 1. Isoflurane increased Aβ and serum deprivation cytotoxicity.** (A) Isoflurane enhanced microinjected intracellular Aβ (iAβ<sub>1-42</sub>, iAβ<sub>1-40</sub> and iAβ<sub>42-1</sub>) peptide toxicity in primary rat neurons. Cell death levels were measured by TUNEL staining. (B) Isoflurane did not enhance extracellular Aβ (eAβ<sub>1-42</sub>, eAβ<sub>1-40</sub> and eAβ<sub>42-1</sub>) peptide toxicity in primary rat neurons. (C) Pre-treatment of isoflurane for 3 hours increased iAβ<sub>1-42</sub> peptide toxicity. (D) Pre-treatment of isoflurane for 3 hours increased eAβ<sub>1-42</sub> peptide toxicity. (E) Isoflurane enhanced the toxicity of the combination of iAβ<sub>1-42</sub> peptide and serum deprivation (-S). (F) Isoflurane enhanced the toxicity of the combination of eAβ<sub>1-42</sub> peptide and serum deprivation (-S). Data represent Means±SE (n=200 for each group). \*\*: p<0.01 compared with the control; ##: p<0.01 compared with the Aβ group. doi:10.1371/journal.pone.0055276.g001



**Figure 2. Bax was upregulated by isoflurane treatment.** (A) Left panel: An example of western blot for total Bax (indicated by 2D2 antibody) suggested that total Bax levels were increased by isoflurane in the absence or presence of eAβ<sub>1-42</sub> peptide. Right panel: Quantitative data showed that isoflurane significantly enhanced total Bax levels. Data represent Means±SE (n=3 repeats for each group). (B) Left panel: Representative images showed that activated Bax (indicated by 6A7 antibody) was upregulated by isoflurane. Scale bar: 100 μm. Right panel: Quantitative data showed that isoflurane significantly enhanced total bax levels. (C) Representative images showed microinjected neurons (indicated by injection marker dye Alex488) with iAβ<sub>1-42</sub> peptide and activated Bax (6A7) staining. Scale bar: 50 μm. (D) Quantitative data showed that isoflurane significantly enhanced total bax levels. (E) Quantitative RT-PCR results showed that there was no significant difference between isoflurane treatment groups on Bax mRNA. (F) Quantitative data showed that isoflurane did not alter exogenous Bax levels at 12 hours after the treatment. Data represent Means±SE (n=200 cells for each group). \*\*: p<0.01 compared with the control. ##: p<0.01 compared with the Aβ group. doi:10.1371/journal.pone.0055276.g002



**Figure 3. Isoflurane decreased miR-214.** (A) Sequence alignment of human and rat miR-214 and Bax 3'UTR. (B) Levels of relative miR-214 in isoflurane-treated primary rat neurons. (C) Representative images showed that in SH-Sy5y cells, the levels of reporter Bax 3'UTR regulated EGFP (green fluorescent) were regulated by miR-214 and its variants. RFP (red fluorescent) indicated the transfected cells. Scale bar: 50  $\mu$ m. (D) Quantitative data showed that miR-214 decreased Bax expression. M-miR-214: mimic miR-214; I-miR-214: miR-214 inhibitor. (E) miR-214 decreased Bax expression in a dose-dependent manner. (F) The time course of miR-214 in suppression of Bax expression. Data represent Means  $\pm$  SE (n=200 cells for each group). \*\*, p<0.01 compared with the control. doi:10.1371/journal.pone.0055276.g003

combination of serum deprivation and A $\beta$  can also be reversed by miR-214 (Fig. 4G–I), suggesting that miR-214 is indeed involved in isoflurane cytotoxicity.

