



FULL LENGTH ARTICLE

The effect of NR4A1 on APP metabolism and tau phosphorylation



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Abstract Alzheimer's disease (AD) is characterized by senile plaques (SP) composed of β -amyloid protein (A β) and neurofibrillary tangles (NFTs) composed of intracellular hyperphosphorylated tau. Recently, nuclear receptor subfamily 4 group A member 1 (NR4A1) was implicated in synaptic plasticity, long-term memory formation, suggesting that it may play a role in the pathophysiology of AD. Here, we showed that the expression of NR4A1 was significantly increased in the hippocampus of APP/PS1 transgenic mice. In addition, NR4A1 overexpression in HT22 cells up-regulated APP and BACE1 levels, down-regulated ADAM10 expression, and promoted amyloidogenesis as indicated by decreased α -CTF levels and elevated β -CTF levels. Furthermore, a raised level of phospho-tau (p-tau, S396) was accompanied by p-GSK3 β (S9) expression reducing, but total tau, p-tau (S262 and T231), CDK5 and ERK remained unchanged in NR4A1-overexpressing cells. Collectively, our results suggest that NR4A1 promotes the amyloidogenic processing of APP by regulating ADAM10 and BACE1 expression in HT22 cells; as well as NR4A1 accelerates tau hyperphosphorylation by GSK3 β signal. Therefore, NR4A1 may play an important role in the pathogenesis of AD.

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, with memory defect, cognitive impairment and

behavioral changes as the main clinical manifestations.^{1,2} The hallmarks of AD pathology are the senile plaques (SP) composed of β -amyloid protein (A β), neurofibrillary tangles (NFTs) composed of intracellular hyperphosphorylated

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microtubule-associated protein tau.^{3,4} A β peptides derive from degradation of β -amyloid precursor protein (APP) by β -secretase (also known as the β -site APP-cleaving enzyme, BACE1) and γ -secretase via the amyloidogenic pathway.^{5,6} APP also undergoes a non-amyloidogenic pathway by α -secretase and γ -secretase. ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10) is the physiologically relevant, constitutive α -secretase of APP.⁷ Moreover, the phosphorylation of tau in NFTs is complex on account of a multitude of phosphorylation sites of tau.^{8,9} Both kinases and phosphatases have been implicated in the appearance of abnormally phosphorylated tau. Among the best studied are the proline-directed kinases glycogen synthase kinase-3 beta (GSK3 β), cyclin-dependent kinases (CDK5), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38⁸.

NR4A1 (also called NGFI-B/NUR77/TR3) belongs to the nuclear receptor subfamily 4, group A subfamily (NR4As) which also comprises NR4A2 (NURR1), and NR4A3 (Nor-1).^{10,11} NR4A1 is implicated in regulating key cellular processes, including inflammation, proliferation, differentiation, and survival.^{10–12} Recent work has shown that NR4A1 plays an important role in the synaptic plasticity, L-LTP, long-term memory formation and neuroprotection.^{13–15} Previous study on RNA-sequencing base identified NR4A1 as a differentially expressed gene by nicotine,¹⁶ indicating NR4A1 may play a key role in cognitive enhancement after nicotine. These results suggest that NR4A1 is closely related to the pathophysiology of AD.

Materials and methods

Antibodies

The anti-APP C-terminal (A8717) antibody was from Sigma (Sigma, St. Louis, USA) and anti-APP (6E10) antibody was from Covance (Princeton, NJ, USA). The anti-total tau, anti-tau-pS396, anti-tau-pT231, anti-ADAM10, anti-BACE1, anti-p-ERK1/2, anti-ERK1/2, anti-GSK3 β , anti-GSK3 β (S9) and anti-CDK5 antibodies were purchased from Abcam (Abcam, Cambridge, UK). The anti-tau-pS262 antibody was obtained from Santa Cruz (Santa Cruz Biotechnology, California, USA). The anti-NR4A1 and GAPDH antibodies were ordered from Proteintech (Proteintech, Wuhan, China). The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Proteintech (Proteintech). The biotinylated secondary goat anti-rabbit antibody was purchased from ZsBio (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China).

