What is the new target inhibiting the progression of Alzheimer’s disease?

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Research Highlights
(1) We investigated the mechanism of action of striatal-enriched phosphatase 61 at the behavioral and signaling molecule levels, using in vivo and in vitro models. We discussed the relationship of striatal-enriched phosphatase 61 with transgene and N-methyl-D-aspartate receptor 2B, and lysed the molecular mechanism by which striatal-enriched phosphatase 61 regulates N-methyl-D-aspartate receptor 2B transport.
(2) Spatial learning and memory deficits in Alzheimer’s disease may be associated with disturbed N-methyl-D-aspartate receptor 2B transport caused by striatal-enriched phosphatase 61.
RNA silencing can effectively inhibit striatal-enriched phosphatase 61 target gene expression and can be used to investigate the function and involvement of striatal-enriched phosphatase 61 in the pathogenesis of Alzheimer’s disease.
(3) This study provides a basic reference for gene therapy for Alzheimer’s disease using striatal-enriched phosphatase 61.

Abstract
To stop the progression of Alzheimer’s disease in the early stage, it is necessary to identify new therapeutic targets. We examined striatal-enriched phosphatase 61 expression in the brain tissues of 12-month-old APPswe/PSEN1E9 transgenic mice. Immunohistochemistry showed that al-enriched phosphatase 61 protein expression was significantly increased but phosphorylated N-methyl-D-aspartate receptor 2B levels were significantly decreased in the cortex and hippocampus of APPswe/PSEN1E9 transgenic mice. Western blotting of a cell model of Alzheimer’s disease consisting of amyloid-beta peptide (1–42)-treated C57BL/6 mouse cortical neurons in vitro showed that valeric acid (AP5), an N-methyl-D-aspartate receptor antagonist, significantly inhibited amyloid-beta 1–42-induced increased activity of striatal-enriched phosphatase 61. In addition, the phosphorylation of N-methyl-D-aspartate receptor 2B at Tyr1472 was impaired in amyloid-beta 1–42-treated cortical neurons, but knockdown of striatal-enriched phosphatase 61 enhanced the phosphorylation of N-methyl-D-aspartate receptor 2B. Collectively, these findings indicate that striatal-enriched phosphatase 61 can disturb N-methyl-D-aspartate receptor transport and inhibit the progression of learning and study disturbances induced by Alzheimer’s disease. Thus, al-enriched phosphatase 61 may represent a new target for inhibiting the progression of Alzheimer’s disease.

Keywords
neural regeneration; brain injury; neurodegeneration; Alzheimer’s disease; striatal-enriched phosphatase 61; amyloid-beta peptide; N-methyl-D-aspartate receptor; GluN2B; RNA interference; immunohistochemistry; western blot; neuroregeneration
INTRODUCTION

Alzheimer’s disease is characterized by the accumulation of amyloid-beta (Aβ) peptide in brains, a process that has been implicated in the progression of the disease[11]. One hypothesis for the pathophysiology of Alzheimer’s disease is that soluble forms of Aβ disrupt synaptic function[2-3]. Amyloid plaque formation occurs subsequent to the loss of synaptic function[4], suggesting that synaptic perturbations are an earlier target of Aβ. The mechanisms underlying Aβ-induced reductions in synaptic function remain a focus of intense research.

Striatal-enriched phosphatase 61 (STEP61; also known as protein tyrosine phosphatase non-receptor 5 (PTPN5)) is a brain-specific tyrosine phosphatase targeted to synaptic compartments in the striatum, hippocampus, cortex, and related brain regions[5]. Alternative splicing produces various STEP family members, and both cytosolic (STEP61) and membrane-associated (STEP62) variants exist[6-7]. STEP opposes the development of synaptic strengthening by dephosphorylating and inactivating key signaling proteins including the mitogen-activated protein kinases extracellular-regulated kinase 1/2 and p38[8], and the tyrosine kinase Fyn. STEP also dephosphorylates the GluR2 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and the GluN2B subunit of the N-methyl-D-aspartate (NMDA) receptor, leading to internalization of the GluN1/GluN2B and GluR1/GluR2 receptors. STEP levels and activity are regulated by phosphorylation, local translation, ubiquitination, degradation and proteolytic cleavage[9-10].