## Discussion

The mechanisms leading to neurodegeneration in the neurodegenerative disease such as AD are of intense interest, among which amyloid- $\beta$  is generally considered to be a pathological marker for AD. In accord with the notion that amyloid- $\beta$  promotes neurodegeneration, our previous studies demonstrate that iA $\beta$ <sub>1-42</sub> and eA $\beta$ <sub>1-42</sub> can induced neuronal cell death through activating Bax [36,37]. In the present study, we show that the commonly used inhaled anesthetic, isoflurane, although do not enhance cell death by themselves, increases vulnerability of the pre-exposed neurons to both iA $\beta$ <sub>1-42</sub> and eA $\beta$ <sub>1-42</sub> insults. Additionally, our data indicate that isoflurane increases both total Bax and activated Bax levels. These data together suggest that inhaled anesthetics do not directly alter cell viability but promote cell vulnerability to A $\beta$  insult. Since isoflurane induces Bax activation without showing significant cell death, our data suggest that Bax activation by the inhaled anesthetics may have destabilized mitochondria which in turn promote cell vulnerability to amyloid- $\beta$  insult and the eventual cell death. Interestingly, isoflurane does not increase neuronal vulnerability to serum deprivation alone although the underlying mechanism for this differential effect remains obscured. Similar to our results, earlier studies also support inhaled anesthetics may promote apoptosis. In postnatal day 7 rat pups, combined application of isoflurane, midazolam and nitrous oxide for 6 hours induces excessive neuronal apoptosis throughout the brain such as the hippocampus and cerebral cortex [7]. In cultured cells, isoflurane, isoflurane with nitrous oxide, sevoflurane, desflurane with hypoxia all have shown to induce apoptosis and increase A $\beta$  formation [25,38,39,40,41].

It has been proposed that general anesthesia induces apoptosis by suppressing neuronal activity, that in turn inhibits neurotrophic factor secretion locally [42,43]. Caspase involvement in anesthesia-induced apoptosis has also been implicated [25]. Although an earlier report indicate that 2% isoflurane increases Bax mRNA levels [44], we did not observe any discernible changes in Bax mRNA following incubation with 0.5% isoflurane. Further investigation of potential underlying mechanism reveals that isoflurane decreases miR-214 level. miR-214 targets Bax 3'UTR and downregulates Bax expression by inhibiting its mRNA translation. Therefore, isoflurane induces cytotoxicity and neuronal cell death in the presence of A $\beta$  through downregulating miR-214 (Fig. 5). This is the first time, to our knowledge, that inhaled anesthetics induces cell death by regulating microRNAs is reported. In addition to Bax expression that may be regulated by miR-214, one single nucleotide polymorphism (SNP) in presenilin-1 (PS1) (rs63750082) was shown to create a potential target site for miR-214 [45]. This may result in the downregulation of PS1 by miR-214 and thereby reduces the production of A $\beta$  and lessens AD pathology. However, our results in the postmortem hippocampal tissues from 8 matched control-AD pairs indicate that there is no discernible change in the miR-214

level in AD. Given that a higher A $\beta$  pathology in the AD brain, our results predict that inhaled anesthetics would have hastened neurodegeneration in AD subjects.

There is evidence that desflurane and isoflurane interact with both nicotinic and muscarinic acetylcholine receptors (AChR) in a dose-dependent manner [46]. Isoflurane treatment significantly decreases the level of choline acetylase (ChAT), but not  $\alpha$ 7 nicotinic AChR in mouse brains [47]. Chronic application of isoflurane also alters the level of muscarinic AChR in the hippocampus [48]. Therefore, it is reasonable to hypothesize that symptoms related to postoperative cognitive dysfunction are possible consequence of inhaled anesthetics interacting with AChR and suppressing central cholinergic transmission [49]. Furthermore, the role for the defected cholinergic function in AD pathogenesis is well-supported by many studies [50], it is also possible that inhaled anesthetics increase AD incidence through inhibiting cholinergic function. Together with our data indicating that isoflurane reduces miR-214 to increase cell death by promoting A $\beta$ <sub>1-42</sub> cytotoxicity, there data support the idea that inhaled anesthetics may increase the risk of AD [33]. Most importantly, our finding in this study suggests that therapies that increase miR-214 may be used to reduce the neurotoxicity and post-operative cognitive dysfunction following the usage of inhaled anesthetics, especially in AD patients.