Experimental animals

All protocols were approved by the Commission of Chongqing Medical University for ethics of experiments on animals and were conducted in accordance with international standards. APPswe/PSEN1dE9 transgenic mice (APP/PS1, $n = 10$) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). Wild-type (WT) mice ($n = 10$) in the C57BL/6 background were obtained from Chongqing Medical University and used as controls. All animals were provided with

a standard diet and housed in an approved facility with climate control and a 12-h light/12-h dark cycle. Six month-old mice were used for all experiments. Mice were humanely killed with an overdose of anesthetics and perfused transcardially with saline, and the brains were collected for Western blot and immunohistochemistry analysis.

Plasmid construction

To construct pCMV6-NR4A1, the mouse *nr4a1* gene was PCR-amplified from mouse hippocampus cDNA with gene-specific primers and sub-cloned into the multiple cloning site (SgfI and MluI) of pCMV6 (Origene, Rockville, USA) (Supplementary Fig. 2). The construct was verified by sequencing.

Cell culture and transfection

Hippocampal neuronal cell line, HT22, was purchased from Shanghai Institute of Biological Sciences (Chinese Academy of Sciences, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C and were transfected with pCMV6-NR4A1 plasmid and pCMV6 (vector) using Lipofectamine 3000 (Invitrogen) for 48 h according to the protocol described previously in our laboratory.¹⁷

Western blot

The cells and tissue samples were lysed in RIPA buffer that included protease inhibitors (Roche, Indianapolis, USA). Protein concentrations were measured using a BCA assay (Dingguo, Beijing, China). Samples were separated on a 12% SDS-PAGE gel, transferred to a PVDF membrane (Millipore, Billerica, MA, USA) and probed with indicated antibodies overnight at 4 °C. The blots were washed and incubated for 1 h with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies. The bands were visualized using an ECL reagent (Thermo, Marina, USA) and a Fusion FX5 image analysis system (Vilber Lourmat, Marne-la-Vallée, France). Relative protein expression levels were calculated using Quantity One software (Bio-Rad) with normalization to the GAPDH signal.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissue samples or cells using Trizol reagent (Takara, Dalian, China) according to the manufacturer's protocol. cDNA synthesis was performed using a PrimeScript RT reagent kit (Vazyme, Nanjing, China). The mRNA expression levels of genes of interest were detected by quantitative real-time PCR (qPCR). Reactions were performed on a Bio-Rad IQ⁵ detection system (Bio-Rad, Hercules, CA, USA) with SYBR green master mix (Takara). The reaction mixture (20 μ l total) consisted of 10 μ l of 2 $\times$ SYBR mix, 7.2 μ l of nuclease-free water, 0.4 μ l of each primer (mouse *nr4a1*: forward primer 5'-GGGGCCGGCTACCTTCAAA ACC-3', reverse primer 5'-TGCTAGGCC CGGAGTCCAAGTGT-

3'; mouse *adam10*: forward primer 5'-ATGGTGTGCCGACAGTGTTA-3', reverse primer 5'-GTTTGGCAGCTGGTGT TTT-3'; mouse *bace1*: forward primer 5'-ACATATC GAGACCTCCGAAAGG-3', reverse primer 5'-AACTTGTCGATTCACTGATGG-3'; mouse *tau*: forward primer 5'-GATCTTAGCAACGTCCAGTCCA-3', reverse primer 5'-TTGCTC AGGTCCACC GGCTGTAG-3'; or mouse *gapdh*: forward primer 5'-CACGATGGAGGGGCGGACTCATC-3', reverse primer 5'-TAAAGA CCTCTATGCCAACACAGT-3') and 2 μ l of diluted cDNA. The reactions were performed using the following steps: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s. A melting curve was run following each assay. The threshold cycle (Ct) value of each sample was calculated, and the relative mRNA level was normalized to the *gapdh* mRNA value. The fold changes were quantified using the $2^{-\Delta\Delta C_t}$ method.

