Characteristics of olfactory ensheathing cells and microarray analysis in *Tupaia belangeri* (Wagner, 1841)

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Abstract. Tree shrews are most closely related to the primates and so possess a number of advantages in experimental studies; they have been used as an animal model in bacterial and virus infection, cancer, endocrine system disease, and certain nervous system diseases. Their olfactory ensheathing cells (OECs) are able to release several cytokines to promote neuronal survival, regeneration and remyelination. The present study used western blot analysis to identify antibody specificity in protein extracts from whole tree shrew brains to identify the specificity of p75 nerve growth factor receptor (NGFR) derived from rabbits (75 kDa). OECs were cultured and isolated, then stained and identified using the antibodies for p75NGFR. To investigate the capacity of OECs to express cytokines and growth factors, microarray technology was used, and the analysis revealed that OECs were able to express 9,821 genes. Of these genes, 44 genes were from the neurotrophic factor family, which may indicate their potential in transplantation *in vivo*. The present study considered the function of OECs as revealed by other studies, and may contribute to future research.

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Introduction

Tree shrews (*Tupaia belangeri*, Wagner, 1841) are small animals originally regarded as either primates or insectivores and later classified as a separate order, Scandentia (1,2). They are most closely related to the primates, with the advantages of being readily available, easy to tame and possessing a strong reproductive capacity (3). Due to these advantages they have been successfully employed as an animal model for bacterial (4) or viral infection (5,6), tumors (7), endocrine system disease (3,8), and certain nervous system diseases, including myopia (9-11), psychosocial stress (12,13), cerebral ischemia (14) and aging (15).

Olfactory ensheathing cells (OECs) are the glial cells that derive from the olfactory placode and envelop olfactory axons in the course of migration from the olfactory epithelium to the bulb (16). A number of studies have identified that OECs can not only release various cytokines (17,18) that can promote neuronal survival (19), regeneration (20,21) and remyelination (22,23), *in vitro* and *in vivo*, but can also migrate into lesion regions (24) and facilitate functional repair (25,26) following injuries. In the present study, OECs of tree shrews were isolated and purified successfully for the first time, to the best of the authors' knowledge. Cytokine or the receptor mRNA expression was also investigated using a microarray. The results of the present study may contribute to the use of OECs in transplantation and the evaluation of their curative effect in fundamental research and future clinical trials.

Materials and methods

Animals. Tree shrews (2 newborn for cell culture and 1 adult male, weight 140±10 g at the start of the experiment) and 1 adult male Sprague-Dawley rats (for western blot analysis, weight 140±10 g at the start of the experiment) were provided by Kunming Medical University Animal Center (Kunming, China) and employed in this study. The animals were raised at 20±5˚C, 40-60% humidity and a 12-h light/dark cycle with

food and water *ad libitum*. All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (approval no. 2006-398), and was approved by the ethics committee of Institute of Neuroscience, Kunming Medical University (Kunming, China). All animals were bred in separated cages.

Primary cell cultures. Primary cultures of OECs were set up from newborn tree shrews and the animals were sacrificed. Olfactory bulbs were aseptically removed. The meningeal layer was stripped off with fine forceps and the tissue was enzymatically (trypsin 0.25%; 1:30 tissues to trypsin ratio) and mechanically dissociated, then incubated in 5% CO_2 at 37°C for 15 min. The cells were recovered by centrifugation (100 x g for 5 min at 37°C) in culture medium [Dulbecco's modified Eagle's medium/F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.)] and seeded at $1x10^5$ cells/ml into culture plates and incubated in 5% CO_2 at 37°C. Cells were cultured for 10 h and then the supernatant was removed to another culture plate.