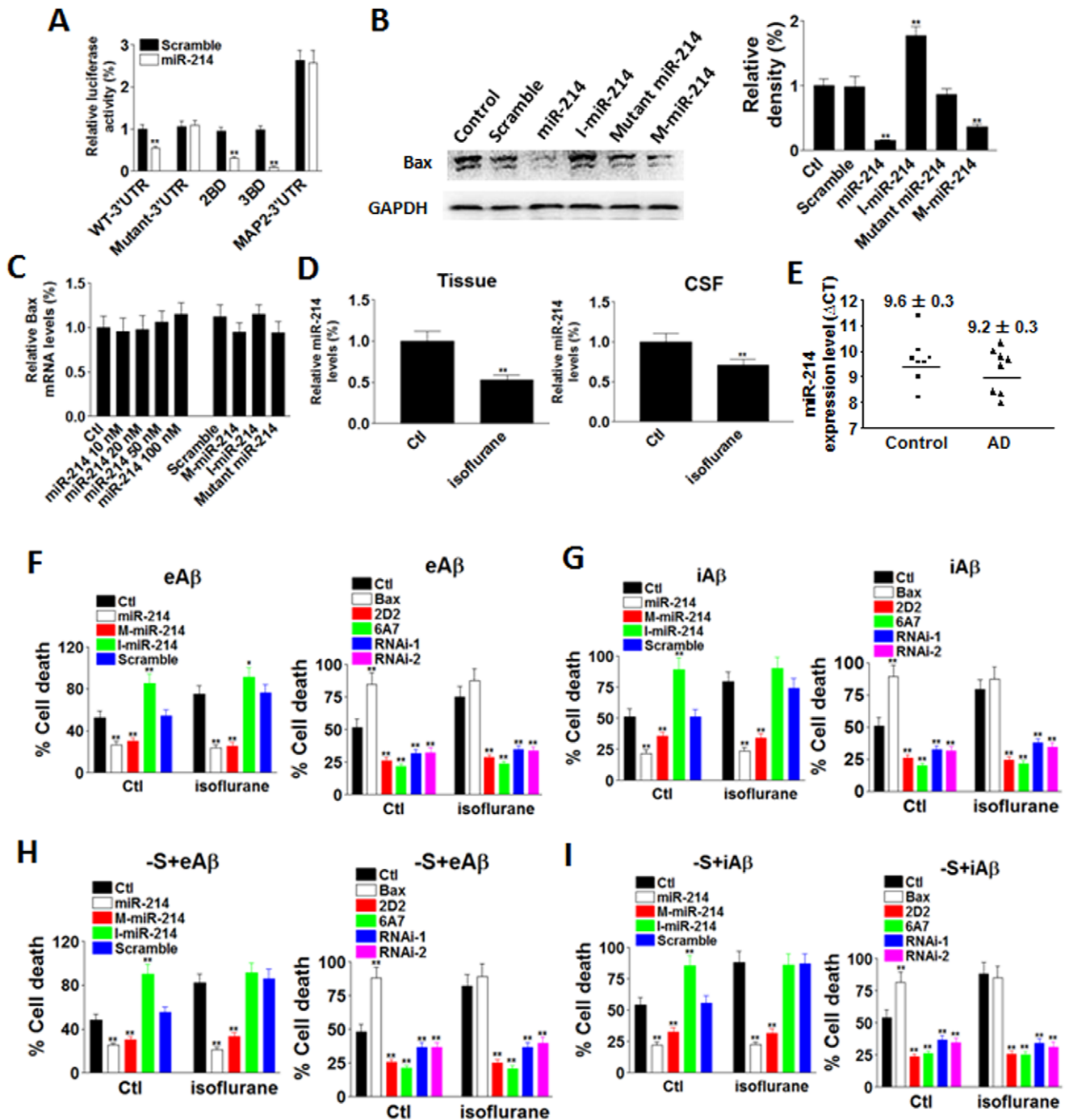
## Materials and Methods

### Cell Culture

Primary neurons were cultured from new born Sprague Dawley (SD) rat hippocampus. The protocol has been approved by the regulations of Peking University Institutional Animal Care and Use Committee (IACUC). In brief, the fresh hippocampal tissues were dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), which was then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone, Logan, UT). The mixture was triturated through pipette to make a homogenous mixture. After filtering the mixture through 70  $\mu$ m sterilized filters, the flow-through was centrifuged. The pellet was then washed once by phosphate buffered saline (PBS, 0.14 M NaCl, 0.003 M KCl, 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, 0.002 M KH<sub>2</sub>PO<sub>4</sub>, 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. Cells were then plated on poly-L-lysine (Sigma, St. Louis, MO) coated plates or glass coverslips at the density of 5  $\times$  10<sup>4</sup> cells/ml. Neurons were incubated at 37°C in DMEM without phenol red with 5% FBS and with 5% circulating CO<sub>2</sub>. Cytarabine was added to culture media 24 hours after plating at 10  $\mu$ M to inhibit dividing cell growth. Medium was changed every 48 hours. SH-Sy5y cells were maintained in DMEM/F12 with 10% FBS.

### Cell and Animal Treatments

A $\beta$  peptides (Bachem, King of Prussia, PA) were dissolved in sterile distilled water at 25  $\mu$ M and immediately frozen at -20°C. IAB peptides were delivered into neurons by microinjection at



**Figure 4. Isoflurane regulated cell death through miR-214.