

# Effect of retinoid X receptor- $\alpha$ nuclear export inhibition on apoptosis of neurons *in vivo* and *in vitro*

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**Abstract.** Alzheimer's disease (AD), which is characterized by excessive apoptosis of neurons, is considered to be a global public health crisis. Retinoid-induced apoptosis is dependent on the orphan nuclear receptor Nur77, a transcription factor that is expressed predominantly in brain tissues. Nur77 nuclear export requires retinoid X receptor- $\alpha$  (RXR $\alpha$ ) as a carrier. However, the involvement of Nur77 in mediating  $\beta$ -amyloid (A $\beta$ )-induced neuronal apoptosis has not yet been elucidated. The primary aim of the present study was to investigate the potential of Nur77 in A $\beta$ -induced neuron apoptosis, and to evaluate the effect of RXR $\alpha$  nuclear export inhibition on neuronal apoptosis. Mouse neuroblastoma Neuro-2a (N2a) cells and mouse hippocampi were treated with A $\beta_{25-35}$  or A $\beta_{25-35}$  combined with a RXR $\alpha$  ligand, 9-cis-retinoid acid (9-cis-RA), while untreated cells and mice served as controls. The expression of RXR $\alpha$  and Nur77 was determined using western blotting and reverse transcription-quantitative polymerase chain reaction analyses, and the translocation of RXR $\alpha$  and Nur77 was detected using confocal microscopy. In addition, the apoptosis and viability of N2a cells was detected using flow cytometry and MTT assays, respectively, and the expression of B cell lymphoma 2 (Bcl-2) and Bcl-2 associated X (Bax) was quantified by western blotting. No significant alterations in the protein or mRNA expression levels of RXR $\alpha$  and Nur77 in N2a cells or mouse hippocampi among the three groups were observed. A $\beta_{25-35}$  treatment resulted in elevated cytoplasmic protein ratios of RXR $\alpha$  and Nur77 in N2a cells when compared with controls, while combined treatment with A $\beta_{25-35}$  and 9-cis-RA reduced cytoplasmic protein ratios of RXR $\alpha$  and Nur77 to 6.67 and 5.44% in N2a cells, respectively. The MTT assay results revealed a significant reduction in the viability of N2a cells following treatment with A $\beta_{25-35}$  for 24 h

when compared with the controls, while the viability of N2a cells treated with A $\beta_{25-35}$  plus 9-cis-RA significantly increased from 53.65 to 84.10%. Western blotting revealed elevated Bax expression and reduced Bcl-2 expression in A $\beta_{25-35}$ -treated N2a cells when compared with controls, while combined treatment with A $\beta_{25-35}$  and 9-cis-RA recovered Bcl-2 expression from 0.46-fold in cells treated with A $\beta_{25-35}$  alone to 2.44-fold (relative to the control) and decreased Bax expression from 2.52-fold in cells treated with A $\beta_{25-35}$  alone to 0.99-fold (relative to the control). Flow cytometry analysis revealed that the apoptotic rate of untreated N2a cells was 4.36%, while a 15.1% apoptotic rate was detected in cells exposed to A $\beta_{25-35}$  for 24 h and a ~5.31% apoptotic rate was observed in N2a cells treated with A $\beta_{25-35}$  plus 9-cis-RA. In conclusion, treatment with A $\beta_{25-35}$  or A $\beta_{25-35}$  plus 9-cis-RA demonstrated no significant effect on the protein and mRNA expression levels of RXR $\alpha$  and Nur77. In addition, inhibition of RXR $\alpha$  nuclear export reduced neuronal apoptosis. The results of the present study may provide novel insight into the development of novel anti-AD agents.

## Introduction

Alzheimer's disease (AD) is the most common form of dementia, and is a complex disease characterized by the formation of senile plaques and neurofibrillary tangles composed of tau amyloid fibrils. These are associated with synapse loss and neurodegeneration, which results in memory impairment and additional cognitive problems (1). It has been estimated that 0.40% of the world population (26.6 million people) were living with AD in 2006, and that the prevalence rate would triple and the absolute number of individuals with the disease would quadruple by the year 2050 (2). Therefore, this disease is considered to be a global public health concern and presents a challenge due to its high prevalence and the burden of the disease (3). However, to date, there are no available treatments that reduce the progression of AD (4). Excessive apoptosis of neurons is a notable pathological feature of AD (5).  $\beta$ -amyloid (A $\beta$ ), the major component of senile plaques, has been demonstrated to contribute to neuronal apoptosis (6). Therefore, anti-apoptotic therapy may be a promising treatment for AD (7-9).

Apoptosis typically occurs via the extrinsic or intrinsic pathway in vertebrate cells (10). It has been demonstrated that the orphan nuclear receptor Nur77, also known as TR3

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or nerve growth factor IB, is a transcription factor expressed predominantly in brain tissues (11) that may be involved in the intrinsic mitochondrial-initiator pathway (12). Nur77 was reported to migrate into the mitochondria to induce conformational alterations in B cell lymphoma-2 (Bcl-2) and contribute to the induction of apoptosis in multiple organs (13). However, Nur77 nuclear export requires retinoid X receptor- $\alpha$  (RXR $\alpha$ ) as a carrier (14).