Immunohistochemistry (IHC)

The brain tissue from WT and APP/PS1 mice was formalin-fixed and embedded in paraffin. For IHC, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol before staining. After antigen retrieval and blocking, the sections were then incubated with anti-NR4A1 antibody at 4 °C overnight. The second day, sections were washed in PBS and incubated with a biotinylated secondary goat anti-rabbit antibody (ZsBio) for 30 min at 37 °C, and then incubated with an avidin-biotin peroxidase complex (ZsBio) for 30 min at 37 °C. The sections were washed in PBS and incubated with 3,3'-diaminobenzidine (DAB, ZsBio) for 3 min. Hematoxylin was used to counterstain nuclei. A LEICA DM6000B

automatic microscope (Leica, Germany) was used to collect images.

Statistical analyses

All data are presented as the mean $\pm$ SEM from three independent experiments and were analyzed using GraphPad Prism software by the independent-samples t-test or one-way ANOVA with a Dunnett's multiple comparison test. Differences were considered to be significant when $P < 0.05$.

Results

Increased NR4A1 expression in the hippocampus of APP/PS1 mice

To determine whether the expression of NR4A1 differed in APP/PS1 mice compared with control, hippocampal samples obtained from 6-month-old APP/PS1 mice and age-matched non-transgenic C57BL/6 controls (WT) were compared. Western blot showed that the levels of APP protein were dramatically increased in the hippocampus of APP/PS1 mice compared with WT control (Fig. 1A). Furthermore, the level of NR4A1 were significantly upregulated 2.11-fold in APP/PS1 mice hippocampus compared with WT (Fig. 1A and B, $P < 0.01$). Accordingly, qPCR analysis showed that *nr4a1* mRNA level was significantly increased in APP/PS1 mice compared with WT (Fig. 1C, $P < 0.05$). As shown in Fig. 1D, in the sections from APP/PS1 mice, strong NR4A1 immunoreactivity was observed,