** (A) miR-214 specifically acted on Bax 3'UTR. (B) In SH-Sy5y cells, miR-214 inhibited total Bax (indicated by 2D2 antibody) expression. (C) miR-214 did not alter Bax mRNA levels. (D) In the rats treated with inhaled isoflurane, miR-214 levels were decreased in the brain tissues and CSF. (E) The expression level of miR-214 in matched Alzheimer's disease and control pairs. RT-PCR results for hippocampal tissues from 8 matched control-AD pairs with miR-214 showed that there was no discernible difference detected between two diagnostic groups ( $p = 0.38281$ , two-tailed Student's  $t$ -test). (F) Left panel: in cultured primary rat neurons, microinjected miR-214 or mimic miR-214 (M-miR-214) reversed cell death induced by isoflurane in the presence of  $eA\beta_{1-42}$ . Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax siRNAs (RNAi-1, RNAi-2) blocked cell death induced by isoflurane in the presence of  $eA\beta_{1-42}$ . (G) Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) reversed cell death induced by isoflurane in the presence of  $iA\beta_{1-42}$ . Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax siRNAs (RNAi-1, RNAi-2) blocked cell death induced by isoflurane in the presence of  $iA\beta_{1-42}$ . (H) Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) reversed cell death induced by isoflurane in the presence of  $eA\beta_{1-42}$  and serum deprivation (-S). Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax siRNAs (RNAi-1, RNAi-2) blocked cell death induced by isoflurane in the presence of  $eA\beta_{1-42}$  and serum deprivation (-S). (I) Left panel: microinjected miR-214 or mimic miR-214 (M-miR-214) reversed cell death induced by isoflurane in the presence of  $iA\beta_{1-42}$  and serum deprivation (-S). Right panel: Bax neutralizing antibodies (2D2 and 6A7) and Bax siRNAs (RNAi-1, RNAi-2) blocked cell death induced by isoflurane in the presence of  $iA\beta_{1-42}$  and serum deprivation (-S). Data represent Means  $\pm$  SE ( $n = 200$  cells for each group). \*\*:  $p < 0.01$  compared with the control. doi:10.1371/journal.pone.0055276.g004