RXR $\alpha$ , a member of the nuclear receptor superfamily, regulates the transcription of target genes by binding to DNA response elements (15). It has been demonstrated that RXR $\alpha$  forms heterodimers with additional members of the steroid/thyroid/retinoid receptor family, including Nur77 (16). Notably, RXR $\alpha$  may additionally be involved in multiple biological processes, including differentiation, inflammation and apoptosis, by translocating from the nucleus to the cytoplasm (17-19). In addition, a previous study revealed that retinoids were unable to induce apoptosis in Nur77 null thymocytes, suggesting that retinoid-induced apoptosis was dependent on Nur77 (20).

It has been demonstrated that Nur77 nuclear export may induce apoptosis of T-cell hybridomas and immature thymocytes (21), as well as various types of cancer cells (22-24); however, the exact function of Nur77 requires further elucidation. In addition, the involvement of Nur77 in A $\beta$ -induced neuronal apoptosis remains unclear. The primary aim of the present study was to examine the potential involvement of Nur77 in A $\beta$ -induced neuronal apoptosis, and to evaluate the effect of RXR $\alpha$  nuclear export inhibition on neuronal apoptosis.

## Materials and methods

**Ethical statement.** All animal experimentation was performed in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals, (Ministry of Science and Technology of the People's Republic of China, Beijing China). The present study was approved by the Ethics Review Committee of Fujian Medical University (permission no. FYD2013-00127).

**Drugs.** A $\beta_{25-35}$  powder (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in ddH $_2$ O, and incubated at 37°C for one week. 9-cis-retinoid acid (9-cis-RA; Sigma-Aldrich; Merck KGaA) was diluted in dimethyl sulfoxide. A $\beta_{25-35}$  and 9-cis-RA stock solutions were stored at -80°C for use in subsequent experiments.

**Cell culture and treatment.** The mouse neuroblastoma Neuro-2a (N2a) cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were seeded onto 3 cell culture dishes (10 mm in diameter) at a density of  $1.6 \times 10^5$  cells/dish, and were cultured in 50% Dulbecco's modified Eagle's medium (Mediatech; Corning Incorporated, Corning, NY, USA) and 50% Opti-minimum essential medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). N2a cells were assigned to the following three groups: i) The A $\beta_{25-35}$  treatment group, where cells

were treated with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 24 h; ii) the A $\beta_{25-35}$ -9-cis-RA treatment group, where cells were incubated in 0.1  $\mu$ mol/l 9-cis-RA solution for 12 h, followed by treatment with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 12 h; iii) Control group, where cells were treated with the same volumes of ddH $_2$ O. The N2a cells were then maintained at 37°C in a humidified incubator with 5% CO $_2$  for 24 h, before they were harvested.

**Animal grouping.** A total of 15 male, 4-month-old C57/BL6 mice (Experimental Animal Center of Fujian Medical University, Fuzhou, China), each weighing ~20 g, were group-caged at a room temperature of 22°C and humidity of 55 $\pm$ 10%, with 10 mice in each cage. All mice were kept under a 12 h light/dark cycle with free access to food and water. Mice were randomly assigned into three equal groups of five as follows: The A $\beta_{25-35}$  treatment group, the A $\beta_{25-35}$ -9-cis-RA treatment group and the sham-operation group. The mice were anesthetized for 3 h with 10% chloral hydrate (Tianjin Beilian Fine Chemical Products Development Co., Ltd., Tianjing, China) at a dose of 300 mg/kg by intraperitoneal injection. Following excision of scalp cutaneous tissue, a bar hole was created at 2 mm posterior to the bregma and 2.5 mm lateral from the midline. Mice in the A $\beta_{25-35}$  treatment group were treated with 1  $\mu$ l A $\beta_{25-35}$  solution (A $\beta_{25-35}$  powder was dissolved in physiological saline to yield a 2  $\mu$ g/ $\mu$ l solution and incubated at 37°C for 7 days) by inserting a microsyringe through the bar hole to reach the hippocampus. Mice in the A $\beta_{25-35}$ -9-cis-RA treatment group were treated with a mixture (1  $\mu$ l total) of A $\beta_{25-35}$  (2  $\mu$ g/ $\mu$ l) and 9-cis-RA solutions (1  $\mu$ g/ $\mu$ l), while mice in the sham-operation group were treated with the same volume of physiological saline. At 24 h after surgery, the mice were anesthetized with 10% chloral hydrate (300 mg/kg) and subjected to cardiac perfusion with 4% paraformaldehyde, and approximately 15 to 20 mg mouse hippocampal tissues were excised, divided into sections, lysed in radioimmunoprecipitation (RIPA) lysis solution (Beyotime Institute of Biotechnology, Haimen, China), heated in a microwave for 5 sec, vortexed for 30 sec (1,500 rpm), incubated on ice for 15 to 30 min and centrifuged at 4°C and 13,000  $\times$  g for 5 min. The supernatant was transferred to sterile Eppendorf tubes for subsequent experiments. Mice that stopped moving and developed complete muscular relaxation were considered to be in deep anesthesia.