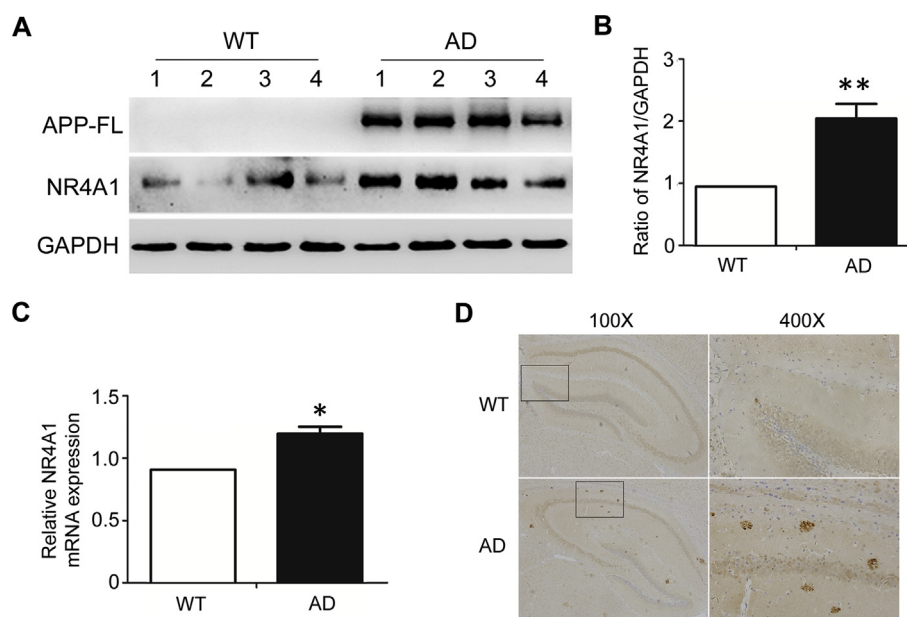


Figure 1 NR4A1 expression in the hippocampus of APP/PS1 mice. (A) Representative Western blot showing APP and NR4A1 protein expression in the hippocampus of 6-month-old APP/PS1 mice, as compared with age-matched wild-type mice (WT). 1–4 indicated the number of brain sample from mice. (B) Quantification of protein level of NR4A1 in the hippocampus of APP/PS1 and WT mice. (C) The relative mRNA expression of *nr4a1* gene measured by quantitative real-time PCR in the hippocampus of APP/PS1 mice and WT mice. (D) Immunohistochemistry of NR4A1 expression in the hippocampus of APP/PS1 and WT mice. Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs control.

whereas only a faint distribution of NR4A1 immunoreactivity was observed in sections from WT. These results suggested that NR4A1 expression was upregulated in the hippocampus of APP/PS1 mice.

NR4A1 overexpression promotes the amyloidogenic processing of APP

To examine the effect of NR4A1 on the APP metabolism, HT22 cells were transfected with mouse pCMV6-NR4A1 plasmid or pCMV6 (vector control). The expression of NR4A1 protein was significantly upregulated in NR4A1-transfected cells compared with control (Fig. 2A). The overexpression of NR4A1 induced a significant increase in the levels of APP protein with A8717 and 6E10 antibodies (Fig. 2A and B). In addition, NR4A1 shifted APP processing from α -to β -

cleavage after transfection, as indicated by decreased α -CTF levels and clearly elevated β -CTF levels (Fig. 2A and B).

We speculated that the decreased α -CTF and elevated β -CTF levels might be caused by the dysregulated expression of APP-relevant enzymes. To test this hypothesis, the protein expression of ADAM10 (α -secretase) and BACE1 (β -secretase) was detected. Quantification revealed a 73% decrease in ADAM10 levels ($P < 0.01$) and 2.30-fold increase in BACE1 levels ($P < 0.05$) in NR4A1-transfected cells (Fig. 2C and D). As shown in Fig. 2E, in NR4A1-overexpressing cells, *adam10* mRNA expression was significantly reduced by 27.5% and *bace1* mRNA level was increased 1.29-fold compared with control ($P < 0.05$). Taken together, these results demonstrated that NR4A1 promoted the amyloidogenic processing of APP in HT22 cells by regulating APP-relevant secretases.

