Low-level Chlorpyrifos Exposure Affects Synaptic Plasticity in Hippocampal Neurons

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Abstract

Chlorpyrifos (CPF) is a widely used organophosphorus pesticide and exhibits environmental persistence and bioaccumulation. Increasing evidence has shown that exposure to CPF in early life might induce neurodevelopmental disorders in adulthood. Synaptic plasticity plays a crucial role in neurodevelopment. This study aimed to investigate the effect of CPF on synaptic plasticity in hippocampal neurons and establish the cellular mechanism of these effects. We analysed the impact of CPF on the expression level of a presynaptic protein, a postsynaptic protein, and ionotropic glutamate receptors, as well as the effects on the Wnt7a/β-catenin pathway in primary hippocampal neurons. We found that CPF decreased the expression of synaptophysin, PSD-95, NMDAR1, GluR1, and Wnt7a, as well as active β-catenin, in primary hippocampal neurons. Our study suggests that low-level CPF exposure affects synaptic plasticity in hippocampal neurons.

Introduction

Chlorpyrifos (CPF) is a widely used organophosphorus pesticide worldwide. More than 200,000 tons of CPF were used worldwide in 2015, and the demand for this insecticide is expected to increase annually [1]. Through decades of tracking, the concentration of CPF had reached 303.8 μg/L in seawater, rivers, groundwater, and even rainwater in different countries [2]. Gurunathan et al. reported that the nondietary dose of CPF in toys for a 3–6-year-old child is 208 μg/kg/day to 356 μg/kg/day [3]. Another study indicated that the ingested CPF via dermal residue contact and nondietary ingestion is approximately 1000 μg/day (median value) [4]. Thus, human exposure to the compound is almost inevitable. Acute high-dose CPF poisoning is associated with cholinergic symptoms caused by acetylcholine accumulation in synapses. Chronic low-level CPF exposure is associated with cognitive impairment, behavioural changes, and decreased working memory [5-7]. A relationship has been established between chronic low-level CPF exposure and developmental disorders, such as attention deficit hyperactivity disorder (ADHD) [8], autism spectrum disorder (ASD) [9], developmental retardation [6] , and Parkinson's disease (PD) [10,11]. Our previous studies also showed that neonatal rats might experience increased anxiety and amnesia in adulthood after exposure to low doses of CPF [12], and CPF combined with lipopolysaccharide was found to induce long-term spatial memory deficits in rats [13]. However, the neurotoxic mechanisms associated with CPF exposure are not completely clear.

Brain plasticity, also known as neuroplasticity, is the brain's capacity to modify neural circuit function and thereby modify subsequent thoughts, feelings, and behaviours in response to an experience. Particularly, synaptic plasticity refers to the activity-dependent modification of the synaptic transmission strength or efficacy at pre-existing synapses [14]. Synaptic plasticity is also thought to be involved in the early development of neural circuitry, and accumulating evidence indicates that the impairment of this process contributes to several prominent neuropsychiatric disorders, including neurodevelopmental disorders, post-injury repair, and learning and memory disorders [15,16].

As one of the main integral proteins in synaptic vesicles, synaptophysin (Syp) plays a central role in synaptic vesicle formation [17] and synapse stabilisation [18], and the levels of this protein can be used as an indirect measure of the number [19], density [20], and transmission efficiency of synapses [21-23]. Importantly, it is one of the most widely used experimental protein markers of synaptic plasticity in the brain [24]Postsynaptic density-95 (PSD-95) is localised to the postsynaptic density of asymmetric synapses [25,26]. PSD-95 reportedly plays a role in the organisation of glutamate receptors and other constituents of the postsynaptic density [27,28], thus determining the size and strength of synapses [29-32], as well as the formation of synapse assemblies [33]. All of these processes contribute to the cellular mechanisms underlying various types of synaptic plasticity. Therefore, PSD-95 is an important regulator of synaptic strength and plasticity [22,34,35].