Antibodies. Antibodies against p75 neurotrophin receptor (p75NGFR) and BrdU were used to study the purity and proliferation of OECs. For each antibody, a minimum of two whole series of different animals were studied. Western blot analysis with protein extracts from whole tree shrew and rat brains ensured specificity of the antibodies used. The tissues were homogenized in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) that contained a mixture of proteinase inhibitors and the supernatant was collected by centrifuging at 4°C at 22,500 x g for 15 min. The protein concentration was determined by the DC protein assay from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and protein samples (100 μg) of each cell lysate in SDS loading buffer (Biosharp) were electrophoresed on 10% SDS-PAGE gel. Electrophoresis was performed and the proteins were transferred onto polyvinylidene difluoride membranes (Pall Life Sciences, Port Washington, NY, USA) using electroblotting apparatus (Bio-Rad Laboratories, Inc.). The membranes were blocked at 37°C for 1 h in TBS containing 0.1% Tween-20 and 5% dried milk, and were then incubated at 4°C overnight with NGFRp75 antibody (1:1,000; cat. no. AB1554; EMD Millipore, Billerica, MA, USA) They were incubated with the IRDye 800-conjugated affinity purified goat anti-rabbit immunoglobulin (Ig)G (1:5,000; cat. no. 611-1302; Rockland Immunochemicals, Inc., Pottstown, PA, USA) for 1 h at room temperature and visualization using an Odyssey laser scanning system (LI-COR Biosciences, Lincoln, NE, USA). Blots were reprobed with the monoclonal mouse anti-β actin antibody (1:5,000; cat. no. A2228; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), followed by reaction with IRDye 800-conjugated affinity purified goat anti-mouse IgG (1:5,000; cat. no. 610-1302; Rockland Immunochemicals, Inc.) at 37°C for 2 h to confirm equal protein loading.

Immunofluorescence. Immunofluorescence was performed on OECs. The cells were fixed with 4% formalin solution at room temperature for 5 min then washed with PBS and
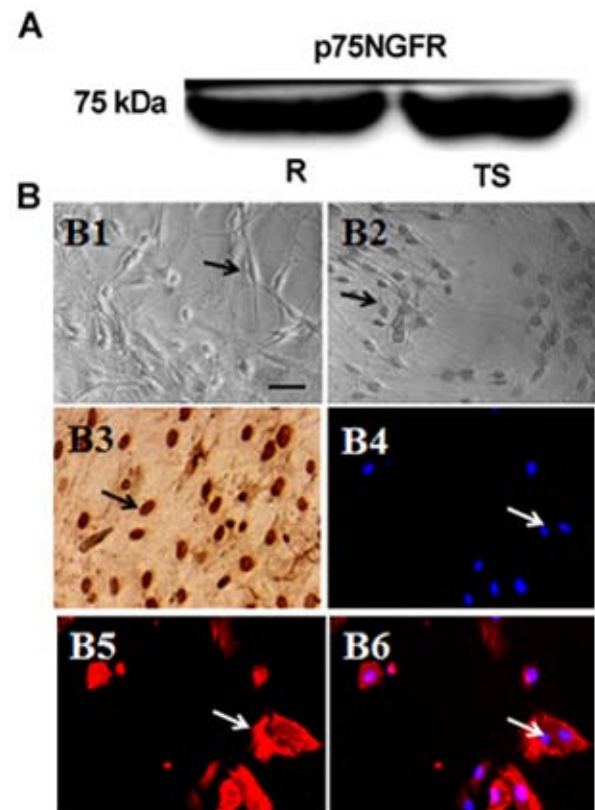

Figure 1. (A) Specificity of the antibody. The bands of p75NGFR with protein extracts from whole rat and tree shrew brain. The molecular marker was a restrained maker and did not react with ECL. (B) OEC biological characteristics *in vitro*. (B1) Morphology of OECs cultured for 24 h (magnification, x200). (B2) Morphology of OECs cultured for 6 days (magnification, x200). (B3) Immunocytochemical staining of BrdU (magnification, x200). (B4) p75NGFR positive cells (magnification, x400). (B5) DAPI positive cells (magnification, x400). (B6) Merged view of the p75NGFR and DAPI double positive cells (magnification, x400). OECs, olfactory ensheathing cells; NGFR, nerve growth factor receptor; R, rat; TS, tree shrew.