**Figure 5. Schematic drawing for isoflurane cytotoxicity mechanism.**

doi:10.1371/journal.pone.0055276.g005

10 nM at the cytosolic area. EA $\beta$  peptides were added to the culture medium at 1  $\mu$ M. For anesthetics treatment on cultured cells, 0.5% isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) (Guoyao Group, Beijing, China) in humidified air with 5% circulating CO<sub>2</sub> was applied to a closed incubator 37°C for 3 hours. For anesthetics treatment on animals, SD rats (male, around 250 g) were anesthetized with humidified 1% isoflurane in 30% O<sub>2</sub> balanced by N<sub>2</sub> for 3 hours in a closed chamber. After exposure, the animals were returned to their cages. For experiments described in Fig. 3, human miR-214, scramble microRNA control, mimic miR-214, miR-214 inhibitor and mutant miR-214 (5'-ACAGACGGCACAGACAGGCAGU-3'), and for experiments described in Fig. 4F–I, rat miR-214, scramble microRNA control, mimic miR-214, miR-214 inhibitor were all purchased from Qiagen (Hilden, Germany). For experiments described in Fig. 4A, several altered human Bax 3'UTR sequences used were as following: mutant Bax 3'UTR: 5'-CCCGAUU-CAUCUACCCGAUUGA-3'; 2BD: 5'-CCCGAUUCAU-CUACCCUGCGCCUGCUGA-3'; 3BD: 5'-CCCGAUU-CAUCUACCCUGCGCCUGCUGCUGCUGCUGA-3'; MAP2-3'UTR: 5'-CATATTCATTCTTTACAAACCATAG-3'. All WT and modified 3'UTR sequences were cloned into pLenti-Luc-UTR vector backbone (Abm, Richmond, BC, Canada). MicroRNAs were transfected into SH-Sy5y cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and microinjected into primary rat neurons. 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 tissue 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 obtain injection needles with a tip diameter of ~0.5  $\mu$ m. Microinjections were performed in the cytosol of each cell using the Eppendorf Microinjector FemtoJet and Eppendorf Micromanipulator (Eppendorf, Hamburg, Germany). Neurons were injected 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, at the soma areas. The solutions were injected at the indicated concentrations with 100  $\mu$ g/ml dextran Texas Red (DTR, MW: 3000, Molecular Probes, Eugene, OR) or Alex488 (Molecular Probes, Eugene, OR) as fluorescent markers to

recognize the injected cells. Approximately 90% neurons survive the injections for at least 16 days [51].

### Measurement of Neuronal Cell Death

Cells were fixed in fresh 4% paraformaldehyde, 4% sucrose in PBS for 20 minutes at room temperature and permeabilized 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 as described by the manufacturer (Roche, Quebec, Canada). The coverslips were then washed once in distilled water for 5 minutes and mounted on glass slides to be observed under a 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. The average of 5 counts was calculated as the percentage of neuronal cell death in a certain treatment. For the injected cells, the percentage of cell death was determined as the ratio of the number of DTR-TUNEL-double-positive cells over the total number of DTR-positive cells.

### Immunostaining

Cells were permeabilized in PBS-Triton at 4°C, blocked 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 the secondary antibody. The nuclei were then staining by Hoechst 33258 (1  $\mu$ g/ml, Sigma, St. Louis, MI) for 15 minutes in dark. The coverslips were mounted with Immunon<sup>TM</sup> mounting medium (Shandon, Pittsburgh, PA) onto glass slides the results were analyzed by using fluorescence microscope (Olympus BH2-RFCA, Olympus, Tokyo, Japan) with digital camera (Olympus DP70 Digital Microscope Camera, Olympus, Tokyo, Japan).

### Postmortem Tissues

This study protocol conformed to the Helsinki (the 4<sup>th</sup> amendment) as reflected in a prior approval by the institution's human research committee. Postmortem brain tissues (frontal cortex) from patients with clinically diagnosed sporadic AD (n = 8, age range = 56–91, mean: 71.1  $\pm$  4.7) and control tissues from normal, age-matched, neurologically normal (n = 8, age range = 55–99, mean: 72.0  $\pm$  5.7) individuals were obtained from the University of Pennsylvania Brain Tissue Resource Center. The postmortem time intervals for collecting these brains were  $\leq$  15 hr (mean postmortem intervals for collection of AD and control brain samples were 9.6  $\pm$  1.5 hr and 9.6  $\pm$  1.8 hr, respectively). There were no discernible differences between diagnostic groups in age (p = 0.90141) and postmortem interval (p = 0.98219).