Glutamatergic receptors (GluRs) are the primary excitatory neurotransmitter receptors in the hippocampus and are divided into two main classes, ionotropic GluRs (iGluRs) and metabotropic GluRs. Several neuronal functions, such as synaptic transmission, neuronal migration, excitability, and plasticity rely on glutamatergic synapses [36]. N-methyl-D-aspartate receptors (NMDARs) and AMPA receptors (AMPAs) are the primary iGluRs in the hippocampus. NMDARs are tetrameric protein complexes, typically comprised of two subunits referred to as N-methyl-D-aspartate receptor 1 (NMDAR1) and N-methyl-D-aspartate receptor 2 (NMDAR2) [37]. In the central nervous system, NMDAR1 is ubiquitous. Based on the four AMPAR subunits, glutamate receptors 1–4 (GluR1–4), heterotetramers are formed by the association of two dimers. The most frequently observed dimers are GluR1/GluR2 and GluR2/GluR3 in the mammalian brain [38]. NMDARs play an important role in synaptogenesis [39], synaptic plasticity [40], and neuropathologies [41] relevant to learning and memory. The levels of AMPARs are considered very important for synapse maturation and plasticity, since they are directly related to the strength of the synaptic electrical response to glutamate [38].

Wnt signalling was recently shown to be involved in the remodelling of pre- and postsynaptic regions, thus regulating synaptic function and plasticity, which are critical for memory and learning [42].The classic Wnt/β-catenin pathway, which has been extensively studied to date, is related to developmental defects [43] and neurodegenerative diseases including Alzheimer's disease and PD [44,45]. Herein, we hypothesised that exposure to low-level CPF would induce Wnt/β-catenin-mediated variations in primary hippocampal neuron synaptic plasticity. By evaluating the expression levels of critical proteins within this pathway, we examined the possible mechanism of CPF-induced neurotoxicity.

Materials And Methods

Animals

All Sprague-Dawley rats used in the experiment were purchased from Changsha Slac Animal Corporation (Hunan, China). The experiments were approved by the Medical Ethics Committee of the Third Xiangya Hospital and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary cell culture, drug treatment, and transfection

The culture method of primary hippocampal neurons was slightly modified based on a previously reported method [46]. The hippocampus of E16–E18 rats was first isolated, and the surrounding vessels were removed. The isolated hippocampus was then digested and separated at 37 °C for 10 min with 0.25% trypsin (Grand Island, NY, USA, Gibco) and 0.2% type Ⅱ collagenase (St. Louis, MO, USA, Sigma) at a 1:1 ratio. The suspension was filtered via a 40 μm filter (Billerica, MA, USA, Millipore) after adding DMEM/F12 (South Logan, UT, USA, HyClone) with 10% foetal bovine serum (FBS, Gibco) supplementation and was centrifuged at 1000 r/min for 5 min. The isolated cells were then stored in DMEM/F12 supplemented with 10% FBS, 1% Glutamax (Gibco), and 1% B-27 supplement (Carlsbad, CA, USA, Invitrogen). According to the applications of the experiment, the cells were plated into different plates with different densities. The 96-well plates were used for subsequent CCK-8 experiments, and the 24-well plates were used for immunofluorescence staining and cell morphology observations. The cell density was 2×10^5/mL. Moreover, the 6-well plates were used for western blotting analysis, and the cell density was 2×10^6/mL. Cells were cultured in an atmosphere of 95% humidified air and 5% CO_2 at 37 °C. Four hours after plating, the culture medium was changed to neurobasal medium with 1% GlutaMax and 2% B27 supplementation, and this was changed every 3 days. The primary hippocampal neurons were mature at 6 days *in vitro* and identified by positive MAP2 expression using immunofluorescence techniques.

To package the *CTNNB1* lentivirus, the sequence containing *CTNNB1* cDNA (NCBI reference sequence: NM_43540-1) was cloned into the Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin plasmid (purchased from Genechem, Shanghai, China). The titres of lentivirus particles were 5×10^8 U/mL. The hippocampal neurons were transfected with the lentivirus on the sixth day *in vitro* and used for drug treatment at 48 h post-viral transfection. Subsequently, the neurons were treated with different concentrations (25, 50, 100, and 200 μM) of CPF for 6, 12, 24, 48, and 72 h, and results were compared to those of control cells treated with 0.1% dimethyl sulfoxide (DMSO) [47].

