

## Original Article

# Activation of muscarinic receptors inhibits glutamate-induced GSK-3 $\beta$ overactivation in PC12 cells

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**Aim:** To investigate the actions of the muscarinic agonist carbachol on glutamate-induced neurotoxicity in PC12 cells, and the underlying mechanisms.

**Methods:** PC12 cells were treated with different concentrations of glutamate for 24 or 48 h. The cell viability was measured using MTT assay, and the expression and activation of GSK-3 $\beta$  were detected with Western blot.  $\beta$ -Catenin translocation was detected using immunofluorescence. Luciferase reporter assay and real-time PCR were used to analyze the transcriptional activity of  $\beta$ -catenin.

**Results:** Glutamate (1, 3, and 10 mmol/L) induced PC12 cell death in a dose-dependent manner. Moreover, treatment of the cells with glutamate (1 mmol/L) caused significant overactivation of GSK-3 $\beta$  and prevented  $\beta$ -catenin translocation to the nucleus. Pretreatment with carbachol (0.01  $\mu$ mol/L) blocked glutamate-induced cell death and GSK-3 $\beta$  overactivation, and markedly enhanced  $\beta$ -catenin transcriptional activity.

**Conclusion:** Activation of muscarinic receptors exerts neuroprotection in PC12 cells by attenuating glutamate-induced GSK-3 $\beta$  overactivation, suggesting potential benefits of muscarinic agonists for Alzheimer's disease.

**Keywords:** muscarinic receptors; carbachol; neuroprotection; glutamate; excitotoxicity; GSK-3 $\beta$ ;  $\beta$ -catenin; PC12 cell; Alzheimer's disease

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## Introduction

Alzheimer's disease (AD), a neurodegenerative disorder affecting patients worldwide, is associated with a loss of cholinergic neurons, which causes profound memory disturbances<sup>[1]</sup>. AD patients benefit from treatment with agonists of the muscarinic receptor (mAChR), which seem to restore lost cholinergic function<sup>[2,3]</sup>. They also benefit from treatment with neuroprotective agents, which can alter the disease progression<sup>[4]</sup>. One novel therapeutic strategy for AD involves not only increasing cholinergic function but also slowing or stopping the pathological progression of the disease. Recent studies elucidating the mechanism by which muscarinic agonists protect cells from apoptosis induced by a variety of stimuli have suggested that the neuroprotective effects of mAChR activation may do more than address the symptoms of cognitive decline<sup>[5–9]</sup>. Rather, these agonists might affect the disease progression itself. The present study addressed the ability of

these agonists to protect PC12 cells from glutamate-induced cell death and the mechanism underlying this process, as the Ca<sup>2+</sup> overload, oxidative stress and neuronal death associated with glutamate-induced excitotoxicity are thought to be key factors in the pathogenesis of AD<sup>[10–15]</sup>.

Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a serine/threonine kinase abundant in the central nervous system, plays an established role in cell survival<sup>[16,17]</sup>. Several apoptotic stimuli appear to be involved in GSK-3 $\beta$  activation<sup>[18]</sup>. Aberrant overactivation of GSK-3 $\beta$  induces neuronal death<sup>[19–23]</sup>. Muscarinic agonists reduce tau hyperphosphorylation and counteract A $\beta$ -toxicity, the hallmarks of AD, by inhibiting GSK-3 $\beta$ <sup>[7,24]</sup>. This effect suggests that overactivation of GSK-3 $\beta$  could be attenuated through muscarinic signaling. The present work investigates whether overexpression or overactivation of GSK-3 $\beta$  plays a role in glutamate-mediated excitotoxicity and whether the GSK-3 $\beta$ / $\beta$ -catenin signaling pathway plays a role in the protective effect of mAChR activation against glutamate-induced neurotoxicity.