Glutamatergic signaling through NMDA receptors is required for synaptic plasticity. Disruptions in glutamatergic signaling are proposed to contribute to behavioral and cognitive deficits[11]. Recent evidence suggests that glutamate receptors are dysregulated by Aβ oligomers, resulting in disruption of glutamatergic synaptic transmission, which parallels early cognitive deficits[12]. NMDA receptors are heteromultimeric complexes comprised of at least two types of subunits, the principal subunit GluN1 and the modulatory subunit GluN2A-D[13]. The GluN2B receptor has been identified as a major tyrosine phosphorylated protein in the postsynaptic density and plays a key role in the signal transduction pathways for NMDA receptor activation[14]. Some studies have suggested a role for tyrosine phosphorylation of GluN2B in the enhancement of synaptic efficacy, and thus, in the development of central sensitization[15], an important synaptic process in Alzheimer’s disease research.

Furthermore, NMDA-receptor-mediated glutamate excitotoxicity plays a major role in Aβ-induced neuronal death. It is thought that NMDA receptors represent a promising target for preventing the progression of Alzheimer’s disease[16]. Excitotoxic neuronal cell death is mediated in part by overactivation of NMDA-type glutamate receptors, which results in excessive Ca2+ influx through the receptor’s associated ion channel. However, whether STEP61 affects phosphorylation of GluN2B via NMDA receptor has not been examined.

In the present study, we characterized the cognitive deficits and the detailed STEP61-related pathologies in the cerebral cortex and hippocampus in 12-month-old APPswe/PSEN1dE9 transgenic (APP/PS1) mice, a well-established transgenic mouse model of Alzheimer’s disease. We further investigated the mechanisms by which STEP61 regulates Aβ-mediated phosphorylation of GluN2B via NMDA receptors in an Alzheimer’s disease cell model.

RESULTS

Quantitative analysis of experimental animals

Eight 12-month-old male APP/PS1 mice were selected, and another eight 12-month-old male wild-type mice (C57BL/6) were used as controls. Sixteen mice were involved in the final analysis.
Spatial learning and memory deficits in APP/PS1 transgenic mice

All APP/PS1 mice used in our study were genotyped using a standardized PCR assay on tail DNA, which confirmed that the mice were APP/PS1 mice (Figure 1). The spatial learning and memory functions of all mice were assessed by Morris water maze. During the training acquisition phase, as shown in Figure 2C, both APP/PS1 transgenic mice and wild-type mice readily learned the location of the hidden platform, as shown by a decrease in the escape latency over training days. One-way analysis of variance results revealed a significant main effect of genotype on the escape latency measured over all days ($P < 0.001$), suggesting that APP/PS1 mice required a significantly longer time to locate the hidden platform relative to wild-type mice, and that spatial learning was impaired in these transgenic mice. Moreover, there was no significant effect of genotype on swimming speed measured over all training days ($P > 0.05$; Figure 2D), suggesting that mean swimming speed did not differ between transgenic and control mice during the spatial learning training phase in the water maze. This indicates that the spatial learning deficits in transgenic mice did not result from their motor deficits.

Spatial memory was evaluated in all mice by probe trial performed 24 hours after the last training session. APP/PS1 mice spent less time in the target quadrant than wild-type mice ($P < 0.001$), indicating impaired spatial memory retention in these transgenic mice (Figure 2A, B, and E). Moreover, the visible platform trials in the water maze revealed no significant differences in escape latency between APP/PS1 mice and wild-type mice ($P > 0.05$; Figure 2F). This implies that the learning and memory impairments of APP/PS1 mice were not caused by vision and motor dysfunctions or a lack of motivation. In summary, 12-month-old APP/PS1 mice showed evident hippocampus-dependent spatial learning and memory deficits in the water-maze.

Figure 1  Amplification products of the APP gene from tail DNA.