Animals and treatment

All Sprague-Dawley rats used in the experiment were purchased from Changsha Slac Animal Corporation (Hunan, China). The experiments were approved by the Medical Ethics Committee of the Third Xiangya Hospital and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimise animal suffering and to reduce the number of animals used. Sprague Dawley rats at postnatal day 11 weighing 17–20 g, regardless of sex, were used for this experiment.

CPF (purity 99.9%, Sigma, St. Louis, MO, USA) was dissolved in DMSO (Sigma) at a concentration of 5 mg/mL and was subcutaneously injected in the abdomen at a dose of 1 mL/kg (5 mg/kg) daily from postnatal day 11 to 14, as in our previous study [48,49]. This dose used did not result in weight loss, impaired viability, or any systemic toxicity including signs of cholinergic hyperstimulation [50-53]. Control animals were administered corresponding vehicle injections, according to the same schedule.

Cell viability assay

A CCK-8 assay was used to evaluate neuron viability. For this, 10 μL CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to the cells after incubation with CPF at different concentrations (0, 25, 50, 100, and 200 μM) for 6, 12, 24, 48, and 72 h. The absorbance at 450 nm was measured using an automated 96-well plate reader after the cells had been incubated for another 2 h. Then, we used the same method to measure the absorbance of the cells treated with CPF at a concentration of 50 μM for 0, 6, 12, 24, 48, and 72 h.

Immunofluorescent and Hematoxylin-eosin (HE) staining

The coverslips with the adhered cells were fixed with 4% paraformaldehyde for 15 min and then treated at room temperature with 0.2% triton X-100 for 20 min. Cells were incubated with an anti-MAP2 antibody (1:500, Abcam, Cambridge, UK) overnight at 4 ℃ after incubation with 5% bovine serum albumin (Geneview) for 30 min at room temperature. The next day, the cells were incubated with appropriate DyLight-labelled secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA, Jackson) for 1 h at room temperature. Finally, DAPI (100 ng/mL, St. Louis, MO, USA, Sigma) was used to counterstain the nuclei. Cells were imaged with standard fluorescence microscopy (Olympus, Tokyo, Japan). ImageJ software (NIH, Bethesda, MD, USA) was used to merge stained images.

The brains of the rats were fixed in 4% paraformaldehyde overnight after perfusion with cold 0.1 M PBS (pH 7.4). For dehydration, the brains tissues were treated with graded ethanol. Then, the brain tissues were embedded with paraffin and cut into 5 μm-thick sections. Sections were stained with HE. Samples were immersed in xylene and alcohol, stained with hematoxylin for 5 min, then stained with eosin for another 3 min and re-immersed in alcohol and xylene. As for immunofluorescent staining, sections were pre-treated with an antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) and a spontaneous fluorescence quenching agent and were incubated in 5% bovine serum albumin for 30 min at room temperature. Followed by incubation with anti-PSD-95 (1:100, Proteintech) antibodies overnight at 4 ℃, the sections were incubated with the secondary antibody (Servicebio) for 1 h at room temperature. Finally, DAPI was used to counterstain the nuclei. Images of the hippocampal CA1, CA3, and DG were acquired under a microscope (magnification 200×; Nikon, Tokyo, Japan) and merged using ImageJ software.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA from the primary neurons was extracted using the High Pure RNA Isolation Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) to synthesise cDNA with the RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Next, 2 μg extracted RNA in a 25 μL reaction system was utilised for the reverse transcription reaction with the following three-step incubation: 25 °C for 5 min, 42 °C for 60 min, and 75 °C for 15 min. The relative expression level of mRNA was then assessed using the SYBR Green Real-time PCR Master Mix Kit (Toyobo, Osaka, Japan). Samples were incubated at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and cDNA

extension at 72 °C for 30 s. The reaction was implemented using the LightCycler R 480II as an analyser (Roche, Mannheim, Germany); *GAPDH* was used as a loading control. The genes were amplified using the following primers: TGGTAGTGCCTGTGATCGTGT (forward) and GGGGAGGGGGTCTTCAAACAA (reverse) for *Syp*, GTCACCCCTGCCCCATCATAA (forward) and GGTGTGTGAAAGACAGGGGAC (reverse) for *PSD95*, CCATCCTTCCTCCAGCCACTA (forward) and GAAATGTCGTGGGAGGGTGGT (reverse) for *NMDAR1*, and GGACAACTCAAGCGTCCAGAA (forward) and ACAGTAGCCCTCATAGCGGTC (reverse) for *GluR1*.