Inhibition of GSK-3 $\beta$  activity and activation of mAChR are both therapeutic methods against AD<sup>[25–27]</sup>. This study may

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reveal a new mechanism by which muscarinic agonist treatment provides therapeutic benefits to AD patients; the agents counteract glutamate-induced neurotoxicity by inhibiting the GSK-3 $\beta$ / $\beta$ -catenin signaling pathway.

## Materials and methods

### Chemicals

RPMI-1640 medium was purchased from Gibco (New York, USA). Carbachol, MTT, and *L*-glutamic acid monosodium were purchased from Sigma (St Louis, MO, USA).

### Cell culture and treatment

PC12 cells were obtained from the Cell Bank of China (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. One day before the experiment, cells were seeded in 96-well (1.0 $\times$ 10<sup>4</sup> cells/well), 24-well (4.0 $\times$ 10<sup>4</sup> cells/well), and 6-well (2.0 $\times$ 10<sup>5</sup> cells/well) plates.

### Cell viability assay

PC12 cells were plated in 96-well culture plates at 1.0 $\times$ 10<sup>4</sup> cells/well. After treatment, cell viability assays were performed using the MTT method. MTT in PBS was added to the cultures at a final concentration of 0.5 mg/mL. After further incubation at 37 °C for 4 h, the medium was carefully removed and formazan crystals were dissolved in 150  $\mu$ L DMSO per well. Absorbance at 490 nm was measured with a plate reader (Bio-tek, VT, USA).

### Immunoblots

In the current work, immunoblots were performed for various purposes and were adjusted for different protein samples. Cells were washed with PBS and lysed with a lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 4  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL aprotinin, and 100  $\mu$ g/mL PMSF). Proteins were loaded onto 10% or 12% SDS-PAGE gels, transferred to nitrocellulose membranes, detected using the proper primary and secondary antibodies, and visualized using chemiluminescence (Pierce Biotechnology, USA). Various primary antibodies were used, including anti-phospho-Ser9-GSK3 $\beta$  (1:500, KeyGEN, China), anti-total-GSK3 $\beta$  (1:500, KeyGEN, China), anti- $\beta$ -catenin (1:1000, Abcam, UK), and anti- $\beta$ -actin (1:5000, Abcam, UK).

### Immunofluorescence

After treatment, PC12 cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Endogenous cellular  $\beta$ -catenin was detected with polyclonal anti- $\beta$ -catenin (1:1000) and fluorescent isothiocyanate (FITC)-conjugated anti-rabbit antibody (1:100, DAKO A/S, Denmark). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were examined under an Axioplan 2 fluorescent microscope (Zeiss, Germany).

### Luciferase reporter assays

PC12 cells were cultured on a 10-cm dish and treated with a DNA mixture consisting of 8  $\mu$ g of the appropriate TOP-luciferase and 800 ng of the effector/control plasmids per well using Lipofectamine 2000 (Invitrogen, CA, USA). Cells were incubated with DNA/lipofectamine complexes for 5 h, then maintained in media with 10% FBS for 16 h and seeded in 24-well plates for post-treatment with 1 mmol/L glutamate. The luciferase (LUC) activity was assayed with the dual-firefly/Renilla luciferase reporter system (Promega, WI, USA) according to the manufacturer's instructions. Relative light units were measured in a Fentomaster FB12 chemiluminometer (Berthold, Pforzheim, Germany).

### RT-PCR

Total RNA was extracted from 0.8 $\times$ 10<sup>6</sup> cells using TRIzol reagent (Gibco, CA, USA) according to the manufacturer's instructions. RNA concentration was measured by spectrophotometric measurement at 260 nm. cDNA was synthesized using random 9-mer primers and AMV reverse transcriptase (Takara, Shiga, Japan).