The arrow indicates the location of the corresponding PCR-signal (270 bp). M: Marker of fragment lengths; lanes 1, 3, 6, 7, 10 represent wild-type mice; lanes 2, 4, 5, 8, 9, 11 represent APP/PS1 mice. Lanes 2, 4, 5, 8, 9, 11 show that the PCR results were as expected. Negative controls were PCRs using DNA from wild-type mice (lanes 1, 3, 6, 7, 10).

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Figure 2  Spatial learning and memory deficits in APPswe/PSEN1ΔE9 (APP/PS1) transgenic mice.

Representative swim traces for each group in probe trials: (A) Wild-type (WT) mice. (B) APP/PS1 mice. (C) In the hidden platform trials in the Morris water maze, APP/PS1 transgenic mice had significantly longer escape latencies than wild-type mice, as demonstrated by a significant effect of genotype. All results are expressed as the mean for each group (six mice for each group). The difference between groups was analyzed using one-way analysis of variance and Student-Newman-Keuls post hoc tests. $^{*} P < 0.001$, vs. wild-type group.

(D) Swimming speed in training trials showed no difference in motor function in each group ($P > 0.05$).

(E) Percent goal quadrant dwell time in the probe trials. In the probe trial, 24 hours after the last training session, the transgenic mice spent less time in the target quadrant compared with wild-type mice. All results are expressed as the mean for each group (six mice for each group). The difference between groups was analyzed using one-way analysis of variance and Student-Newman-Keuls post hoc tests. $^{*} P < 0.001$, vs. wild-type mice.

(F) No significant differences were detected between APP/PS1 and wild-type mice in the visible platform trials ($P > 0.05$). All tests were performed at least three times to ensure reproducibility.

Expression of STEP$_{61}$ in the cortex and hippocampus of APP/PS1 transgenic mice

To assess the distribution of STEP$_{61}$ in the cortex and...
hippocampus, we performed immunohistochemistry analyses. Dense staining for STEP$_{61}$ was observed in the cortex and hippocampal CA1 region of APP/PS1 transgenic mice (Figure 3A). The STEP$_{61}$ HSCOREs in the cortex and hippocampal CA1 region from APP/PS1 mice were significantly higher than those in the same region from wild-type controls (APP/PS1: cortex = 1.64 ± 0.76, hippocampus = 1.83 ± 0.61; wild-type: cortex = 0.89 ± 0.44, hippocampus = 0.94 ± 0.35; $P = 0.003$ and $P = 0.000$, respectively; Figure 3B).

![Image](https://example.com/image.png)

Figure 3  Expression of STEP61 in the cortex and hippocampus from APP/PS1 transgenic mouse brains.

(A) STEP$_{61}$ expression in the cortex and hippocampal CA1 region (immunohistochemistry). In contrast to less immunoreactivity in the wild-type (C57BL/6) mouse brain (a, b, c), more neurons were strongly stained in the APP/PS1 transgenic mouse brain (d, e, f). a, d: × 100, scale bars = 50 µm; b, c, e, f: × 200, scale bars = 25 µm. Arrows indicate STEP61-positive cells. Insets: high magnification (×400) of STEP$_{61}$-positive cells.

(B) STEP$_{61}$ HSCOREs in the cortex and hippocampal CA1 region. The STEP$_{61}$ HSCOREs in the cortex and hippocampal CA1 region from APP/PS1 mice were significantly higher than those in the same region from wild-type controls ($^aP < 0.01$, vs. wild-type mice, one-way analysis of variance and Student-Newman-Keuls post hoc tests; STEP$_{61}$ HSCOREs from five randomly selected sections). The HSCORE was calculated using two indices, proportion ($P_\pi$) and intensity ($i$). $P_\pi$ was estimated after taking into account the percentage of positive cells. $i$ was judged as 0 (no staining), or 1$^+$ (light brown staining), or 2$^+$ (brown staining), or 3$^+$ (heavy brown staining). The STEP$_{61}$ HSCORE was derived by summing the proportion of cells stained at each intensity, multiplied by the intensity of staining. HSCORE = $\sum P_\pi \times i$.