Western blot analysis

Protein was isolated from the hippocampus neurons and was resolved with 10% SDS-PAGE gels. After migration, proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) and blocked in tris-buffered saline containing 0.2% tween-20 and 5% skim milk powder for 1 h at room temperature. Then, the membrane incubated overnight at 4 ℃ with the primary antibodies at specific dilutions as follows: anti-NMDAR1 (1:1000, Abcam), anti-glutamate receptor 1 (1 μg/mL, Abcam), anti-synaptophysin (1:20,000, Abcam), anti-PSD95-specific (1:1000, Proteintech), anti-active-β-catenin (anti-ABC; 1:1000, Millipore), and anti-Wnt7a (1:500, Proteintech). β-Tubulin (1:1000, Cell Signaling Technology, Danvers, MA, USA) was used as the gel loading control. After incubation with primary antibodies overnight at 4 ℃, the blots were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000, Proteintech) for 1 h at room temperature. An enhanced chemiluminescence reagent was used to detect the proteins. Quantity One software was used for the analysis of the band intensity.

Statistical analysis

Data were analysed using the program Prism (GraphPad, Inc., San Diego, CA, USA). Significant differences were identified using two-way analysis of variance followed by Bonferroni post hoc tests or an unpaired Student's *t*-test. Results were expressed as the mean ± SEM from three independent experiments. The level of statistical significance was set to $p < 0.05$.

Results

Effect of CPF on the cell morphology and viability of primary hippocampal neurons

The primary hippocampal neurons were mature at 6 days *in vitro* and were identified by positive MAP2 expression using immunofluorescence techniques (Figure 1A). Primary hippocampal neurons were treated with 50 μM CPF for different times. Microscopic observations revealed no obvious change in the number of neurons. The cell body was still large and full, with a halo around it, and the network between synapses could still be seen. However, compared with those in the control group, the cell body was slightly shrunken, the synapse was shortened, and the intersynaptic network was relatively sparse. Moreover, the change was more significant as the exposure time increased (Figure 1B). Through HE staining, we can also see that the neuronal synapses in each zoomed hippocampal region of the rat in the CPF treated group was shortened (HE staining # in Figure 2E). Concerning cell viability, CCK-8 assays

revealed a concentration-dependent decrease. When the concentration of CPF was 25 or 50 μM, regardless of the intervention time (6, 12, 24, 48 or 72 h), the cell viability of neurons was not obviously changed compared with that in the control group ($P > 0.05$; Figure1C). However, when the concentration of CPF reached 100 μM or more, the cell viability began to decrease significantly ($P < 0.05$; Figure1C). Moreover, no significant time-dependent change in cell viability was observed after CPF treatment (50 μM; $P > 0.05$; Figure 1D).

CPF exposure affects the expression level ofpresynaptic and postsynaptic markers

We next analysed the expression levels of Syp and PSD-95, representing presynaptic and postsynaptic markers of primary hippocampal neurons, respectively. Protein levels of Syp and PSD-95 in cells exposed to CPF for 12–72 h were lower than those in corresponding controls (Figure 2A,B).The expression levels of the proteins gradually decreased between 12 and 48 h of CPF treatment, reaching their lowest point at 48 h, which was followed by an increase at 72 h. Further, the mRNA levels of *Syp* and *PSD-95* in cells exposed to CPF was lower than that in corresponding controls (Figure2C,D). Moreover, through HE staining, we can clearly distinguish the zoomed hippocampal region. And the expression level of PSD-95 in the CA1, CA3, and DG regions of the rat hippocampus was decreased according to the immunofluorescence results (Figure 2E).

CPF exposure affects the expression level of iGluRs in hippocampal neurons

We also analysed the expression levels of NMDAR1 and GluR1 in primary hippocampal neurons. We found that the protein levels of NMDAR1 and GluR1 in cells exposed to CPF for 12–72 h were lower than those in corresponding controls (Figure 3A–C). The expression levels of the proteins gradually decreased between 12 and 48 h of CPF treatment, reaching their lowest point at 48 h, which was followed by an increase at 72 h. Similarly, *NMDAR1* and *GluR1* mRNA levels in cells decreased significantly after CPF treatment (Figure 3D).