**Western blotting assay.** Cell and hippocampal tissue lysates were prepared in RIPA lysis buffer as described previously (25). The nuclear and cytoplasmic extracts from N2a cells and mouse hippocampal tissues were prepared using the Nuclear Extraction kit (Merck KGaA). Equal quantities of protein (25  $\mu$ g/per lane) were resolved on a 4-20% Tris-glycine gel, and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in 0.05% TBS-Tween-20 (TBST) containing 5% skim milk at room temperature, and subsequently incubated at 4°C overnight with the following primary antibodies: Rabbit anti-Bcl-2 (cat. no. D160117; 1:500; Sangon Biotech Co., Ltd.; Shanghai, China), rabbit anti-Bcl-2 associated X (Bax; cat. no. D120073; 1:500; Sangon Biotech Co., Ltd.), rabbit anti-RXR $\alpha$  (cat. no. sc-553; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and rabbit anti-Nur77 (cat. no. sc-5569; 1:1,000; Santa

Cruz Biotechnology, Inc.), while rabbit anti- $\beta$ -actin (13E5) antibody (cat. no. 4970; 1:3,000; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-poly (ADP-ribose) polymerase (cat. no. 9532; 1:2,000; Cell Signaling Technology, Inc.) and rabbit anti-heat shock protein 60 (cat. no. ab46798; 1:2,000; Abcam, Cambridge, UK) served as loading controls. After washing in TBST, the membranes were incubated with the goat anti-rabbit horseradish peroxidase-conjugated IgG antibody (cat. no. 7074; 1:8,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. Detection of proteins was performed by using an EasyBlot ECL kit (Sangon Biotech Co., Ltd.) according to the manufacturer's protocol. The band density was quantified using ImageJ 1.49 (National Institutes of Health; Bethesda, MD, USA). Experiments were performed in triplicate.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was prepared using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from 1  $\mu$ g total RNA using a High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed using Power 2X SYBR Real-time PCR Premixture (BioTek Instruments, Inc., Winooski, VT, USA) according to the manufacturer's protocol. The following primer sequences were used: RXR $\alpha$ , forward, 5'-TCAAGCGCAGACAAGCAGC-3', and reverse, 5'-GCCAGGAGAATCCCATCT-3'; Nur77 forward, 5'-GAAGCTCAGGCAGTTTGC-3', and reverse, 5'-CGCTCTGGTCCTCATCAC-3'; GAPDH, forward, 5'-AGCCTCCTTGATGGCCTCCTTG-3', and reverse, 5'-AGAACATCATCCAGCAGC-3'. PCR amplification was performed in a 25  $\mu$ l reaction containing 12.5  $\mu$ l of 2X Premix, 1  $\mu$ l forward and 1  $\mu$ l reverse primers, 1  $\mu$ l cDNA template, and 9.5  $\mu$ l ddH<sub>2</sub>O under the following conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 15 sec, 60.5°C for 60 sec, and 60.5°C for 30 sec. The relative quantity of mRNA expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (26).

**Immunohistochemistry and immunocytochemistry.** Following perfusion with 4% paraformaldehyde/phosphate-buffered saline (PBS), mouse brains were cryopreserved in 30% (w/v) sucrose/PBS at 4°C overnight. Brain samples embedded in optimal cutting temperature compound (Sangon Biotech Co., Ltd.) were divided into 20- $\mu$ m coronal sections and mounted on glass. Every tenth section was collected in sequential order (starting at bregma-1.46 mm). The total number of sections was 4 per animal. Brain sections were blocked at room temperature for 1 h in a solution containing 10% normal goat serum (Sigma-Aldrich; Merck KGaA), 0.2% Triton X-100 (Sangon Biotech Co., Ltd.) and 0.02% NaN<sub>3</sub> (Kegonghua Chemical Technology Co., Beijing, China) in Tris-buffered saline, and incubated with primary antibodies at 4°C overnight. Brain sections were then incubated with fluorescence-conjugated goat secondary antibodies for 1 h. To visualize the nuclei, sections were counterstained with DAPI (2  $\mu$ g/ml; Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min in the dark at room temperature. Following incubation with antibodies, sections were washed three times with PBS containing 0.5% Tween-20 for 10 min each time. The sections were visualized using laser confocal microscopy (Leica TCS SP5, Leica Microsystems

GmbH, Wetzlar, Germany). Acquired images were analyzed with Leica Application Suite X software 4.2 (version 4.2; Leica Microsystems GmbH). A total of four visual fields (magnification, x200) of each coronary section were randomly selected, and a total of four sections from each animal were used for assessment.

For immunocytochemical analysis, drug-treated N2a cells ( $2 \times 10^5$  cells/well) were rinsed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. N2a cells were permeabilized in 0.1% Triton X-100 for 25 min, and fixed N2a cells were blocked with 10% normal goat serum in PBS for 1 h at room temperature prior to incubation with primary antibodies at 4°C overnight. N2a cells were then incubated with fluorescence-conjugated secondary antibodies at room temperature for 1 h, and counterstained with DAPI to visualize the nuclei. A total of four visual fields (magnification, x200) of each section were randomly selected for visualization using laser confocal microscopy (Leica TCS SP5; Leica Microsystems GmbH) and assessment by using Leica Application Suite X software (version 4.2; Leica Microsystems GmbH). For immunohistochemical and immunocytochemical analysis, the primary antibodies used were rabbit anti-RXR $\alpha$  (cat. no. sc-553; 1:200; Santa Cruz Biotechnology, Inc.) and goat anti-Nur77 (cat. no. sc-7014; 1:200; Santa Cruz Biotechnology, Inc.). The secondary antibodies were goat anti-rabbit IgG DyLight<sup>®</sup> 594 (cat. no. 35560; 1:200; Thermo Fisher Scientific, Inc.) and donkey anti-goat IgG DyLight<sup>®</sup> 488 (cat. no. SA5-10086; 1:200; Thermo Fisher Scientific, Inc.).