(C) Immunoblotting and quantitation of STEP$_{61}$ levels (61 kDa) normalized to β-actin levels (42 kDa).

(D) STEP$_{61}$ levels were significantly increased in APP/PS1 mice brains compared with wild-type controls. Results are presented as percentages of the absorbance for wild-type controls. ($^aP < 0.001$, vs. wild-type mice; mean ± SD of eight mice for each group; one-way analysis of variance and Student-Newman-Keuls post hoc tests).

(E) pGluN2B levels (190 kDa) were analyzed in APP/PS1 transgenic mice. Equal amounts of protein from each sample were processed for immunoblot analysis using anti-pGluN2B, GluN2B antibodies.

(F) Quantitative analysis results for levels of pGluN2B. Results are presented as percentages of the absorbance for wild-type controls ($^aP < 0.001$, vs. wild-type mice; mean ± SD of eight mice for each group; one-way analysis of variance and Student-Newman-Keuls post hoc tests). All tests were performed at least three times to ensure reproducibility.

APP/PS1: APPswe/PSEN1delE9e; HSCORE: histological score.
We also performed western blot analyses to examine STEP$_{61}$ protein levels in synaptosomes in the hippocampus and cortex from APP/PS1 transgenic mice. The results showed that STEP$_{61}$ levels were significantly elevated in the hippocampus and cortex of APP/PS1 mice compared with the same brain regions in wild-type mice (Figure 3C). Quantitative analysis revealed that the STEP$_{61}$ level was significantly increased in the hippocampus of APP/PS1 mice compared with wild-type controls ($P < 0.001$; Figure 3C). STEP$_{61}$ levels were markedly increased in the cortex of APP/PS1 mice compared with wild-type control mice ($P < 0.001$; Figure 3C). We next examined the level of pGluN2B in the hippocampus and cortex in APP/PS1 mice (Figure 3D). Notably, as in other Alzheimer’s disease mouse models, the genotype significantly affected the level of pGluN2B. Student-Newman-Keuls post hoc tests revealed that basal expression of pGluN2B was reduced in APP/PS1 mice relative to wild-type mice ($P < 0.001$). These results reveal increased STEP$_{61}$ levels and decreased pGluN2B levels in the middle-aged APP/PS1 mouse brain.

**RNAi silencing of STEP$_{61}$ decreases STEP$_{61}$ mRNA levels in cortical neurons**

Cells were harvested 48 hours after transfection with STEP$_{61}$ siRNA and non-silencing siRNA, and were subjected to real-time reverse transcription-PCR analysis. The level of mRNA for STEP$_{61}$ was decreased by 83% in cells transfected with STEP$_{61}$ siRNA compared with the level in cells transfected with the non-silencing control siRNA (Figure 4A). We then examined whether RNAi silencing of STEP$_{61}$ could affect the viability of cortical neurons incubated with Aβ$_{1-42}$ 1 μmol/L for 1 hour. Cells were treated with Aβ$_{1-42}$ before transfection with STEP$_{61}$ RNAi for 48 hours. MTT analysis revealed that the cell viabilities were all dramatically increased in cortical neurons treated with STEP$_{61}$ siRNA as compared with controls (Figure 4B).

**STEP$_{61}$ protein levels in Aβ$_{1-42}$-induced cortical neurons**

The Aβ-induced increase in STEP$_{61}$ levels and the amount of dephosphorylated STEP$_{61}$ in cortical neurons was mediated by the NMDA receptor; RNAi silencing of STEP$_{61}$ increased pGluN2B levels in Aβ-treated cortical neurons.