### Plasmid constructs

The cDNA constructs for rat  $\beta$ -catenin were generated by PCR using the following primers containing *Sal* I and *Bam*H I restriction sites:  $\beta$ -catenin-*Sal* I-forward: 5'-GCGTCCGACACAATGGCTACTCAAG-3';  $\beta$ -catenin-*Bam*H I-reverse: 5'-CGCGGATCCCTTACAGGTCCGGTATC-3'. The PCR-amplified fragments were cut with *Sal* I/*Bam*H I and cloned into the pEGFP-C1 plasmid (Clontech, CA, USA).

### Real-time PCR

Up to 1  $\mu$ L template cDNA was used. Thermal cycling conditions were 95 °C for 60 s, followed by 45 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 35 s. Primer efficiency was >90%, which was confirmed with a standard curve spanning four orders of magnitude. After the reactions, the data were exported using 7300 System Software v1.3.0 (Applied Biosystems, CA, USA) and analyzed. The following primers were used (Table 1).

### Statistical analysis

The data are presented as the mean $\pm$ SD. Statistical signifi-

**Table 1.** Primers for real-time PCR.

Gene	Sequences
Cyclin D1	5'-TGCTGCAAATGGAAGTCTCTGG-3' 5'-AAGGTCTGTGCATGTTTGC GGATG-3'
Engrailed-1	5'-AGAAGGATCCAAGTCCAGCGAGA-3' 5'-TGAAGAAGTTGGTGATGCGATGCG-3'
Connexin43	5'-TCATCTTCATGCTGGTGGTGTCT-3' 5'-TGGTGAGGAGCAGCCATTGAAGTA-3'
$\beta$ -Actin	5'-TTGCTGATCCACATCTGCT-3' 5'-GACAGGATGCAGAAGGAGAT-3'

cance of differences between groups was assessed by using a Student-Newman-Keuls test, where  $P < 0.05$  indicated a significant difference.

## Results

### Glutamate-induced neurotoxicity was accompanied by GSK-3 $\beta$ overactivation and decreased $\beta$ -catenin nuclear translocation in PC12 cells

Glutamate treatment at 1, 3, or 10 mmol/L for 24 or 48 h induced excitotoxicity in PC12 cells. The viability of PC12 cells treated with glutamate significantly decreased in a time- and dose-dependent manner relative to the control group. The cellular survival rates after 48 h treatment with 1, 3, and 10 mmol/L glutamate were  $82.9 \pm 2.4\%$ ,  $41.1 \pm 3.9\%$ , and  $23.2 \pm 1.1\%$ , respectively (Figure 1A). We next assayed for GSK-3 $\beta$  phosphorylation in PC12 cells by immunoblotting with antibodies against phosphorylated GSK-3 $\beta$  (Ser9). After treating PC12 cells with 1, 3, or 10 mmol/L glutamate for 8 h, GSK-3 $\beta$  was overactivated, as indicated by decreased phosphorylation levels (Figure 1B). These results are consistent with previous reports demonstrating that GSK-3 $\beta$  could be negatively regulated by phosphorylation at Ser9<sup>[28, 29]</sup>. However, phosphorylation levels at Ser9 did not change after 24 or 48 h glutamate treatment (data not shown).

GSK-3 $\beta$  participates in the Wnt pathway, which regulates  $\beta$ -catenin stability. The present work evaluated the intracel-