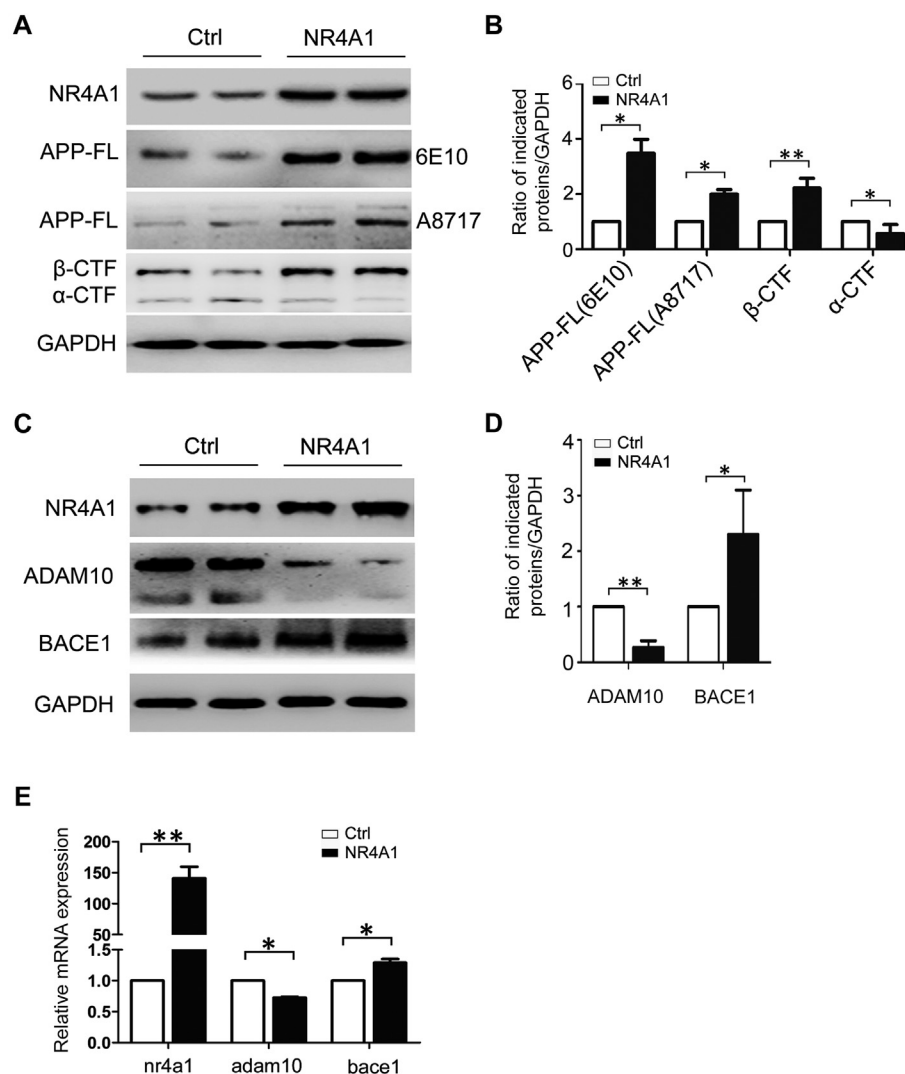


Figure 2 NR4A1 overexpression promotes APP amyloidogenic processing in HT22 cells. (A) Representative western blot of NR4A1, APP, α -CTF and β -CTF expression in HT22 cells transfected with vector or NR4A1. The "two bands" represented for two different sample. (B) Quantification of protein levels of APP, α -CTF and β -CTF in NR4A1-transfected cells compared with control. (C) BACE1 and ADAM10 expression in HT22 cells transfected with NR4A1 or vector. (D) Quantification of protein levels of ADAM10 and BACE1 in NR4A1 overexpressing cells compared with control. (E) The relative mRNA expression of *nr4a1*, *adam10* and *bace1* measured by qRT-PCR. Data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs control.

NR4A1 increased the levels of phosphorylated tau (S396) in HT22 cells

To determine the effects of NR4A1 on the clearance of endogenous total and phosphorylated tau, HT22 cells were transfected with pCMV6-NR4A1 plasmid or pCMV6 (vector control, Ctrl) for 48 h. The levels of total tau and different phosphorylated tau species were determined by immunoblotting. We examined three phosphorylated tau species (pS262, pS396 and pT231), which are all increased in the AD mice brains and are found in NFTs.⁹ As shown in Fig. 3, NR4A1 overexpression significantly increased the levels of p-tau (S396) compared with control (3.97-fold, $P < 0.01$), but the levels of total tau (t-tau), p-tau (S262 and T231) were not changed. Furthermore, NR4A1 over-expression did not affect *tau* mRNA levels (Supplementary Fig. 1), indicating that the reduction in p-tau (S396) levels was due to degradation, rather than transcriptional inhibition.

NR4A1 regulated the activity of GSK3 β

GSK3 β , CDK5 and ERK are the major kinases that involved in tau hyperphosphorylation.^{18,19} Therefore, we analyzed the effect of NR4A1 over-expression on the levels of these kinases. Western blot results showed that levels of phosphorylated forms of GSK3 β (S9) was reduced after NR4A1 transfection (Fig. 4, $P < 0.01$), indicating enhanced activity of GSK3 β . Subsequently, we also tested whether NR4A1 affected the levels of phosphorylated tau by regulating the function of CDK5 and ERK, another kinase that had been implicated in tau hyperphosphorylation. The level of CDK5 and the activity of ERK (p-ERK/ERK) remained unchanged after NR4A1 transfection (Fig. 4). These findings suggested that GSK3 β might contribute to NR4A1-induced increased of phosphorylated tau.