We then investigated the effects of Aβ$_{1-42}$ on STEP$_{61}$ levels. Previous studies have shown that STEP$_{61}$ is present as a doublet and that the upper band (phosphorylated form) constitutes the principal component of the doublet under basal conditions$^7$. Immunoblotting revealed a marked increase in STEP$_{61}$ levels by 225 ± 8.9% following Aβ$_{1-42}$ treatment ($P < 0.001$; Figure 5B). Aβ$_{1-42}$ treatment reduced the proportion of phosphorylated STEP$_{61}$ (phosphorylated/total STEP$_{61}$) to 72% of the control level ($P < 0.001$; Figure 5C; the proportion of dephosphorylated STEP$_{61}$ was significantly increased). Pretreatment with the NMDA receptor antagonist valeric acid (AP5) before treatment with Aβ$_{1-42}$ treatment compared with controls ($P < 0.001$). However, the decreased GluN2B phosphorylation was significantly restored after treatment of cells with STEP$_{61}$ siRNA ($P < 0.001$; Figure 5D, E). These results suggest that NMDA receptor activity is necessary for the Aβ-induced increase in STEP$_{61}$ expression and activation, that STEP$_{61}$ negatively regulates Aβ-mediated phosphorylation, and that it possibly also regulates the activity of GluN2B.
DISCUSSION

In the present study, we used APP/PS1 mice as an Alzheimer’s disease mouse model. The transgenic mice expressed a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and mutant human presenilin 1 (PS1-dE9). Both mutations are associated with early onset of Alzheimer’s disease. The Mo/HuAPP695swe transgene causes mice to secrete a human Aβ peptide. As previously reported, STEP is expressed in both the soma and the processes of all neurons. The present findings revealed elevated STEP61 levels in the cortex and hippocampal CA1 region of middle-aged APP/PS1 transgenic mouse brains. We speculated that the increase in STEP61 levels is likely caused by increases in Aβ levels or overexpression of the APP or PS1 mutant. In addition, cognitive deficits are normally present in aged APP/PS1 transgenic mice. Therefore, there are possible correlations between STEP61 and cognitive deficits in APP/PS1 mice. It has been reported that genetic reduction of STEP in 3xTg-Alzheimer’s disease mice results in significant improvements in three cognitive tasks. These findings demonstrate the importance of STEP61 in normal cognitive functions.

Figure 5  The amyloid-beta (Aβ1–42)-induced increase in STEP61 levels and the proportion of dephosphorylated STEP61 in cortical neurons were mediated by the glutamate/NMDA receptor.

(A) Cortical neurons were pre-incubated with control buffer, NMDA receptor antagonist valeric acid, AP5 (200 μmol/L), non-NMDA receptor antagonist CNQX (20 μmol/L), AP5 + CNQX 10 minutes before 1 μmol/L Aβ1–42 treatment for 1 hour and processed to obtain synaptosomes. A western blot was performed for STEP61 (61 kDa). The re-probed membrane with β-actin (42 kDa) served as a control for loading.

(B, C) Measurement of STEP61 levels and the proportion of phosphorylated STEP61 after treatment of cultured neurons with control buffer, AP5, CNQX, AP5 + CNQX 10 minutes before Aβ1–42 treatment (aP < 0.001, vs. control group; mean ± SD of eight mice for each group; one-way analysis of variance and Student-Newman-Keuls post hoc tests).

(D) RNAi silencing of STEP61 leads to high expression of pGluN2B in cortical neurons cultured in Aβ1–42-added medium. The cells were transfected with STEP61 siRNA for 48 hours, and western blotting was performed to assess the phosphorylation of GluN2B (pGluN2B; Tyr1472, 190 kDa). Total GluN2B (190 kDa) was not affected. Reprobed membrane with β-actin served as a control for loading.

(E) Quantification of pGluN2B levels from five separate experiments (mean ± SD, aP < 0.001, vs. control group; one-way analysis of variance and Student-Newman-Keuls post hoc tests of eight mice for each group). All tests were performed at least three times to ensure reproducibility.

STEP61: Striatal-enriched phosphatase 61; NMDA: N-methyl-D-aspartate; AP5: valeric acid; CNQX: 6-cyano-T-nitroquinoxaline-2,3-dione.
function, and its possible involvement in cognitive disorders such as Alzheimer’s disease.