permeabilized with 0.1% Triton X-100. The cells were then blocked with 1% bovine serum albumin (10082-139; Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min and incubated for 18 h at 4°C with anti-p75NGFR (1:500; AB1554; EMD Millipore) and anti-BrdU (1:200; mAb #5292; Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies. Following washing with PBS, the cells were incubated at 37°C for 2 h with respective anti-Mouse IgG H&L (Alexa Fluor 647; 1:500; ab150115; Abcam, Cambridge, MA, USA). Following incubation, the slides were washed with PBS, mounted and examined under a fluorescence microscope (Leica TCS SP2; Leica Microsystems GmbH, Wetzlar, Germany). Fluoroshield mounting medium with DAPI (Sigma-Aldrich; Merck KGaA) was used for DAPI staining and the staining was performed according to the manufacturer's protocol.

Microarray analysis. Total RNA from OECs was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.) and RNA integrity was assessed using standard denaturing agarose gel electrophoresis (27) (performed by KangChen Biotech Co., Ltd., Shanghai, China). Total RNA from each sample was used for labeling and array hybridization as
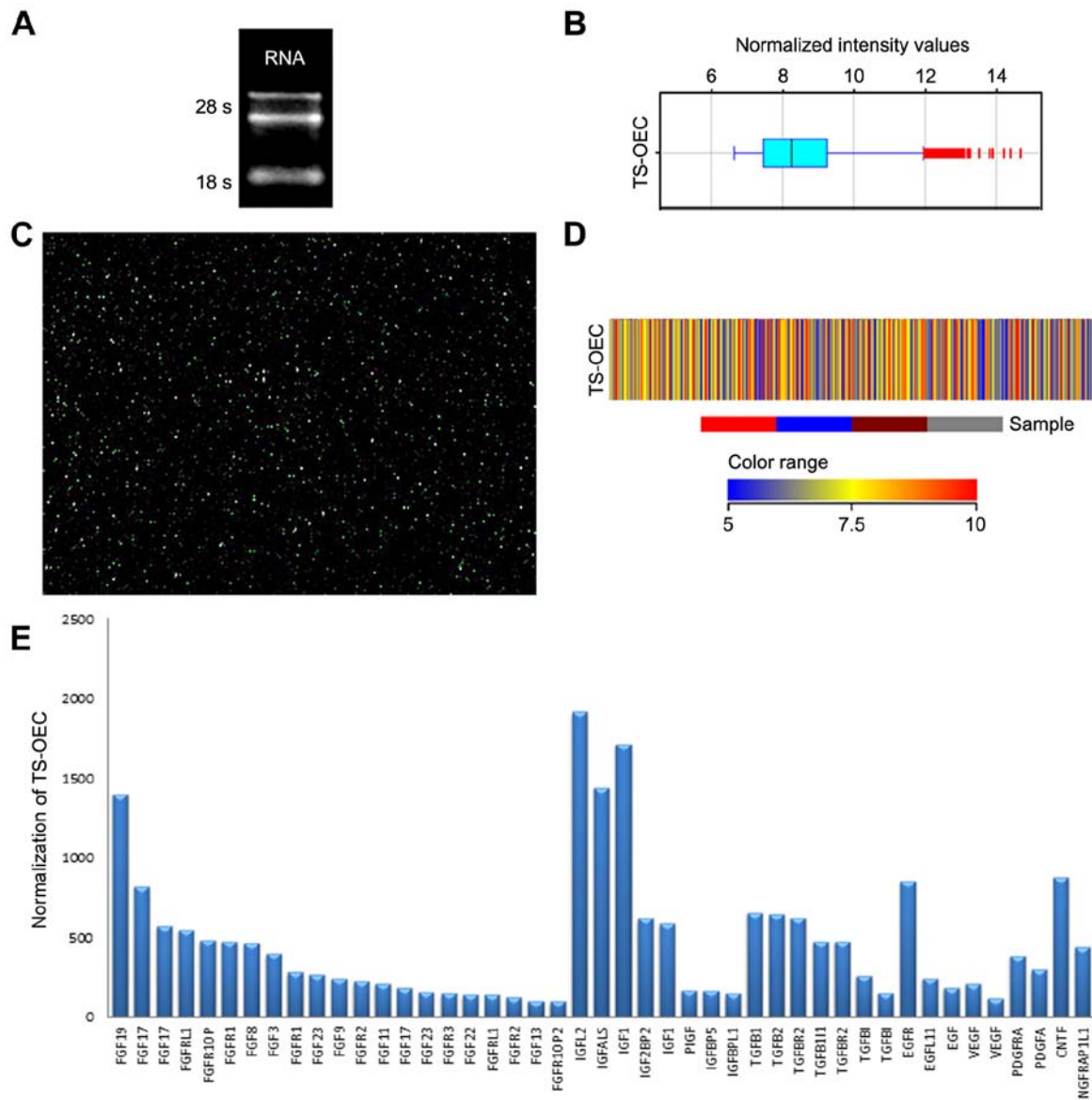
Figure 2. Gene chip assay. (A) Bands of RNA agarose gel electrophoresis. (B) Boxplot of the samples. (C) Map of gene chip assay. (D) Heat map of OECs expressed genes. (E) Bar chart of 45 important genes. OECs, olfactory ensheathing cells; NGFR, nerve growth factor receptor.