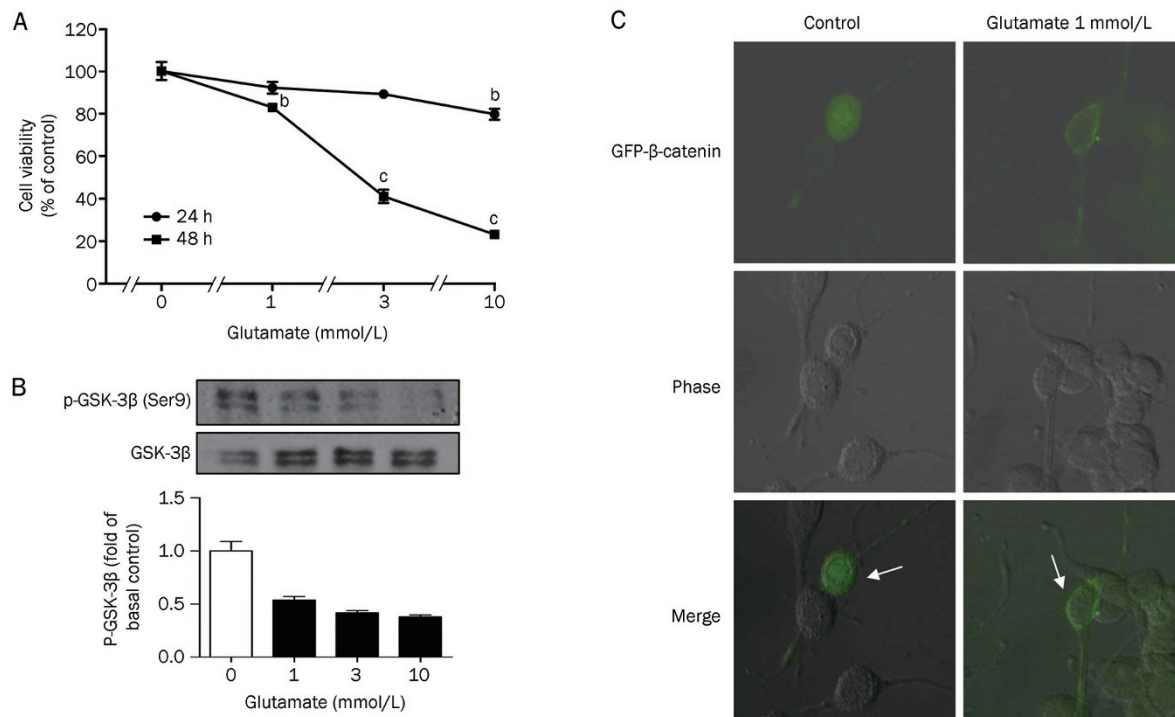
lular distribution of  $\beta$ -catenin in PC12 cells during glutamate treatment.  $\beta$ -catenin nuclear localization was detected in transiently transfected PC12 cells using GFP- $\beta$ -catenin. Cells incubated with 1 mmol/L glutamate displayed less  $\beta$ -catenin nuclear localization (Figure 1C).

### mAChR activation protected PC12 cells from glutamate-induced cell death and GSK-3 $\beta$ overactivation

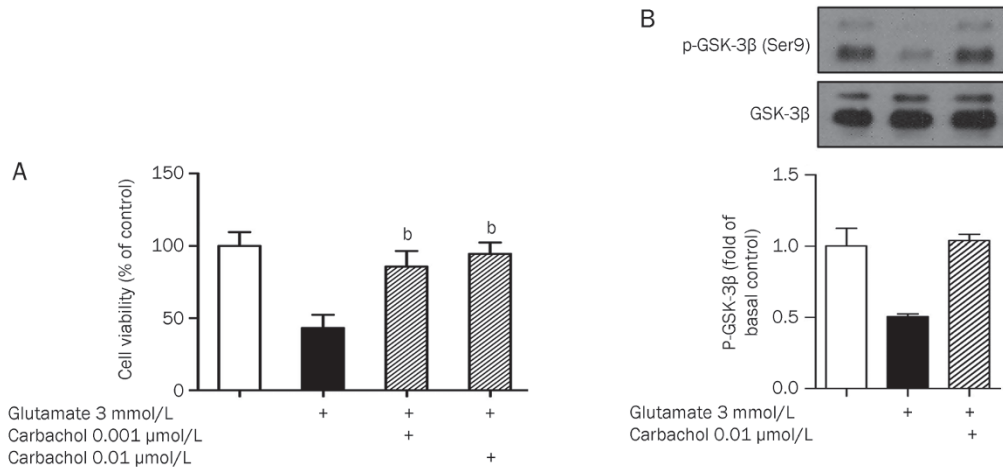
PC12 cells were pretreated with 0.001  $\mu$ mol/L or 0.01  $\mu$ mol/L muscarinic agonist carbachol for 24 h, followed by 3 mmol/L glutamate treatment for 48 h. The 0.001  $\mu$ mol/L or 0.01  $\mu$ mol/L carbachol pretreatment increased cell viability by  $85.71 \pm 4.9\%$  and  $94.55 \pm 3.7\%$ , respectively (Figure 2A). As 0.01  $\mu$ mol/L carbachol elicited a more effective response, this concentration was used in subsequent cell signal transduction experiments. Carbachol treatment (0.01  $\mu$ mol/L) diminished glutamate-evoked Ser9 GSK-3 $\beta$  phosphorylation deregulation (Figure 2B).