**MTT assays.** The procedures were performed by using MTT Cell Proliferation and Cytotoxicity assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. N2a cells were seeded onto 96-well plates at a density of  $2 \times 10^3$  cells/well, and cells were assigned to the following 3 groups as follows: The A $\beta_{25-35}$  treatment group, where cells were treated with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 24 h; the A $\beta_{25-35}$ -9-cis-RA treatment group, where cells were incubated in 0.1  $\mu$ mol/l 9-cis-RA solution for 12 h, followed by treatment with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 12 h; the control group, where cells were treated with the same volume of ddH<sub>2</sub>O. The N2a cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 24 h. MTT solution (10  $\mu$ l) was then added to each well, and cells were incubated for a further 4 h. Formazan diluent solution (100  $\mu$ l) was subsequently added to each well, and the plates were placed in a humidified incubator with 5% CO<sub>2</sub> at 37°C until the purple formazan crystals were completely dissolved. Cell viability was measured using the absorbance value at 570 nm by using a BioTek ELx808 Absorbance Microplate reader (BioTek Instruments, Inc.).

**Flow cytometric assay.** Cell apoptosis was determined using an Annexin V-FITC Apoptosis kit (BioVision, Inc., Milpitas, CA, USA). N2a cells were seeded onto 6-well plates at a density of  $1 \times 10^5$  cells/well and were assigned to the following 3 groups: The A $\beta_{25-35}$  treatment group, where cells were treated with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 24 h; the A $\beta_{25-35}$ -9-cis-RA treatment group, where cells were incubated in 0.1  $\mu$ mol/l 9-cis-RA solution for 12 h, followed by treatment with 25  $\mu$ mol/l A $\beta_{25-35}$  solution for 12 h; the control group, where cells were treated



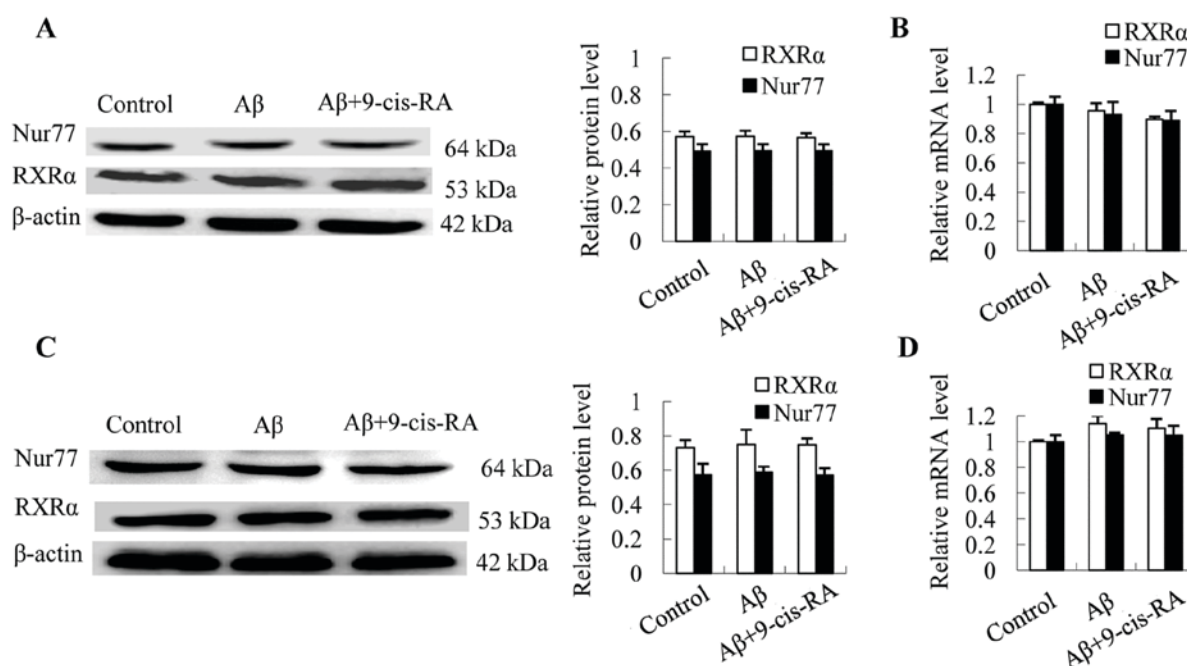


Figure 1. RXR $\alpha$  and Nur77 (A) protein and (B) mRNA expression levels in Neuro 2a cells, and the (C) protein and (D) mRNA expression levels of these factors in C57BL/6 mouse hippocampi. Data are representative of three independent experiments, and are expressed as the mean  $\pm$  standard deviation. RXR $\alpha$ , retinoid X receptor- $\alpha$ .