follows: i) Reverse transcription using the Superscript ds-cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.); ii) ds-cDNA labeling using the NimbleGen One-Color DNA Labeling kit (Roche NimbleGen, Inc., Madison, WI, USA); iii) array hybridization using the NimbleGen Hybridization System, followed by washing with the NimbleGen wash buffer kit (Roche NimbleGen, Inc.); and iv) array scanning using the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5; Roche NimbleGen, Inc.) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level (*_norm_RMA.pair) files and Gene level (*_RMA.calls) files were generated following normalization. All gene level files were imported into Agilent GeneSpring GX software (version 11.5; Agilent Technologies, Inc., Santa Clara, CA, USA) for further analysis. Finally, hierarchical clustering was performed using

Gene Ontology (http://www.geneontology.org) to show distinguishable gene expression profiling among samples.

Results

Specificity of the antibody. Western blotting with protein extracts from whole tree shrew and rat brains ensured specificity of the antibodies used in the present study. Western blotting demonstrated that the antibodies produced bands that were located at the same level in the gel for rat and tree shrew brain protein extracts. Notably, the western blots also demonstrated that these bands had the proper molecular weight for the p75NGFR antibody examined (Fig. 1A).

OEC biological characteristics in vitro. To investigate the method of culturing OECs from tree shrews, the bulbus olfactorius of newborn tree shrews was used. Cultured for 24 h, certain cells began to demonstrate adherence and assume a round or star-like shape (Fig. 1B1). Then, 6 days following

Table I. Genes with significant expression difference.

