# The Expression and Regulation of GSK-3β, CDK-5 and PP2A in Differentiated Neural Stem Cells of Rats

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【**Abstract**】 **Objective:** To explore the expression of GSK-3β, CDK-5 and PP2A and the regulation of them by $A\beta_{25\sim35}$ and ginsenoside Rb1 after NSCs are transformed into neurons. **Methods** Neural stem cells of the third passage were induced towards neurons; the expressions of GSK-3β (pTyr279, 216), PP2A and the regulation of them by $A\beta_{25\sim35}$ and ginsenoside Rb1 were tested by the immunofluorescence cytochemical staining after NSCs had been induced for one week; The expressions of GSK-3β, CDK-5, PP2A and the regulation of them by $A\beta_{25\sim35}$ and ginsenoside Rb1 were detected with RT-PCR assays. **Results:** Immunofluorescence cytochemisty showed that neural cells differentiated from NSCs can express GSK-3β (pTyr279, 216) and PP2A. $A\beta_{25\sim35}$ can enhance the expression of GSK-3β (pTyr279, 216), meanwhile it also inhibited the expression of PP2A. Moreover ginsenoside Rb1 can alleviate the affect of $A\beta_{25\sim35}$. RT-PCR results showed that neural stem cells differentiated from NSCs can express GSK-3β, CDK-5, PP2A. The expression of GSK-3β and CDK-5 rose up and the expression of PP2A weakened when they were treated by $A\beta_{25\sim35}$. However, the effect of $A\beta_{25\sim35}$ was restrained when they were pretreated by ginsenoside Rb1. **Conclusions** NSCs which were cultured and induced in vitro can express GSK-3β, CDK-5 and PP2A; Additionly $A\beta_{25\sim35}$ and ginsenoside Rb1 can regulate the expressions of GSK-3β, CDK-5 and PP2A. Our results suggest that cells which differentiated from neural stem cells in vitro have protein phosphorylation regulation system of normal cells.

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**Key words:** neural stem cells; GSK-3β; CDK-5; PP2A; ginsenoside Rb1; $A\beta_{25\sim35}$

## Introduction

Neural stem cells (NSCs) are cells that have self-renewal ability and multidifferentiation potential. NSCs are transformed into neurons as its immature forms changed to mature forms, which is coincident with the change of the tau protein's location and expression. Tau protein is an important microtubule-associated protein, and localizes primarily in the axon of neurons. Its major role is to promote the formation and increase the stability of microtubules, therefore it plays an important part in maintenance of the morphology and function of cells. The normal tau protein is a phosphoprotein, and it can not only influence the formation of microtubules when it is hyperphosphorylated, but also can cause various kinds of depositions to localize in the neurons, which will cause damage of the neurons and diseases of the nervous system[1].

Tau protein's phosphorylation is regulated by the relative activity of protein kinases (which catalyze hyperphosphorylation) and protein phosphatases (which catalyze dephosphorylation) [2-3]. Glycogen synthakinase-3β (GSK-3β) and cyclin- dependent kinase-5 (CDK-5) belong to Ser/Thr kinase, which can promote tau protein hyperphosphorylation in vivo and vitro and play a very important role in the regulation of tau phosphorylation[1]. The decreasing activity of protein phosphatases, especially the activity of protein phosphatase-2A (PP2A), also plays a key part in the regulation of tau phosphorylation [4-5]

It has been proved that the mature neurons in the brain can express GSK-3β、CDK-5 and PP2A . $A\beta_{25\sim35}$ and ginsenoside Rb1 can also regulate the expression of GSK-3β[6], CDK-5[7] and PP2A. However, there is still no evidence of the neurons which are differentiated from NSCs. Thus, in this study we intend to investigate the expression of GSK-3β、CDK-5 、PP2A after NSCs are transformed into neurons and the regulation by $A\beta_{25\sim35}$ and ginsenoside Rb1

## 1. Materials and methods

### 1.1 Main reagents

$A\beta_{25\sim35}$ (sigma); GSK-3β (pTry279, 216) antibody (sigma); ginsenoside Rb1 (Chinese Medicine Identification Institute); Rabbit Anti –PP2A antibody (Beijing Zhongshan); DMEM/F12 culture medium (GIBCO); Fetal bovine serum (Hangzhou Chinese Holly Bioengineering Material Research Institute); gelose (Sigma); Marker (TaKaRa Biotechnology); AMV First Strand cDNA Synthesis Kit and PCR Kit (Shanghai Bioengineering Company).