The behavioral impairments in Alzheimer’s disease transgenic mice are consistent with the biochemical data. The pGluN2B level is significantly lowered in the cortex and hippocampus from APP/PS1 transgenic mice. Recent studies have suggested that NMDA receptor mislocalization is a dominant factor contributing to glutamatergic dysfunction and pathogenesis in neurological disorders such as Alzheimer’s disease and ischemia. Therapeutic approaches that specifically rectify receptor mislocalization or target downstream apoptotic signaling pathways could be beneficial for preventing disease onset or progression across many disorders that are commonly caused by NMDA receptor dysfunction. NMDA receptors are ion channels gated by glutamate; they are widespread in the central nervous system and are involved in numerous physiological and pathological processes including synaptic plasticity, chronic pain and psychosis. Aberrant NMDA receptor activity also plays an important role in the neuronal loss associated with ischemic insults and major degenerative disorders including Parkinson’s and Alzheimer’s diseases. Agents that target and alter NMDAR function may, thus, have therapeutic benefit. Previous studies have shown that STEP associates with the NMDA receptor complex, reduces NMDA receptor activity, and opposes the induction of long-term potentiation through a process whereby STEP dephosphorylates a regulatory tyrosine site (Tyr1472) on the GluN2B subunit, leading to internalization of GluN1/GluN2B receptor complexes. In the present study, the pGluN2B level was significantly decreased in the cortex and hippocampus from APP/PS1 mice, and this decrease was accompanied by an increase in STEP levels. Decreased pGluN2B levels were also observed in an AD cell model. The changes in STEP protein level as well as the proportion of dephosphorylated STEP could possibly be involved in the decreased phosphorylation of GluN2B. Importantly, knockdown of STEP enhanced the pGluN2B level. It has been reported that phosphorylation of GluN2B at Tyr1472 is correlated positively with NMDA currents. This implies that STEP is possibly involved in the trafficking of NMDA receptors, and that it is inappropriately activated by Aβ.

Furthermore, we extended these findings by demonstrating a role for NMDA receptors in the pathological pathways affecting STEP regulation in Alzheimer’s disease, which has not previously been reported. Pretreatment with the NMDA receptor antagonist valeric acid (AP5), but not the non-NMDA receptor antagonist CNQX before treatment with Aβ1-42 inhibited the increase in STEP levels and activation. Thus, NMDA receptor-mediated Ca2+ influx appears to be responsible for the effects of Aβ-induced activation of STEP. Taken together, the results of the present study indicate that STEP has potential to negatively regulate the activation of GluN2B via NMDA receptors that may be associated with the pathology of Alzheimer’s disease.

MATERIALS AND METHODS

Design
A randomized, controlled, in vivo and in vitro study.

Time and setting
The experiments were performed at the Central Laboratory of China Medical University, China from May to November 2011.

Materials
A total of 16 healthy, 12-month-old, male APP/PS1 mice (Shenyang, China; license No. SYXK (Liao) 2008-0005) and age-matched C57BL/6 mice, weighing 30.0 ± 6.5 g, were used. Throughout the experiments, the animals were maintained in stainless-steel cages in a controlled environment (22–25°C, 40–60% relative humidity, 12-hour light-dark cycle), with free access to food and water.

All procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

Methods
Morris water maze test for spatial learning and memory deficit in transgenic mice
The apparatus consisted of a circular water tank (120 cm in diameter and 50 cm high). The water was rendered opaque by the addition of nontoxic white paint and the water temperature was maintained at 23 ± 1°C. The mice were required to find the hidden platform using spatial cues available in the testing room. They were given 60 seconds to find the submerged platform. The mice were allowed to stay on the platform for 20 seconds. The time that an individual mouse took to reach the hidden platform was recorded as the escape latency (second). The swimming speed was recorded and analyzed.

The average time that an individual mouse spent in the target quadrant that previously contained the hidden
platform was respectively recorded as a measure of spatial memory. The visible platform tests (four trials per day for 2 days) were performed 24 hours after the probe trial. In this trial, the visible platform was positioned 1 cm above the water surface. The time to reach the visible platform and the percentage of time spent in the correct quadrant were recorded and analyzed.