### mAChR activation reversed glutamate repression of $\beta$ -catenin nuclear translocation in PC12 cells

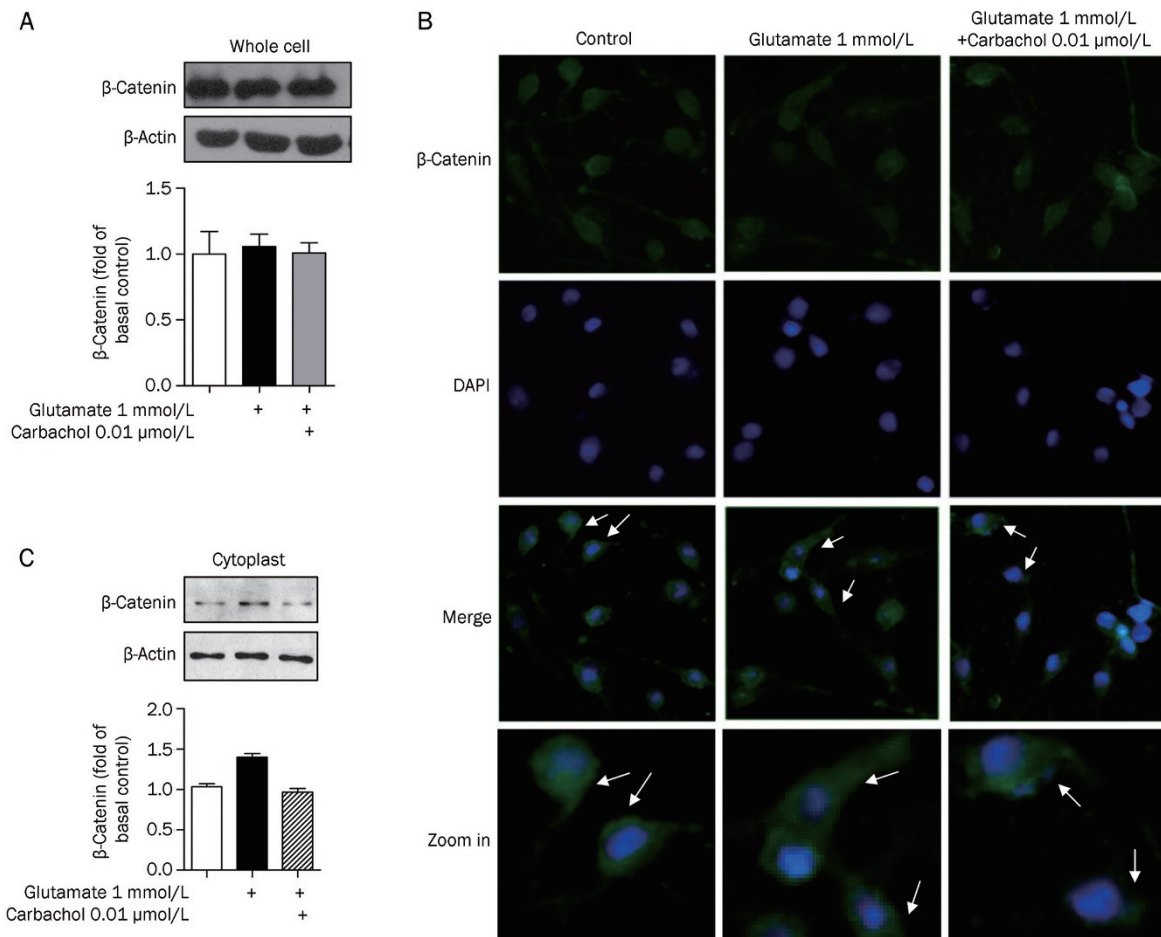
There was no change in  $\beta$ -catenin protein levels as detected by immunoblotting (Figure 3A). Immunofluorescence analysis with antibodies specific to  $\beta$ -catenin and the nuclear marker DAPI revealed less nuclear  $\beta$ -catenin in cells treated with 1 mmol/L glutamate than in untreated cells. Pretreatment