**1.2 Primer design and synthesis**

RT-PCR primer was designed by corresponding software: primer of GSK-3β (Forward: 5'AACACCAACAAGGGAGCAAA 3'; Reverse: 5'GAGCGTGAGGAGGGATAA GG3'), primer of CDK-5 (Forward: 5'ATTGGGGAAGGCACCTACGG 3'; Reverse: 5'TCCAGGTCACCATTGCAGCT 3'), primer of PP2A (Forward: 5'AGGTGGGAG AGTCGTCATCT3'; Reverse: 5' GTGGTAGGTATGGGCGTTGG 3') and primer of β-actin for intra-contrast (Forwar: 5'GAGCTGCGTGTGGCCC CT AG3'; Reverse: 5'AGTTTCATGGATGCCACAGG3'). All primers were synthesized by Shanghai Bioengineering Company.

**Table 1. Sequences of primers**

| Gene | GeneBankNo. | Primer sequence | Product size |
|---|---|---|---|
| *CDK-5* | NM－080885 | Forward:5'ATTGGGGAAGGCACCTACGG 3'<br>Reverse:5'TCCAGGTCACCATTGCAGCT 3' | 249bp |
| *GSK-3β* | NM－032080 | Forward:5'AACACCAACAAGGGAGCAAA 3'<br>Reverse:5'GAGCGTGAGGAGGGATAAGG 3' | 326bp |
| *PP2A* | M83297 | Forward:5'AGGTGGGAGAGTCGTCATCT 3'<br>Reverse:5'GTGGTAGGTATGGGCGTTGG 3' | 455bp |
| *β-actin* | AY039651 | Forward:5'GAGCTGCGTGTGGCCCCTGAG3'<br>Reverse:5'AGTTTCATGGATGCCACAGG3' | 554bp |

**1.3 Source of animals**

The newly born 24h old SD rats were afforded by Animal Center of Zhengzhou University.

**1.4 Isolation, culture and differentiation of NSCs**

NSCs of 24h old SD rats were isolated from the dentate gyrus of the hippocampus and cultured with DMEM/F12 culture medium containing 2%B27, bFGF(20 ng/ml) and EGF (20 ng/ml) at 37℃ and 5% $CO_2$. The cultured cells were replaced with fresh medium every three days. NSCs began to accumulate and formed neurospheres after one week. We treated NSCs to the next passage by centrifuging the cell's suspension for 5min (1000 rpm). The third passage NSCs were induced into neurons by adding 10% fetal bovine serum and removing mitogens. Undifferentiated neural stem cells, neurons and astrocytes were identified separately with Nestin, NSE and GFAP antibodies by using immunocytochemistry.

**1.5 The experimental groups**

The cells were divided into 3 experimental groups after NSCs had been induced for one week.

(1) The control group: the cells were cultured for another 36h without additional treatment.

(2) $A\beta_{25\sim35}$ treatment group: the cells were cultured for another 24h and added $A\beta_{25\sim35}$ (20 μmol/L) for 12h.

(3) Ginsenoside Rb1 pre-treatment group: the cells were pre-treated with ginsenoside Rb1 (10 μmol/L) for 24h and added $A\beta_{25\sim35}$ (20 μmol/L) for 12h.

After total 36h, each group of cells were collected.

**1.6 Immunofluorescence cytochemistry**

Each group of cells were collected from 6-well plates.The operational procedure according to the instruction of reagent kit. The morphological changes of cells were observed under fluorescence microscope.