Discussion

Despite emerging evidence of NR4A1 functional roles in learning and memory, there have been no study of NR4A1

expression and regulation in brain with direct relevance to AD during disease progression. Evidences showed that NR4A1 expression was decreased in peripheral blood of AD patients compared to healthy controls.²⁰ Strikingly, our results demonstrated that NR4A1 was highly expressed in the hippocampus of the APP/PS1 mice. These implied that NR4A1 may be directly or indirectly involved in the pathological process of APP/PS1 mice.

Although the exact mechanisms of AD pathogenesis are not fully understood, A β and tau are considered to have critical roles in AD-related pathology.²¹ BACE1 and ADAM10 are enzymes involved in A β generation.^{5,7} Experimental studies had shown that BACE1 protein was significantly increased and ADAM10 expression was decreased in AD brains of patients and experimental mice.^{22–24} Extensive evidences have focused on the development of secretase modulators as a therapeutic approach for AD^{5,7,25,26}. Thus, identifying novel molecules that regulate BACE1 and/or ADAM10 activity remains critical. In present study, we found that NR4A1 overexpression shifted APP processing from α -to β -cleavage, as indicated by decreased α -CTF levels and elevated β -CTF levels (Fig. 2A and B). The mechanism research of APP metabolism suggested that BACE1 protein and mRNA levels were highly expressed, whereas ADAM10 was reduced in NR4A1-overexpressing cells (Fig. 2). Taken together, we demonstrated that NR4A1 may promote the amyloidogenic processing of APP by down-regulating the expression of ADAM10 and/or up-regulating the expression of BACE1.

Recently, the accumulation and aggregation of tau has been reported to be involved in the pathogenesis of AD and other tauopathies.^{8,9,27} Deficiency or inhibition of tau clearance has been proposed to be a risk factor in AD. Abundant evidence indicated that abnormal tau phosphorylation was closely associated with dementia and cognitive disorder.⁹ Western blot results of this study showed that NR4A1 over-expression significantly increased the levels of endogenous phosphorylated (S396) tau in HT22 cells (Fig. 3). However, the levels of endogenous total and phosphorylated (S262 and T231) tau remained unchanged. Besides, NR4A1 did not affect *tau* mRNA levels

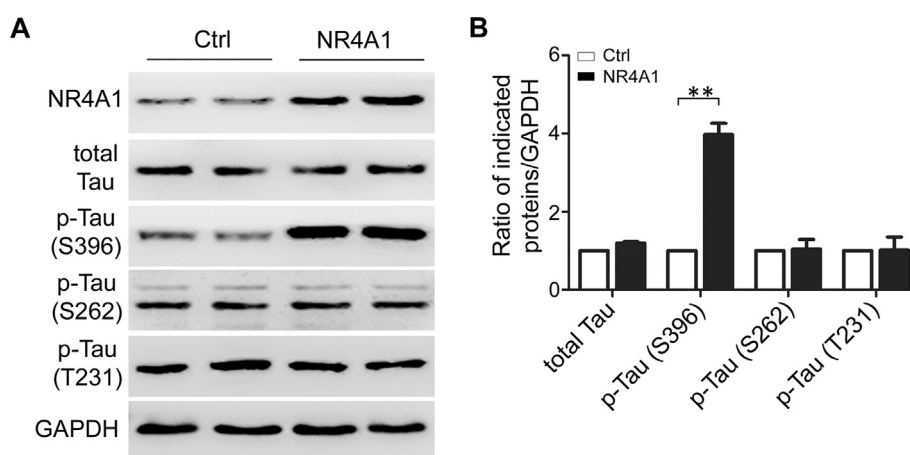


Figure 3 NR4A1 increases the level of phosphorylated tau (S396). (A) Representative western blot of endogenous total and phosphorylated (S396, S262 and T231) tau expression. (B) Quantification of protein levels of t-tau, p-tau (S396, S262 and T231) in NR4A1 overexpressing cells compared with control. Data are presented as means $\pm$ SEM. ** $P < 0.01$ vs control.