**Figure 1.** Glutamate-induced PC12 cells death was accompanied with GSK-3 $\beta$  overactivation. (A) PC12 cells were incubated with 1, 3, and 10 mmol/L glutamate for 24 or 48 h. Data are reported as the percentage of cells compared with control group (100%), and represent mean  $\pm$  SD of three independent experiments performed with 5 replications per group. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs control group. (B) After 8 h treatment with glutamate (1, 3, and 10 mmol/L) on PC12 cells, the activated GSK-3 $\beta$  [p-GSK-3 $\beta$  (Ser9)] and total GSK-3 $\beta$  was determined by Western blotting. (C) PC12 cells were transfected with GFP- $\beta$ -catenin. Cells were incubated with or without 1 mmol/L glutamate 24 h after transfection.



**Figure 2.** Activation of mAChR protected PC12 cells from glutamate-induced cell death and GSK-3β overactivation. (A) After pre-treatment with 0.001 μmol/L, 0.01 μmol/L carbachol for 24 h, cultures were exposed to 3 mmol/L glutamate for 48 h and the cell viability was analyzed by MTT. Data are reported as the percentage of cells compared with control group (100%), and represent the mean±SD of three independent experiments performed with 5 replications per group. <sup>b</sup>P<0.05 vs injury group. (B) Pre-treatment of PC12 cells with 0.01 μmol/L carbachol for 24 h, after 8 h treatment of 3 mmol/L glutamate, the up-regulated GSK-3β activation can be reduced.



**Figure 3.** Carbachol rescued the loss of nuclear β-catenin induced by glutamate. PC12 cells were exposed to 1 mmol/L glutamate with or without pre-treatment of 0.01 μmol/L carbachol for 24 h. (A) Corresponding to the prior treatment, total amount of β-catenin in PC12 cells was detected by Western blotting. (B) Immunofluorescence analysis showed the β-catenin (FITC, green) and nuclei (DAPI, blue). (C) The amount of β-catenin in the cytoplasm was detected by Western blotting.

with 0.01  $\mu\text{mol/L}$  carbachol increased nuclear localization of  $\beta$ -catenin compared to 1 mmol/L glutamate treatment alone (Figure 3B). Cytoplasm was extracted using a nuclear and cytoplasmic protein extraction kit and probed for  $\beta$ -catenin protein levels. Consistent with the decreased nuclear localization detected by immunofluorescence, higher levels of  $\beta$ -catenin were found in the cytoplasm after 1 mmol/L glutamate treatment by immunoblot. Carbachol attenuated this effect (Figure 3C).

#### Carbachol alleviated glutamate inhibition of $\beta$ -catenin transcriptional activity in PC12 cells

After accumulating and stabilizing in the cytoplasm,  $\beta$ -catenin translocates to the nucleus where it interacts with Tcf/LEF transcription factors, inducing the expression of Wnt target genes. These are important events in neural development and maintenance<sup>[30]</sup>.