**1.7 RT-PCR**

Abstraction of RNA and synthesis of cDNA were processed according to the instruction of reagent kit. Amplification by PCR was carried on after synthesis of cDNA : Put the following materials into Ependoff tubes in turn: 2mmol/L dNTP 2 μl, Taq enzyme (5U/ μl) 1 μl, cDNA 5 μl, specific primer 1,2 each of 0.5 μl, β-actin 0.5 μl, 10×PCR buffer 3 μl, ddH2O 17 μl. Conditions of amplification for PCR: 94℃ for 3min, 94℃ for 45s, 55℃ for 45s,72℃ for 1min, 35 cycles later, extending at 72℃ for 7min. 5 μl amplification product mixed with buffer solution was put into 1.8% gelose gel which contained EB, after electrophoresing by voltage 5-10V/cm for 50minutes, the results were observed by gel scan imaging system.

**1.8 Statistics analysis**

Results expression by $\overline{X} \pm S$, analysis data by ANOVA. Comparison between groups by LSD, $p$ <0.05 is significant.

**2. Results**

**2.1 Isolation, *ex vivo* culture and differentiation of NSCs**

Freshly isolated single NSCs from the dentate gyrus of newborn rats hippocampus were small, round and contained much opaque particles. After 3 passages, the neurospheres grew significantly bigger than before and the particles mostly disappeared. The specific markers of the NSCs(Nestin) were expressed on primary culture and the differentiated cells. After 3

days induction, most floating neurospheres began to adhere to the bottom of the bottle and grew outwards like in the shape of a thorn. After 7 days induction, most cells of the neurospheres grew outwards and formed axons which were interlaced with each other. Immunocytochemistry found that the differentiated cells showed the specific markers of neurons (NSE) and astrocytes (GFAP).