with the same volume of ddH<sub>2</sub>O. The N2a cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 24 h before they were harvested. Cells were subsequently rinsed in cool PBS, and re-suspended in 250  $\mu$ l binding buffer (1X). A total of 100  $\mu$ l cell suspension (cell density of  $1 \times 10^5$  cells/well) was transferred to a 5 ml tube, and 5  $\mu$ l Annexin V-fluorescein isothiocyanate and 5  $\mu$ l propidium iodide was added, mixed and incubated in the dark at room temperature for 15 min. A total of 400  $\mu$ l binding buffer (1X) was then added, and apoptosis of N2a cells was analyzed using an EPICS Altra Flow Cytometer (Beckman Coulter, Inc.; Brea, CA, USA) and CellQuest software (version 5.1; Beckman Coulter, Inc.).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation, and statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between the means of groups were tested for statistical significance using the one-way analysis of variance followed by Tukey's multiple comparisons test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**A $\beta_{25-35}$  treatment demonstrates no significant effect on RXR $\alpha$  and Nur77 expression.** In order to evaluate the effect of A $\beta_{25-35}$  treatment on the expression of RXR $\alpha$  and Nur77, N2a cells were treated with 25  $\mu$ mol/l A $\beta_{25-35}$  alone or in combination with 0.1  $\mu$ mol/l 9-cis-RA, while untreated cells served as controls. Western blotting and RT-qPCR analyses revealed comparative protein and mRNA expression levels of RXR $\alpha$  and Nur77 relative to the controls (Fig. 1). These results indicated that treatment with A $\beta_{25-35}$  alone or in combination with 9-cis-RA demonstrated no significant effect on the expression

levels of RXR $\alpha$  and Nur77, at the translational (Fig. 1A) or transcriptional levels (Fig. 1B). In addition, mouse hippocampi were injected with 2  $\mu$ g A $\beta_{25-35}$  or 2  $\mu$ g A $\beta_{25-35}$  combined with 1  $\mu$ g 9-cis-RA, while untreated mice served as controls. No significant difference in the expression of RXR $\alpha$  and Nur77 among the three experimental groups at the translational (Fig. 1C) or transcriptional (Fig. 1D) levels were observed, indicating that treatment with A $\beta_{25-35}$  alone or A $\beta_{25-35}$ -9-cis-RA combination demonstrated no significant effects on RXR $\alpha$  and Nur77 mRNA and protein expression levels.

**Nuclear-cytoplasmic translocation of RXR $\alpha$  is required for cytoplasmic targeting of Nur77 in N2a cells and the mouse hippocampus.** RXR $\alpha$  and Nur77 are primarily located in the nuclei of untreated N2a cells, and the cytoplasmic protein ratios of RXR $\alpha$  and Nur77 were 5.26 and 4.85%, respectively (Fig. 2A). Treatment of N2a cells with 25  $\mu$ mol/l A $\beta_{25-35}$  for 24 h resulted in significantly reduced RXR $\alpha$  and Nur77 expression in the nucleus (Fig. 2A) and significantly elevated expression in the cytoplasm when compared with the controls (RXR $\alpha$ , 8.41-fold,  $P < 0.001$ ; Nur77, 7.33-fold,  $P < 0.001$ ; Fig. 2A). In order to investigate whether RXR $\alpha$  nuclear export is required for Nur77 cytoplasmic targeting in neurons, N2a cells were pretreated with 0.1  $\mu$ mol/l 9-cis-RA or control medium for 12 h, followed by treatment with 25  $\mu$ mol/l A $\beta_{25-35}$  for 24 h. Western blot analysis demonstrated that the cytoplasmic protein ratios of RXR $\alpha$  and Nur77 were significantly reduced in the A $\beta_{25-35}$ -9-cis-RA group compared with A $\beta_{25-35}$ -treated N2a cells [RXR $\alpha$ , A $\beta_{25-35}$  (42.22%) vs. A $\beta_{25-35}$ -9-cis-RA (6.67%),  $P < 0.001$ ; Nur77, A $\beta_{25-35}$  (35.49%) vs. A $\beta_{25-35}$ -9-cis-RA (5.44%),  $P < 0.001$ ; Fig. 2A]. Confocal microscopy revealed that Nur77 and RXR $\alpha$  proteins were predominantly localized in the nuclei of untreated N2a cells,