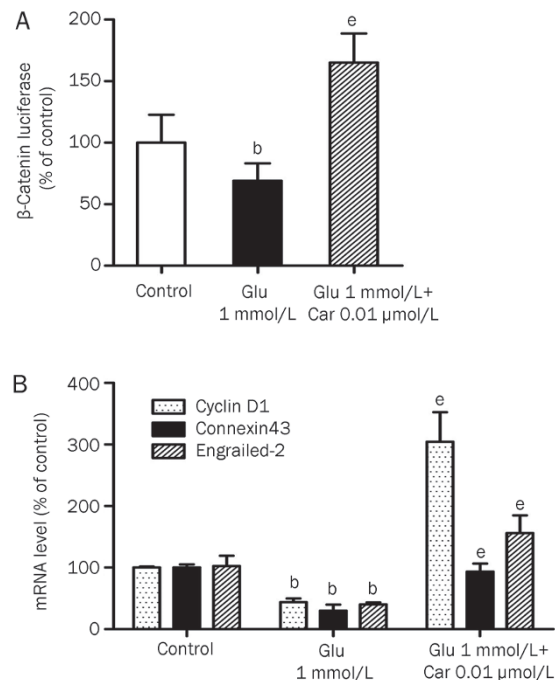
Glutamate inhibited  $\beta$ -catenin translocation to the nucleus.  $\beta$ -Catenin transcriptional activity was evaluated with a luciferase assay and by measuring mRNA levels of Wnt target genes after 1 mmol/L glutamate treatment for 8 h. PC12 cells were transfected using TOP-FLASH, which drives high levels of luciferase activity in response to Wnt signaling activity. Glutamate (1 mmol/L) treatment for 8 h decreased  $\beta$ -catenin transcriptional activity to 69.02% of control levels (Figure 4A). Exposure to glutamate reduced engrailed-2, connexin43, and cyclin D1 mRNA levels to 40.17%, 28.81%, and 43.60% of control levels, respectively. Pretreatment with 0.01  $\mu\text{mol/L}$  carbachol increased transcriptional activity to 165.10% of control levels and increased engrailed-2, connexin43, and cyclin D1 mRNA levels to 304.56%, 93.36% and 156.23% control levels, respectively (Figure 4B).

#### Discussion

The hypothesis of glutamate excitotoxicity argues that excessive glutamate causes neuronal dysfunction and degeneration<sup>[14]</sup>. Understanding the mechanism underlying the excitotoxic process may lead to a better understanding of neurodegenerative disorders. The GSK-3 $\beta$ / $\beta$ -catenin pathway has recently been shown to modulate many fundamental cell processes, and GSK-3 $\beta$  itself is involved in the pathology of AD<sup>[31, 32]</sup>.

Among cell lines of neuronal origin, PC12 cells are selectively affected by the cytotoxic effect of glutamate<sup>[33]</sup>. The present work utilized a well-established experimental method where PC12 cells are treated with glutamate. Glutamate-induced neurotoxicity was accompanied by GSK-3 $\beta$  overactivation, which was inhibited by mAChR.

Previous studies demonstrated that glutamate can decrease GSK-3 $\beta$  activity in hippocampal slices, which may contribute to neurotoxicity in the hippocampus<sup>[34]</sup>. The present work established that glutamate causes PC12 cell death in a dose-dependent manner, accompanied by GSK-3 $\beta$  overactivation. Unlike most kinases, GSK-3 $\beta$  is constitutively active in cells. Activity can be dynamically inhibited by phosphorylation at Ser9 and stimulated by phosphorylation at Tyr216<sup>[35]</sup>. Unlike