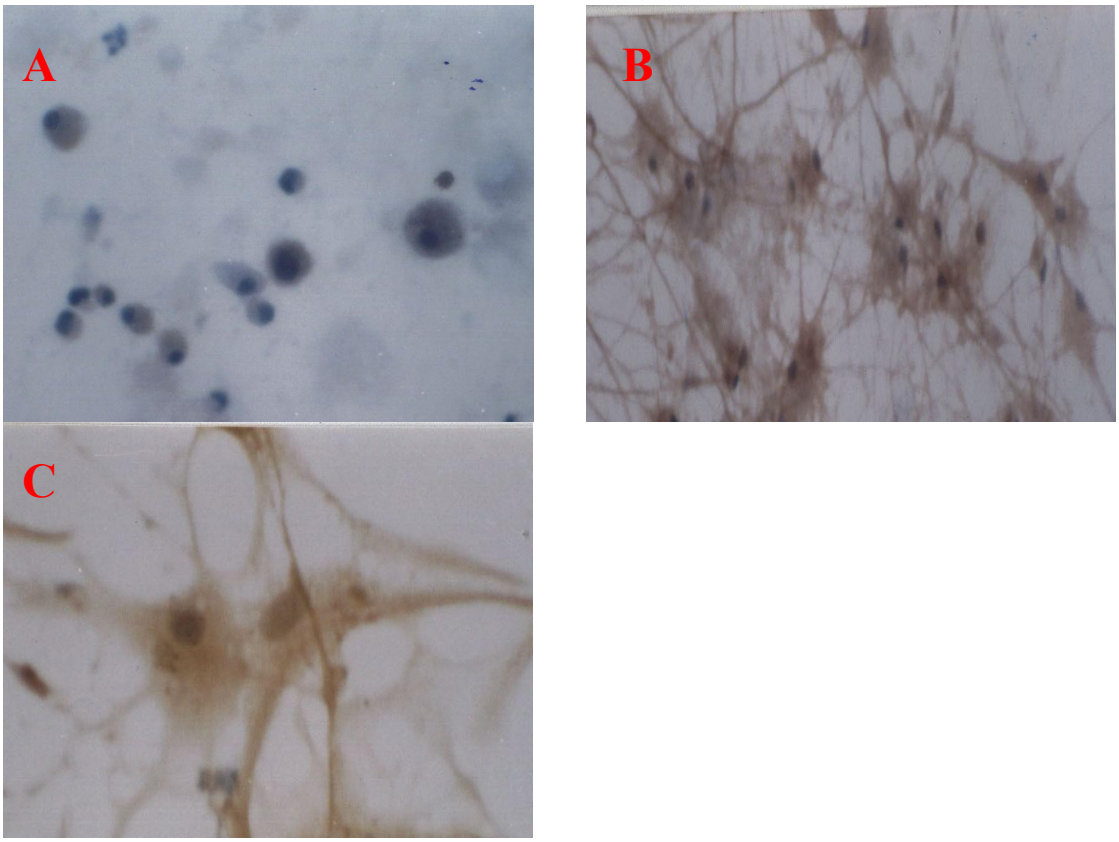


Figure 1. Immunocytochemistry results of NSCs and the cells differentiated form NSCs
A: NSCs have expression of Nestin (×100)
B: The cells differentiated form NSCs have expression of NSE (×100)
C: The cells differentiated form NSCs have expression of GFAP (×200)

**2.2 Immunofluorescence cytochemistry**

Compared with control group, the expressions of PP2A were all reduced in the $A\beta_{25\sim35}$ and the ginsenoside Rb1 treatment groups by immunofluorescence cytochemisty assays ,especially in the $A\beta_{25\sim35}$ group (Figure 2 A, B, C). However, the expressions of GSK-3β (pTyr279,216) in the $A\beta_{25\sim35}$ and the ginsenoside Rb1 treatment groups were all increased compare with which in the control group, especially in the $A\beta_{25\sim35}$ group (Figure 2 D, E, F).

**2.3 Results of gene expression of GSK-3β、CDK-5 and PP2A**

The cells of the control group expressed GSK-3β、CDK-5 and PP2A. The expressions of GSK-3β and CDK-5 in the $A\beta_{25\sim35}$ and the ginsenoside Rb1 groups were more than those in the control group. Moreover, the expressions of GSK-3β and CDK-5 were the most in the $A\beta_{25\sim35}$ treatment group. The difference was considered significantly ($p<0.01$); However, the expression of PP2A was lower in the $A\beta_{25\sim35}$ and the ginsenoside Rb1 teatment groups than the control group, the expression of PP2A were the lowest in the $A\beta_{25\sim35}$ group. The difference was considered significantly ($p<0.01$).