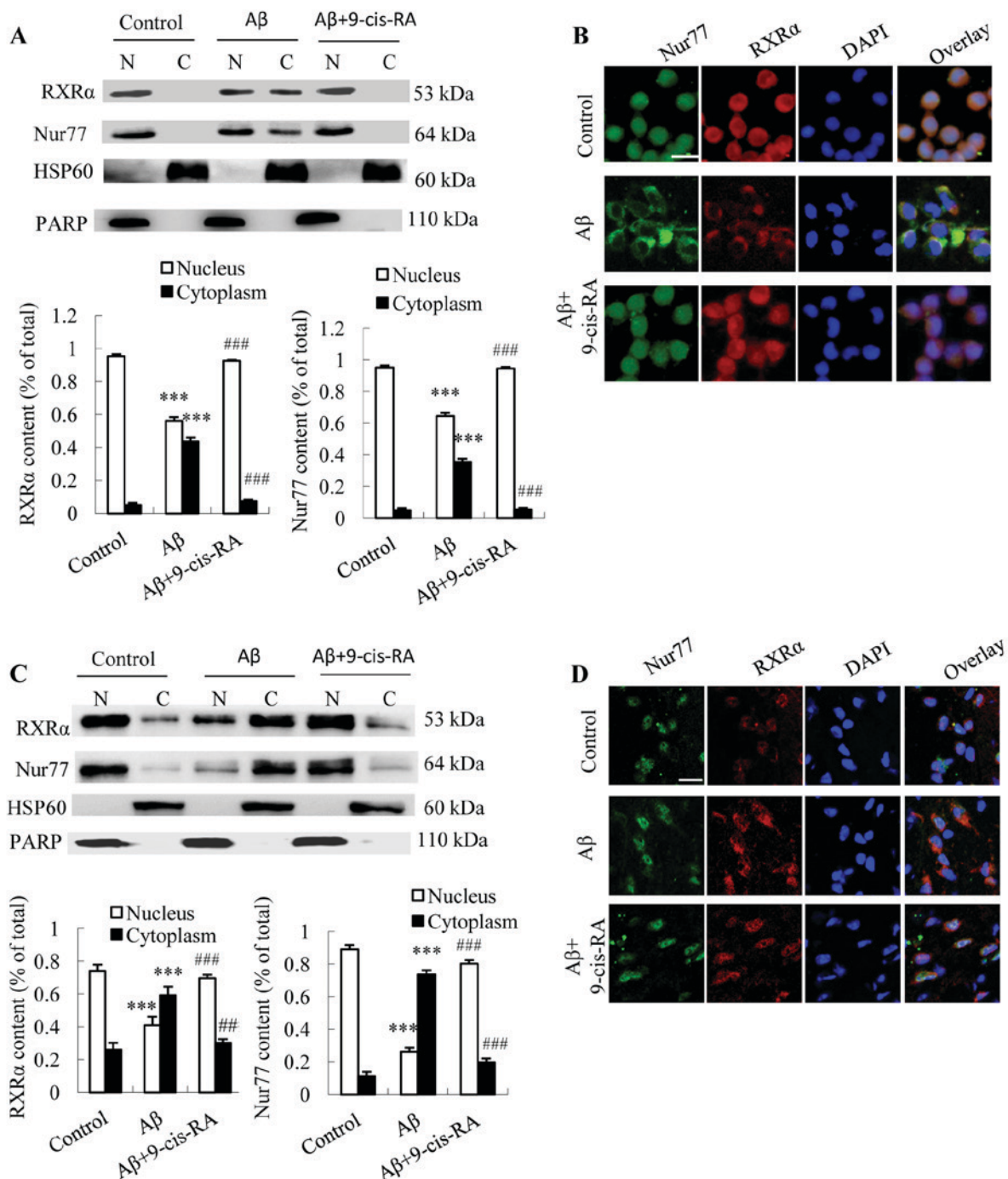


Figure 2. Effect of Aβ<sub>25-35</sub> treatment or Aβ<sub>25-35</sub> plus 9-cis-RA treatment on the cellular location of RXRα and Nur77 in N2a cells and the mouse hippocampus. (A) RXRα and Nur77 nuclear and cytoplasmic protein expression in N2a cells, normalized to the expression of cytoplasmic protein HSP60 and nuclear protein PARP (n=3). (B) Representative fluorescence microscope images showing immunocytochemical staining of RXRα and Nur77 expression in N2a cells (scale bar, 10 μm). (C) RXRα and Nur77 nuclear and cytoplasmic protein expression in mouse hippocampus tissue was normalized to the expression of cytoplasmic protein HSP60 and nuclear protein PARP (n=5). (D) Representative fluorescence microscope images showing immunohistochemical staining of RXRα and Nur77 in the CA1 region of C57BL/6 mouse hippocampi (scale bar, 10 μm). Values are presented as the mean ± standard deviation of ≥3 independent experiments. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control group; #P<0.05, ##P<0.01 and ###P<0.001 vs. Aβ group. Aβ, β-amyloid; 9-cis-RA, 9-cis-retinoid acid; RXRα, retinoid X receptor-α; N2a, Neuro-2a; HSP60, heat shock protein 60; PARP, poly(ADP-ribose) polymerase.

while RXRα and Nur77 were predominantly co-localized in the cytoplasm of Aβ<sub>25-35</sub>-treated N2a cells (Fig. 2B). In addition, the distribution of RXRα and Nur77 overlapped, suggesting an interaction between RXRα and Nur77 in the cytoplasm (Fig. 2B). However, the nuclear export of RXRα and Nur77 was visibly inhibited in N2a cells treated with Aβ<sub>25-35</sub> plus 9-cis-RA (Fig. 2B).

The dependence of the cytoplasmic localization of Nur77 on RXRα localization *in vivo* was then evaluated. The hippocampi of C57BL/6 mice were injected with 2 μg Aβ<sub>25-35</sub> or 2 μg Aβ<sub>25-35</sub> plus 1 μg 9-cis-RA for 24 h, while sham-operated mice served as controls. Western blotting analysis revealed that Aβ<sub>25-35</sub> treatment was associated with a marked increase in the translocation of RXRα and Nur77 from the nucleus to the cytoplasm