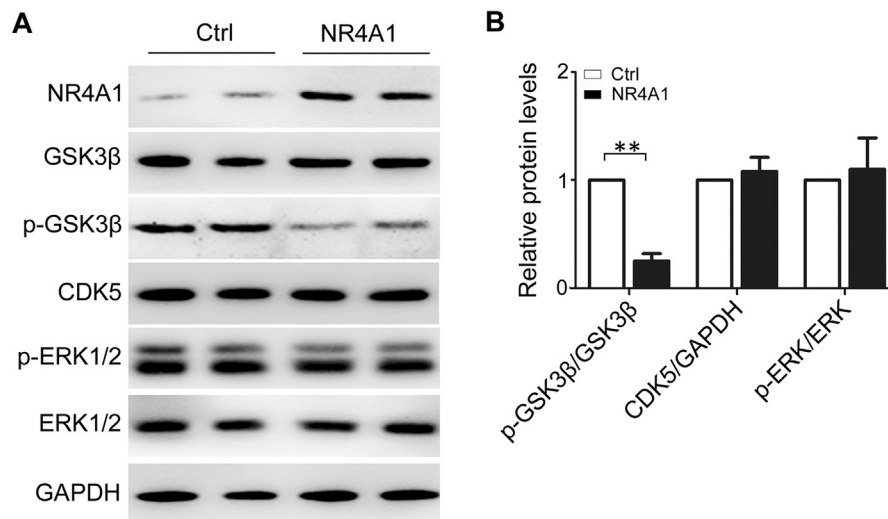


Figure 4 NR4A1 regulates the activity of GSK3β. (A) Representative western blot of GSK3β, CDK5 and ERK expression in HT22 cells transfected with pCMV6-NR4A1 or pCMV6 vector. (B) Quantification of protein levels of GSK3β, CDK5 and ERK. Data are presented as means ± SEM. **P < 0.01 vs control.

(Supplementary Fig. 1), suggesting the reduction in tau occurred via degradation. In cells, tau S396 was mainly mediated by GSK3β, while tau S262 was mainly regulated by protein kinase A (PKA).²⁸ So, we tested the possibility that NR4A1 facilitated the clearance of phosphorylated tau by modulating the activity of tau kinases. The levels of phosphorylated GSK3β (S9) reduced and the levels of total GSK3β, CDK5 and the activity of ERK remained unchanged after NR4A1 transfection (Fig. 4). The differences in the phosphorylation level of tau protein on varying sites are noticeable. The regulation mechanism of tau phosphorylation is rather complicated because each of the phosphorylation site of tau was regulated by several protein kinases. Giving that S396 is the best phosphorylation site of tau catalyzed by p-GSK3β, our results demonstrate that the phosphorylation of tau in NR4A1-overexpressing cells was mainly mediated by p-GSK3β.

Conclusions

In summary, our data indicated that NR4A1 was involved in the pathological process of AD, including APP metabolism and tau phosphorylation. NR4A1 overexpression promoted the amyloidogenic pathway of APP by down-regulating the expression of ADAM10 and/or up-regulating BACE1 expression. In addition, NR4A1 enhanced the level of tau phosphorylation through p-GSK3β signal. The current findings suggested that the upregulation of NR4A1 may represent a novel risk factor for the onset and progression of AD. We propose that targeting NR4A1 may provide an alternative strategy in the therapeutic intervention for AD or other tauopathies.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.gendis.2018.04.008>.

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