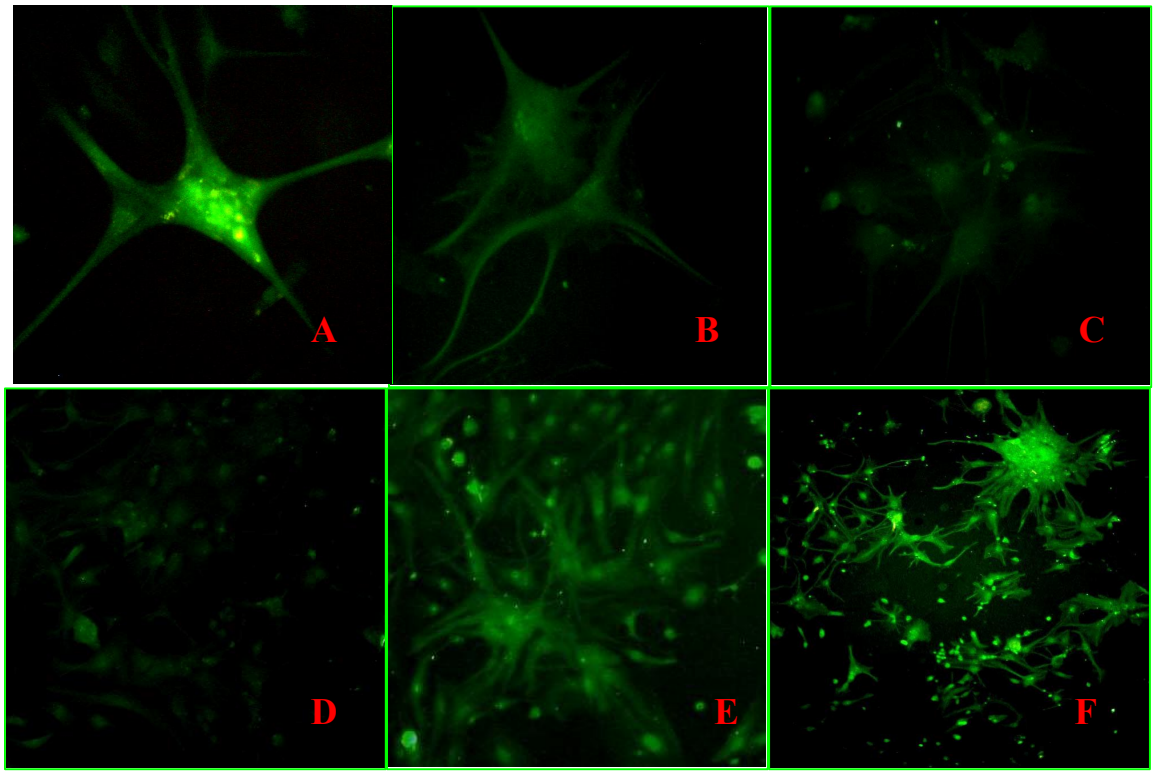


Figure 2. Immunofluorescence cytochemical staining after NSCs had been induced for one week.
A, B, C: The cells were stained with GSK-3β (pTry279,216) (×100)
D, E, F: The cells were stained with PP2A (×400)
A and D: Control group; B and E: Ginsenoside Rb1group; C and F: $A\beta_{25\sim35}$group

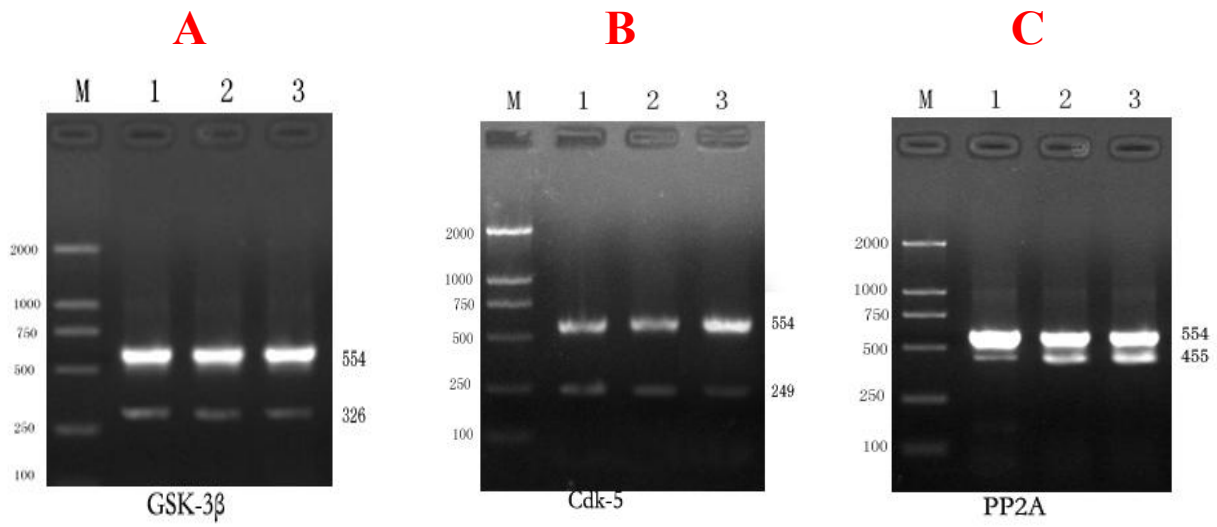


Figure 3. Expression of mRNA after NSCs had been differentiated for one week.
A: Expression of GSK-3β; B: Expression of CDK-5; C: Expression of PP2A .
M: Marker;1: $A\beta_{25\sim35}$group2: Ginsenoside Rb1group; 3: Control group

**Table 2. The expression of GSK-3β/CDK-5/PP2A mRNA after NSCs had been differentiated for one week** ($\bar{X} \pm S$, n=5)

| group | GSK-3β | CDK-5 | PP2A |
|---|---|---|---|
| Control | 0.074±0.011 | 0.084±0.011 | 0.714±0.087 |
| $A\beta_{25\sim35}$ | 0.246±0.011 | 0.324±0.011 | 0.116±0.011 |
| Ginsenoside Rb1 | 0.128±0.008 | 0.208±0.008 | 0.408±0.072 |

The difference of compare with each other is significant, $p$ <0.01.