All cases of AD dementia studied met clinical criteria for that disorder specified by NINCDS-ADRDA [52] as determined in consensus conferences after review of medical records, direct clinical assessments, and interviews of care providers. Clinical diagnosis requires that an individual showed clear cognitive decline from his or her previous levels as verified in tests of memory and in at least one other cognitive domain (e.g., perceptual speed). The diagnoses were confirmed by postmortem examination of neuritic plaque densities in midfrontal gyrus (dorsolateral prefrontal cortex), superior+inferior temporal gyrus, inferior parietal gyrus, hippocampus, and substantia nigra as specified by the Consortium to Establish a Registry for AD (Mirra et al., 1991). The final diagnoses were consistent with Braak scores for neurofibrillary tangle pathology as recommended by the NIA-Reagan Institute consensus on diagnosis of AD [53]. All



postmortem tissues were identified by an anonymous identification number, and experiments were performed as a best matched pair without knowledge of clinical information.

### Tissue Samples and Extraction

Frozen hippocampal formation was lysed in QIAzol lysis reagent (Qiagen, Hilden, Germany) and homogenized with Kontes pestle. Extraction of RNA was performed by using miRNeasy kit (cat# 217004, Qiagen, Hilden, Germany). Briefly, for 700 l of QIAzol, 140 l of chloroform was added and mixed for 15 seconds. After 3 minutes incubation at room temperature, the samples were centrifuged at 12,000 g (4°C) for 15 min. The upper aqueous layer containing RNA was transferred to another microcentrifuge tube for RNA extraction. 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)

For brain tissues, RT-PCR was performed using the QIAGEN MicroRNA Reverse Transcription kit (cat# 218161, Mispript II RT). cDNA concentrations were determined by Qubit fluorometer. QIAGEN Mispript SYBR Green PCR kit (cat # 218073) was used to detect miR-214 expression. Forward primer Micro RNA 214, 5'-acagcaggcacagacaggcagu-3', was purchased from QIAGEN (cat# MS00031605). 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 in each individual sample was calculated by the delta Ct method.

For RT-PCR with cellular extracts, cells were harvested and total RNA was isolated with TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China). Total RNA (2 µg) were reversely transcribed using TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). Real-time PCRs were done by using TransStart Green qPCR SuperMix UDG<sup>TM</sup> (Beijing TransGen Biotech Co., Ltd., Beijing, China). Sequences of primers 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 and the average were determined. In order to use the comparative Ct method for relative quantification, the amplification efficiency of target and housekeeping gene must be approximately equal. Quantification was done using the comparative Ct method, expression levels for the target gene was normalized to the GAPDH of each sample [ $2^{-\Delta Ct} = 2^{-(Ct(\text{target gene}) - Ct(\text{GAPDH}))}$ ]. 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 in the cell lysis buffer (50 mM Tris, pH8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS) and 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 70 volts for about 2 hour. Proteins were transferred to Immobilon-P<sup>TM</sup> polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) at 100 milliamps for 2 hours. The membrane was blocked with 5% non-fat milk in Tris buffered saline (TBS) with 0.1% tween20 (TBS-T) 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 for Western blots as primary antibodies. After 3 washes of 10 minutes each with TBS-T, 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 calculated by the total 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 shown by ANOVAs. A p value of less than 0.05 was used as an indicative of statistical significance.

### Author Contributions

Conceived and designed the experiments: YZ. Performed the experiments: HY TX KL HZ. Analyzed the data: HY YZ. Contributed reagents/materials/analysis tools: TX HW. Wrote the paper: YZ HW.

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