**Figure 4.** Effects of glutamate and pre-treatment of carbachol on  $\beta$ -catenin transcriptional activity and Wnt target gene expression in PC12 cells. PC12 cells were exposed to 1 mmol/L glutamate with or without pre-treatments of 0.01  $\mu\text{mol/L}$  carbachol for 24 h. (A) TOP-FLASH was transfected into PC12 cells and the cells were treated as described above. Luciferase activity was determined after 8 h. Relative changes in expression are shown compared with the levels of TOP-FLASH in untreated cells. (B) After 8 h, PC12 cells were harvested and RNA was prepared. mRNA levels for cyclin D1, connexin43, engrailed-2 were evaluated by real-time PCR analysis. Data represent mean $\pm$ SD of three independent experiments. <sup>b</sup> $P < 0.05$  vs control group. <sup>e</sup> $P < 0.05$  vs injury group.

previous publications, which measured phosphorylation at Tyr216, the present work evaluated phosphorylation at Ser9. Ser9 phosphorylation renders GSK-3 $\beta$  inactive and thus is implicated in neuronal survival<sup>[21]</sup>. Glutamate decreased GSK-3 $\beta$  phosphorylation at Ser9, indicating that GSK-3 $\beta$  is overactivated during glutamate-induced neurotoxicity. Consistent with these findings, glutamate led to GSK-3 $\beta$  activation, resulting in reduced accumulation of nuclear  $\beta$ -catenin and reduced enhancement of transcriptional activity.

Next, we demonstrated that the muscarinic agonist carbachol protects PC12 cells from glutamate-induced cytotoxicity. mAChR is primarily responsible for these neuroprotective effects, as these effects can be antagonized by an mAChR or M1 mAChR antagonist. This finding is consistent with the view that activation of  $G_{q/11}$ -coupled mAChR can protect cells against apoptosis induced by variety of stimuli<sup>[8]</sup>. This finding is also consistent with recent reports stating that mAChR activation has neuroprotective effects in attenuating the progression of AD<sup>[5]</sup>. More importantly, the present work established that the neuroprotective effect of carbachol occurs through the inhibition of GSK-3 $\beta$  during glutamate treatment. These find-

ings are highly interesting in light of the well-established link between cell survival and GSK-3 $\beta$ : while apoptotic processes involve GSK-3 $\beta$  activation, anti-apoptotic signaling includes GSK-3 $\beta$  inhibition<sup>[21, 36, 37]</sup>.

Inactivation of GSK-3 $\beta$  by phosphorylation at Ser9 can increase the stabilization of  $\beta$ -catenin, which promotes its nuclear translocation<sup>[38]</sup>. Augmented  $\beta$ -catenin signaling is an anti-apoptotic stimulus<sup>[39]</sup>. To further examine the finding that mAChR activation can inhibit glutamate-induced GSK-3 $\beta$  overactivation, we assayed the stability and localization of  $\beta$ -catenin. After treatment with 1 mmol/L glutamate, the total quantity of  $\beta$ -catenin did not change, but less  $\beta$ -catenin localized to the nuclei, which decreased its transcriptional activity. Carbachol reversed these effects of glutamate.

In summary, AD is a complex disorder of the central nervous system. Different etiological hypotheses on the origins of AD are based on different histopathological lesions found in the brains of AD patients. mAChR has long been a high-profile target for AD treatment because its manipulation may restore cholinergic hypofunction. Small-molecule inhibitors of GSK-3 $\beta$  have also attracted significant attention because GSK-3 $\beta$  is a key signaling molecule in the etiology of AD<sup>[40, 41]</sup>. Our study provides evidence that GSK-3 $\beta$  may be directly involved in pathways leading to glutamate-induced cell death and that mAChR activation exerts a neuroprotective effect by inhibiting GSK-3 $\beta$  overactivation. These results provide a new explanation for the benefits experienced by AD patients treated with mAChR activation – mAChR activation inhibits the overactivation of the GSK-3 $\beta$  signaling pathway.

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### Author contribution

Ke MA performed research and wrote the paper; Li-min YANG contributed new reagents; Hong-zhuan CHEN contributed to the experimental design; Yang LU is the corresponding author.

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