## 3. Discussion

Since a long time ago it has been argued that the functional status of the cells differentiated from neural stem cells(NSCs) in rats. Our laboratory has already proved that the neural cells differentiated from NSCs in rats have been found $K^+$ current by using patch clamp technique (PCT). But it is various to identify the function of neural cells. In this investigation, the neural cells which were differentiated from NSCs in rats expressed GSK-3β, CDK-5 and PP2A by using immunofluorescence cytochemisty and RT-PCR, that is, these neural cells had regulation system of tau protein phosphorylation as same as the mature neural cells in rat's brain. So this experiment identified function of the neural cells which were differentiated from NSCs in another way.

Neuropathologically, Alzheimer's disease (AD) is now defined by the accumulation of two types of insoluble fibrous material---extracellular amyloid protein in the form of senile plaques and intracellular neurofibrillary lesions (NFL) which were made by abnormal and hyperphosphorylated tau protein. In addition to the neurofibrillary tangles (NFTs) [8].The NFL consists neuropil threads and dystrophic neurites which were associated with senile plaques. Although AD and its main brain histopathology, that is, senile plaques and neurofibrillary tangles (NFTs), were described a century ago, The discoveries of the major protein components of senile plaques as amyloid β-peptide and of NFTs as abnormally hyperphosphorylated tau in the 1980s initiated a new era of AD research. Recent studies demonstrate that it is the abnormal hyperphosphorylation that makes tau protein lose its normal function to stimulate microtubule assembly, gain toxic activity, and aggregate into NFTs [9], upregulation of tau phosphorylation could lead to neurofibrillary degeneration [10].

To understand the mechanism leading to abnormal tau hyperphosphorylation in AD, protein kinases and phosphatases which regulate tau phosphorylation level must be identified firstly.

The kinases that most likely play a role in tau phosphorylation in the brain include glycogen synthase kanase-3β (GSK-3β), cyclin-dependent kinase 5 (CDK-5) [11]. The activity of GSK-3β is regulated by Ser and tyrosine phosphorylation. Tyr279, 216 phosphorylation may increase the activity of GSK-3β, however, Ser 9 phosphorylation will down-regulate the activity of GSK-3β[12-13]. Our results showed that in $A\beta_{25\sim35}$ treatment group the expression of GSK-3β mRNA and CDK-5 mRNA, GSK-3β (pTyr279, 216) all raised up, but in GinsenosideRb1 pre-treatment group they were restrained. This result is coincidence with that of in AD brain GSK-3β showed tendency of up-regulation..

Among protein phosphatases, PP2A has been shown to be the major tau phosphatase in the brain[14-17] . It has been reported the okadaicacid-induced inhibition of PP2A activity and prevents tau hyperphosphorylation in hippocampal slice cultures from adult rats[18]. Our results also showed in $A\beta_{25\sim35}$ treatment group the expression of PP2A mRNA cut down, but in Ginsenoside Rb1 pre-treatment group it was up-regulation.

These results may due to the following factors: $A\beta_{25\sim35}$ might activate the activity of GSK-3β、CDK-5 and inhibit the activity of PP2A ， which could result in the expression of GSK-3β mRNA and CDK-5 mRNA raise up and the expression of PP2A mRNA cut down in $A\beta_{25\sim35}$ treatment group. However ginsenosideRb1 might activate the activity of PP2A and inhibit the activity of GSK-3β and CDK-5 ,which would lead to the expression of PP2A mRNA up-regulated and the expression of GSK-3β mRNA and CDK-5 mRNA down-regulated. in GinsenosideRb1 pre-treatment group. In conclusion, $A\beta_{25\sim35}$ and GinsenosideRb1 could regulate the expression of GSK-3β, CDK-5 and PP2A in the cells differentiated from NSCs..

Inhibition of dysregulation of protein phosphorylation/dephosphorylation is a promising target to treat AD. Further investigation of new compounds that could inhibit abnormal hyperphosphorylation of tau will likely provide new treatments for AD.

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