Sequence identification	Normalized tree shrew OECs	Gene name	Synonyms
NM_001002915	1914.4823	IGFL2	VPRI645
M37484	1709.5454	IGF1	IGFI
NM_004970	1441.7056	IGFALS	ALS
NM_005117	1399.2097	FGF19	n/a
NM_201284	849.4621	EGFR	ERBB, ERBB1, mENA
NM_000614	879.9232	CNTF	HCNTF
BC105131	818.3038	FGF17	FGF-13
BC000125	658.60596	TGFB1	CED, DPD1, TGFB
M19154	643.18677	TGFB2	MGC116892, TGF-β2
BC040499	623.899	TGFBR2	AAT3, HNPCC6, MFS2, RIIC, TGFR-2, TGFβ-RII
NM_001007225	620.82745	IGF2BP2	IMP-2, IMP2, VICKZ2, p62
M29644	592.314	IGF1	IGFI
AY358869	576.01324	FGF17	FGF-13
NM_001004356	551.01306	FGFRL1	FGFR5, FHFR
BC037785	484.15298	FGFR1OP	FOP
AK024388	476.04077	FGFR1	BFGFR, C-FGR, CD331, CEK, FLG, FLT2, H2, H3, H4, H5, KAL2, N-SAM
NM_015927	474.31787	TGFB1I1	ARA55, HIC-5, HIC5, TSC-5
NM_001024847	474.17993	TGFBR2	AAT3, HNPCC6, MFS2, RIIC, TGFR-2, TGFβ-RII
NM_006119	463.30542	FGF8	AIGF, HBGF-8
NM_001012978	436.89304	NGFRAP1L1	BEX5, MGC104434, MGC126446
NM_005247	398.42584	FGF3	HBGF-3, INT2
NM_006206	383.68063	PDGFRA	CD140A, MGC74795, PDGFR2
NM_002607	299.87314	PDGFA	PDGF-A, PDGF1
M63888	280.45587	FGFR1	BFGFR, C-FGR, CD331, CEK, FLG, FLT2, H2, H3, H4, H5, KAL2, N-SAM
NM_020638	267.7028	FGF23	ADHR, HPDR2, HYPF, PHPTC
BC004972	260.64957	TGFBI	BIGH3, CDB1, CDG2, CDGG1, CSD, CSD1, CSD2, CSD3, LCD1
NM_198283	246.34807	EGFL11	KIAA0663, dJ1018A4.2
BC069692	240.3946	FGF9	GAF, HBFG-9, MGC119914, MGC119915
NM_023028	225.70663	FGFR2	BEK, BFR-1, CD332, CEK3, CFD1, ECT1, JWS, K-SAM, KGFR, TK14, TK25
BC032502	210.7425	FGF11	FHF3, FLJ16061, MGC102953, MGC45269,
S85192	207.95761	VEGF	MGC70609, VEGFA, VPF
BC093731	185.4517	EGF	URG
NM_003867	183.04973	FGF17	FGF-13
NM_002643	168.185	PIGF	MGC32646, MGC33136
BC011453	165.53284	IGFBP5	IBP5
BC098252	157.41748	FGF23	ADHR|HPDR2|HYPF|PHPTC|
BC026352	151.22711	TGFBI	BIGH3, CDB1, CDG2, CDGG1, CSD, CSD1, CSD2, CSD3, LCD1
NM_000142	151.22061	FGFR3	ACH, CD333, CEK2, HSFGFR3EX, JTK4
NM_001007563	149.79308	IGFBPL1	IGFBP-RP4, bA113O24.1
NM_020637	140.37721	FGF22	n/a
AK172829	139.57779	FGFRL1	FGFR5, FHFR
NM_022976	125.33181	FGFR2	BEK, BFR-1, CD332, CEK3, CFD1, ECT1, JWS, K-SAM, KGFR, TK14, TK25
M27281	120.924126	VEGF	MGC70609, VEGFA, VPF
NM_004114	102.02009	FGF13	FGF2, FHF2
NM_015633	100.07872	FGFR1OP2	DKFZp564O1863, HSPC123-like

OECs, olfactory ensheathing cells; n/a, not applicable.

adherent culture, three classic morphologies of OECs were observed: Bipolar or fusiform and oblate (Fig. 1B2). Among these shapes, fusiform and oblate were more common, and an evident three-dimensional appearance. After 14 days, the number of cells did not significantly increase, although the cells were still growing rapidly (data not shown). Cells were stained against BrdU (Fig. 1B3) to confirm their capacity for proliferation, which demonstrated that all stages of OECs nuclei were positively stained, demonstrating that they possessed proliferative ability. Immunocytochemical staining against p75NGFR (Fig. 1B4) demonstrated that the fusiform cells or their nuclei were positively stained, demonstrating that they were OECs. All cell nuclei were positively stained with DAPI (Fig. 1B5) and the merged image is demonstrated in Fig. 1B6.