**PCR for detection of APP expression in APP/PS1 transgenic mice**

A fragment of the APP gene was amplified by PCR from transgenic mouse tail genomic DNA using Taq DNA polymerase. After initial denaturation at 94°C for 5 minutes, 30 cycles of amplification were performed. DNA was annealed at 55°C for 30 seconds, and the samples were incubated at 72°C for 5 minutes to ensure complete strand extension.

| Gene   | Sequence (5’–3’)                  | Product size (bp) |
|--------|-----------------------------------|-------------------|
| APP    | Upstream: GAC TGA CCA             | 270               |
|        | CTG GAC CAG GTT CTG               |                   |
|        | Downstream: CTT GTA AGT           |                   |
|        | TGG ATT CCT CAT ATC CG            |                   |

**Culture of primary cortical neurons from C57BL/6 mice**

Primary cortical neuronal cultures were prepared from timed-pregnant E15–16 wild-type (C57BL/6) mice[28]. The brains were removed and the cortices dissected out under a dissecting microscope. Tissue was trypsinized with 0.025% trypsin-ethylenediamine tetraacetic acid (Ybio-tech, Shanghai, China) and dissociated in Hanks balanced salt solution (Beyotime, Jiangsu, China) by titration to isolate cells. Cortical cells were resuspended in neurobasal medium containing B27 supplement and 5% fetal bovine serum (HyClone, Shanghai, China) and plated in 12-well culture dishes containing poly-D-lysine (Sigma, St. Louis, MO, USA)-coated 18-mm German-glass coverslips. The cells were maintained in neurobasal medium without fetal bovine serum for 14–15 DIV at 37°C in a humidified 5% CO₂ incubator.

**Immunohistochemistry for distribution and expression of STEP₆₁ in the cortex and hippocampus**

Serial coronal sections (5 μmol/L thickness) were cut from various sections of the brain. After the coronal sections were rinsed in PBS three times, citrate buffer was used for endogenous antigen retrieval. Peroxidase activity was blocked by incubation with 3% H₂O₂ for 10 minutes. The sections were incubated with 10% normal goat serum. After the blocking serum was removed, sections were immunostained overnight at 4°C using a rabbit anti-STEP₆₁ polyclonal antibody (1:150; Abgent, San Diego, CA, USA), and then with biotinylated secondary antibody goat anti-rabbit IgG at 37°C for 20 minutes (Beijing Zhongshan Biotechnology Co., Ltd.). STEP₆₁ positive cells were detected using streptavidin-biotin complex and 3,3’-diaminobenzidine kits (Zhongshan, Beijing, China). Primary antibodies were replaced with PBS buffer as a negative control. Immunohistochemical results were judged by HSCORE (histological score)[29]. The HSCORE was calculated using two indices, proportion (Pi) and intensity (i). The Pi was estimated based on the percentage of positive cells. The i was judged as 0 (no staining), 1+ (light brown staining), or 2+ (brown staining). The STEP₆₁ HSCORE was derived by summing the proportion of cells stained at each intensity, multiplied by the intensity of staining.

\[
HSCORE = \sum P_i \times i, \text{ where } i = 0, 1, 2 \text{ or } 3 \text{ and } Pi \text{ varied from 0 to 1.0. }
\]

STEP₆₁ HSCOREs ranged from a minimum of zero in cases with no staining to a maximum of 3.0 in cases in which all the cells stained with maximal intensity. We judged HSCORE > 0 as positive and HSCORE = 0 as negative. HSCORE ≥ 2.0 was judged as highly positive. In each STEP₆₁ section, the stained cells were counted in three visual fields (n = 5) from each of the cortex and hippocampal CA1 regions.