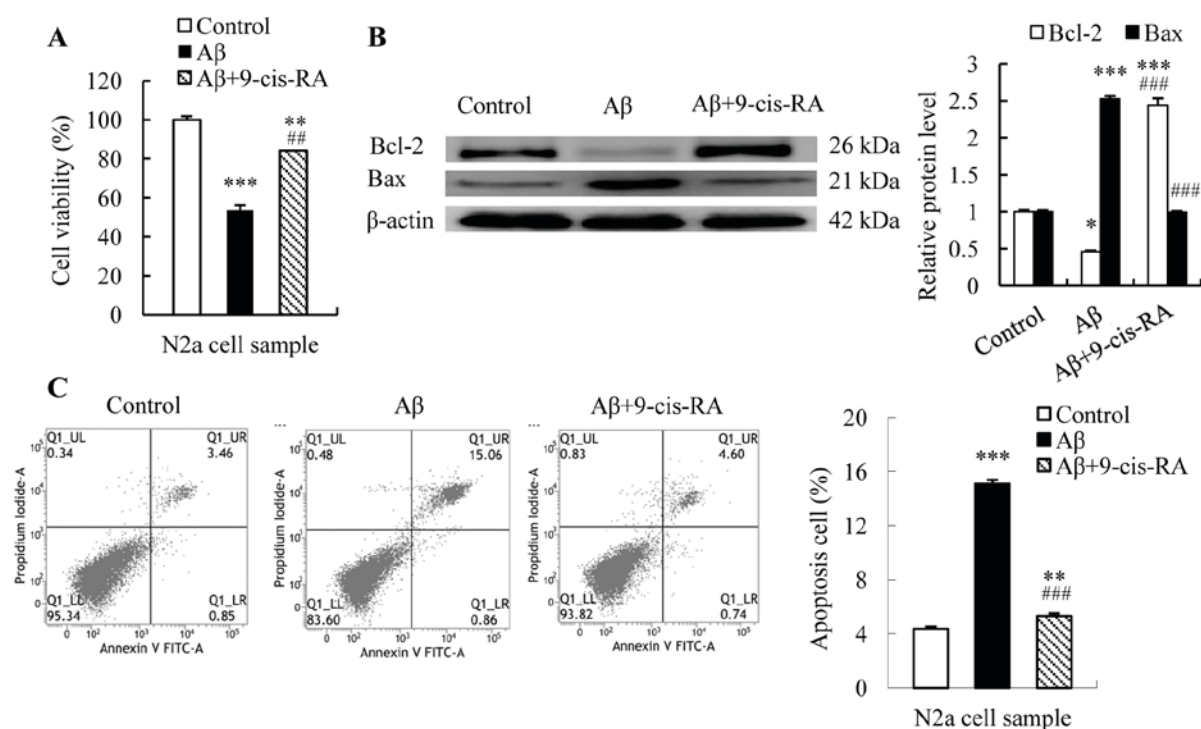


Figure 3. 9-cis-RA induces cell proliferation and inhibits apoptosis of N2a cells in the presence of A $\beta_{25-35}$ . (A) N2a cell viability, as determined using an MTT assay. (B) Western blotting analysis and quantification of Bcl-2 and Bax protein expression levels. (C) The apoptosis rate of N2a cells as determined by flow cytometry analysis. Data are representative of three independent experiments, and values are presented as the mean  $\pm$  standard deviation. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. control group; # $P$ <0.05, ## $P$ <0.01 and ### $P$ <0.001 vs. A $\beta$  group. 9-cis-RA, 9-cis-retinoic acid; N2a, Neuro-2a; A $\beta$ ,  $\beta$ -amyloid; Bcl-2, B cell lymphoma-2; Bax, Bcl-2 associated X.

of hippocampus cells, and RXR $\alpha$  and Nur77 protein expression increased from 26.1 and 11.1% to 59.2 and 73.7%, respectively, in the cytoplasm relative to the controls ( $P$ <0.001 and  $P$ <0.001, respectively; Fig. 2C). In addition, the cytoplasmic protein ratios of RXR $\alpha$  and Nur77 decreased from 59.2 and 73.7% in the hippocampal tissues of A $\beta_{25-35}$ -treated mice to 30.2 and 19.8% in A $\beta_{25-35}$  plus 9-cis-RA co-treated mice, respectively, when compared with mice treated with A $\beta_{25-35}$  alone ( $P$ <0.01 and  $P$ <0.001, respectively; Fig. 2C). Confocal microscopy revealed that RXR $\alpha$  and Nur77 were primarily localized to the nucleus and the nuclear periphery of untreated mice, while an increase in RXR $\alpha$  and Nur77 fluorescence in the cytoplasm of A $\beta_{25-35}$ -treated mice was observed (Fig. 2D). In the mice treated with 2  $\mu$ g A $\beta_{25-35}$  plus 1  $\mu$ g 9-cis-RA, RXR $\alpha$  and Nur77 were observed to reside in the nucleus and nuclear periphery when compared with the A $\beta_{25-35}$  treatment group (Fig. 2D).

**9-cis-RA improves cell viability and inhibits apoptosis of N2a cells in the presence of A $\beta_{25-35}$ .** The results of the MTT assay revealed a significant reduction in the viability of N2a cells following treatment with A $\beta_{25-35}$  for 24 h when compared with the controls (from 100 to 53.65% cell viability;  $P$ <0.001; Fig. 3A). By contrast, the viability of N2a cells treated with A $\beta_{25-35}$  plus 9-cis-RA significantly increased from 53.65 to 84.10% ( $P$ <0.01 vs. A $\beta_{25-35}$ -treated cells; Fig. 3A).