Gene chip assay. In order to investigate RNA integrity and genomic DNA contamination, the denaturing agarose gel electrophoresis test was used. The result demonstrated that optical density (OD) A260/A280 ratio was 2.00 and the OD A260/A230 ratio was 2.35. These results confirmed that the RNA was pure [for spectrophotometer, the O.D. A260/A280 ratio should be close to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable) The OD A260/A230 ratio should be >1.8]. The 28S and 18S ribosomal RNA bands were fairly sharp, intense bands and there was a diffuse smear of ethidium bromide-stained material migrating between the 18S and 28S ribosomal bands, possibly comprising mRNA and other heterogeneous RNA species (Fig. 2A). Prior to analysis a boxplot was performed (Fig. 2B). Then, gene chip assay suggested that OECs expressed 9,821 genes (Fig. 2C) and 45 important genes are listed in Table I and Fig. 2D and E.

Discussion

Specificity of the antibodies. Tree shrews possess a number of advantages as animal models, thus, studies on them are popular. However, there are no specific reagents for tree shrews, particularly antibodies. The present study used western blot analysis on protein extracts from whole tree shrew and rat brains to ensure specificity of the antibodies used. Protein extracts from rat brain were used as a positive control, as the specificity of the antibodies has already been verified in this species. The results demonstrated that the antibodies produced bands that were located at the same level. Notably, the molecular weight for p75NGFR was ~75 kDa (28). These data suggested that the antibodies used were specific and the results reliable.

OECs biological characteristics in vitro. The present study was the first to successfully isolate and purify tree shrew OECs, to the best of the authors' knowledge. As in a previous study, the cultured cells exhibited the three classical morphologies of OECs: Bipolar or fusiform, multi-ecphyma shape and oblate (26). Previous studies have demonstrated that p75NGFR was the specific marker of OEC (29,30) and that the antibody to BrdU is useful in detecting S-phase cells, as BrdU-positive cells are proliferating (31,32). The present study demonstrated that the majority of cells were p75NGFR positive and BrdU positive. These data further proved that the

majority of the cultured cells were OECs and the majority of them proliferated well.

Gene chip assay. The microarray technique allows the monitoring of the expression of tens of thousands of genes simultaneously in one hybridization experiment. DNA segments are closely arranged on a slide. cDNA was reverse transcribed from mRNA derived from tree shrew OECs, labelled with a Cyanine3 (Cy3) fluorophore and hybridized on the microarray slide. Through this technique, the detection of differentially expressed genes is greatly facilitated. In the present study, prior to the microarray analysis, RNA quantification and quality assurance was performed to ensure reliability of the results (33-35). Prior to the statistical analysis, a boxplot test was performed. The boxplot is a traditional method for visualizing the distribution of a dataset (36) and is useful for comparing the distributions of several datasets (37). In the present study, a boxplot was used to observe the distributions of expression values for the samples in the experiment following normalization. The data demonstrated that tree shrew OECs can express 9,821 genes, containing 44 cytokines, including the fibroblast growth factors family (38), the insulin-like growth factor family (39-42), the transforming growth factor family (38), epidermal growth factor (43,44), ciliary neurotrophic factor (40), vascular endothelial growth factor (45) and platelet derived growth factor receptor α (46,47), which were hypothesized as possessing the capacity to promote neuronal survival, regeneration, remyelination or function improvement.

In conclusion, tree shrew OECs were isolated and purified successfully for the first time, to the best of the authors' knowledge, and the cells were able to secrete a number of cytokines that may contribute to nervous system diseases. Although there was no specific kit, tests were performed to ensure that the data were reliable. These results may lead to future fundamental research or clinical trials.

Availability of data and materials

All the materials included in the manuscript, including all relevant raw data, will be made freely available to any researchers who wish to use them for non-commercial purposes, while preserving any necessary confidentiality and anonymity.

Authors' contributions

T-HW, NL and H-YW designed the study. T-YW performed cell cultures, X-JD, W-JH, and T-YW performed immunofluorescence, NL and JW performed microarray analysis,

western blotting and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (approval no. 2006-398), and was approved by the ethics committee of Institute of Neuroscience, Kunming Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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