**Preparation of synaptosomes from cortex and hippocampus**

Homogenates were prepared from brain tissue using glass homogenizers in homogenization buffer: 0.35 mol/L sucrose, 10 mmol/L Tris, 0.5 mmol/L EGTA solution brought to pH 7.4 with KOH. The homogenates were centrifuged at 2 000 × g for 1 minute on a Beckman model J-21B centrifuge using a JA-20 fixed angle rotor to remove the nuclear pellet (PI). PI was washed once by resuspension in the homogenizing buffer and repelleted. The supernatant was combined with the supernatant from the first centrifugation, and the pellet was discarded. The pooled supernatant (SI) was centrifuged at 23 000 × g for 4 minutes to yield a crude mitochondrial pellet (P2), which was washed once by resuspension in homogenizing buffer and repelleted. The pellet was then brought up to 6 mL total volume with the same buffer. This suspension was layered onto a discontinuous gradient of 5% and 13% Ficoll in 0.35 mol/L sucrose (1 mL suspension/2 mL each of 5 and 13% Ficoll) that had been allowed to equilibrate at 4°C for 1 hour prior to loading the sample. The gradient was centrifuged at 45 000 × g 45 minutes on a Beckman L5-75 ultracentrifuge (Oregon, USA) using an SW 50.1 swinging bucket rotor. A synaptosomal fraction was collected from the 5–13% interface and
washed by diluting in 0.35 mol/L sucrose/10 mmol/L Tris and centrifuging at 23,000 × g for 20 minutes. The synaptosomal pellet was resuspended in incubation buffer[30].

Assessment of cell viability by MTT assay
Cell viability was measured in 96-well plates by quantitative colorimetric assay with MTT (KeyGEN Biotech, Nanjing, China), which is an indicator of the mitochondrial activity of living cells. Cell viability was expressed as the ratio of the signal obtained from treated cultures and control cultures.

Quantitative real-time reverse transcription-PCR
1 μg of total RNA was reverse transcribed to cDNA in a total volume of 20 μL using an RT (reverse transcriptase) reaction kit (TaKaRa, Tokyo, Japan). Real-time PCR was performed using a Thermal Cycler Dice® real-time system (TaKaRa Code: TP800) according to the manufacturer’s instruction and SYBR® Premix Ex Taq (TaKaRa) as a DNA-specific fluorescent dye. PCR was carried out for 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All reactions were repeated at least three times. The primers used for analysis of β-actin mRNA are as follows:

| Gene       | Gene sequence (5’→3’)                | Tm (°C) | Product size (bp) |
|------------|--------------------------------------|---------|-------------------|
| STEP       | Upstream: TTC CTA CAT CAA TGC CAA CTA C | 55.0    | 194               |
|            | Downstream: GCT CCT CCG GCC AAT AC   |         |                   |
| β-actin    | Upstream: TCG TGC GTG ACA TTA ACG AG | 55.0    | 304               |
|            | Downstream: ATG CCA GGG TAC ATG GTG GT |         |                   |

Tm: Temperature.

Western blot analysis for STEP81, pGluN2B and GluN2B protein expression in the cortex and hippocampus
Protein concentrations were determined by bicinechonic acid assay. Membrane proteins (100 μg for each sample) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% gradient Tris/glycine gels. Then, proteins were transferred to polyvinylidene difluoride membrane (Millipore, Temecula, CA, USA). Blots were incubated with the following primary antibodies: rabbit polyclonal anti-STEP81 (1:300; Abgent, San Diego, CA, USA), rabbit anti-pGluN2B, -GluN2B polyclonal antibody (1:500; Cell Signaling Technology, Beverly, MA, USA), and rabbit anti-β-actin monoclonal antibody (1:500; Biosynthesis Biotechnology Co., Beijing, China). The samples were detected using a chemiluminescent detection system (Pierce Technology, Rockford, IL, USA), and the absorbance of each band was measured using Image J software (Chicago, IL, USA). Western blot was performed at least three times to ensure reproducibility.

Statistics analysis
Data were expressed as mean ± SD and were analyzed by one-way analysis of variance and Student-Newman-Keuls post hoc test using SPSS 18.0 software (SPSS, Chicago, IL, USA). Differences were considered statistically significant if the P value was less than 0.05.

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