Mitochondrial outer membrane permeabilization, which is involved in the induction of apoptosis, is controlled by pro- and anti-apoptotic factors (27). Therefore, the expression of mitochondrial membrane permeabilization factors Bcl-2 and Bax was examined in the present study. N2a cells were treated

with 25  $\mu$ mol/l A $\beta_{25-35}$  for 24 h alone or in combination with 0.1  $\mu$ mol/l 9-cis-RA for 12 h, while untreated cells served as controls. Bcl-2 and Bax protein expression levels were subsequently quantified. Western blotting analysis revealed elevated Bax expression and reduced Bcl-2 expression (Bax, 2.52-fold,  $P$ <0.001; Bcl-2, 0.46-fold,  $P$ <0.05; Fig. 3B) in A $\beta_{25-35}$ -treated N2a cells when compared with controls. Combined treatment with A $\beta_{25-35}$  and 9-cis-RA recovered Bcl-2 expression from 0.46-fold in A $\beta_{25-35}$ -treated cells to 2.44-fold relative to controls ( $P$ <0.001; Fig. 3B), and decreased Bax expression from 2.52-fold in A $\beta_{25-35}$ -treated cells to 0.99-fold relative to controls ( $P$ <0.001; Fig. 3B).

Flow cytometry analysis revealed that the apoptotic rate of untreated N2a cells was 4.36%, while an apoptotic rate of 15.1% was detected in cells cultured in the presence of 25  $\mu$ mol/l A $\beta_{25-35}$  for 24 h ( $P$ <0.001; Fig. 3C). In addition, combined treatment with 25  $\mu$ mol/l A $\beta_{25-35}$  plus 0.1  $\mu$ mol/l 9-cis-RA resulted in a ~5.31% apoptotic rate, which was a significant reduction when compared with cells treated with A $\beta_{25-35}$  alone ( $P$ <0.001; Fig. 3C).

## Discussion

A $\beta$  accumulation is generally accepted to be critical for the development of AD dementia (28,29). A $\beta$  has been confirmed to exhibit neurotoxic effects on neurons (30). In the present study, an increased apoptotic rate of A $\beta_{25-35}$ -treated N2a cells was observed, and cell viability decreased when compared with untreated controls. These results are consistent with the notion that A $\beta$  is toxic to neurons. However, these results were



obtained from cell and animal experiments, and further human studies are therefore required to validate this conclusion.

Modulation of RXR $\alpha$  levels has been previously reported to directly affect A $\beta$  generation (25). Given the involvement of RXR $\alpha$ /Nur77 in the regulation of cell death, the effect of A $\beta$ <sub>25-35</sub> treatment on RXR $\alpha$  and Nur77 expression at translational and transcriptional levels was investigated in the present study. Western blotting and RT-qPCR analysis revealed no significant difference in the protein and mRNA levels of RXR $\alpha$  and Nur77 between the A $\beta$ <sub>25-35</sub>-treated and untreated control groups. This is inconsistent with the report demonstrating that higher levels of RXR $\alpha$  gene expression occur in AD (19). It is possible that a single injection was insufficient to alter RXR $\alpha$  expression significantly.

RXR $\alpha$  has been demonstrated to regulate Nur77-dependent apoptosis by modulating Nur77 nuclear export (8). However, the involvement of RXR $\alpha$  in the regulation of neuronal apoptosis is unclear. In the present study, this potential function of RXR $\alpha$  was confirmed using *in vivo* and *in vitro* experimental systems, which mimic the pathologic condition of AD. In response to A $\beta$ <sub>25-35</sub> stimulation RXR $\alpha$ /Nur77 were co-transported into the cytoplasm in N2a cells and hippocampal neurons, as the rate of apoptosis increased. Further studies to validate the co-localization of RXR $\alpha$ /Nur77 in mitochondria and investigate the mechanisms underlying the association between RXR $\alpha$ /Nur77 nuclear export and apoptosis are required.

In the present study, the role of RXR $\alpha$  nuclear export in mediating Nur77 nuclear export was investigated. In the presence of 9-cis-RA, A $\beta$ <sub>25-35</sub>-induced RXR $\alpha$ /Nur77 nuclear export was inhibited both *in vivo* and *in vitro*. The results of the present study provide some evidence to suggest that Nur77 may be transported to the cytoplasm via its interaction with RXR $\alpha$ , which is consistent to a previous report (14). The pro-apoptotic Bax and anti-apoptotic Bcl-2 protein expression levels were then analyzed. A $\beta$ <sub>25-35</sub> treatment was demonstrated to result in elevated Bax expression and reduced Bcl-2 expression, accompanied by RXR $\alpha$ /Nur77 nuclear export and an increased apoptotic rate in N2a cells. The expression of Bax and Bcl-2 was consistent with previous observations that Bcl-2 inhibits apoptosis and Bax promotes apoptosis (31). However, treatment with 9-cis-RA plus A $\beta$ <sub>25-35</sub> resulted in a decreased apoptotic rate of N2a cells, and inhibition of RXR $\alpha$ /Nur77 nuclear export coincided with the decreased Bax and increased Bcl-2 expression. These results suggested that A $\beta$ -induced RXR $\alpha$  and Nur77 co-translocation was associated with the alterations in Bcl-2 and Bax expression, which may have initiated apoptosis in neurons.

In conclusion, the results of the present study demonstrated that A $\beta$ -mediated neuronal death may be dependent on Nur77, and RXR $\alpha$  and its ligands may be involved in regulating Nur77-dependent apoptosis in neurons. Elucidation of the mechanisms underlying the inhibition of RXR $\alpha$  translocation may be of significance for the development of anti-AD agents that suppress neuronal apoptosis.

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