CPF induces the down-regulation of Wnt/β-catenin signalling in hippocampal neurons

The present study showed that Wnt7a and active-β-catenin expression decreased upon treatment with CPF (50 μM) within the range of 12–72 h, as compared to levels in corresponding controls (Figure 4A,B). Similar to these results, Wnt7a and active-β-catenin expression levels gradually decreased between 12 h and 48 h of CPF treatment, reaching their lowest point at 48 h, which was followed by an increase at 72 h. To verify that Wnt/β-catenin signalling is involved in the effect of CPF on synaptic plasticity, we over-expressed β-catenin (Figure 4C,D). However, β-catenin over-expression did not compensate for the CPF-induced reduction in the expression levels of the pre- and postsynaptic proteins studied (Figure 4E,F).

Discussion

Despite increasing concerns related to developmental neurotoxicity associated with chronic low-level exposure to CPF, this agent remains one of the most widely used organophosphate pesticides. In the

present study, we analysed the impact of low-level CPF on the expression of a presynaptic protein, a postsynaptic protein, and iGluRs in primary hippocampal neurons. At the same time, we tried to determine whether the changes in synaptic plasticity caused by CPF are affected by the Wnt signalling pathway. In this study, we demonstrated that in addition to the decreased connections between neurons, the cell numbers and viability of primary hippocampal neurons were unchanged upon treatment with ≤ 50 μM CPF, regardless of the treatment time. And 50–150 μM CPF has been routinely used in previous related studies to evaluate the mechanisms underlying CPF-induced neurotoxicity [54-58]. Therefore, this concentration (50 μM) was regarded as 'low-level' and was used in all subsequent experiments to evaluate the risks associated with this exposure level. Moreover, CPF decreased the expression of Syp, PSD-95, NMDAR1, and GluR1in primary hippocampal neurons. We also found that the expression of Syp and PSD-95 in the hippocampus of rats decreased after CPF exposure. Syp and PSD-95 are the most widely used experimental protein markers of synaptic plasticity in the brain [22,34,35]. Moreover, both Syp and PSD-95 are associated with neurodevelopmental and neurodegenerative diseases. NMDARs and AMPARs play an important role in synaptic plasticity [40] and neuropathologies [41] relevant to learning and memory. The present study suggested that CPF exposure affects synaptic plasticity in hippocampal neurons. This might suggest a possible mechanism of low-level CPF-induced neurotoxicity.

A relationship has been established between low-level CPF exposure and developmental disorders, such as ADHD [8], ASD [9], and developmental retardation [6]. Reduced Syp was found in a mouse model of ADHD [59]. Recently, genomic studies have linked PSD-95 dysfunction to neurodevelopmental disorders such as ASD and intellectual disorder (ID) [60]. Moreover, NMDAR dysfunction has been linked to mental disorders including ADHD [61,62] and ASD [63]. Finally, a significant reduction in surface GluR1 subunits was observed in an animal model of ADHD [54]. A relationship has been also established between low-level CPF exposure and neurodegenerative diseases. CPF exposure increases PD risk [10,11]. Further, the expression of PSD-95 and Syp was found to be significantly decreased in a mouse model of PD [64]. Some studies have also found that the expression of NMDARs is decreased in the PD brain [65]; in addition, neuronal AMPAR levels were found to be decreased in brain tissue from knockout mice and human patients with PD[66-69]. However, some studies in humans present contrasting results. Some researchers reported that NMDA-binding sites are increased in several brain regions with PD ([70-72], as well as NMDAR levels [73,74,76,77]. However, alterations in NMDARs and AMPARs in PD in both experimental models and human tissue have shown contradictory results, and the altered expression levels have been confirmed by many studies.

Overall, Syp and PSD-95 expression decline in PD, ADHD, autism, and ID, consistent with the effect of CPF on Syp and PSD-95 in this study. However, except for a few studies showing that the expression of NMDARs and AMPARs in PD is increased, most have concluded that the expression of these markers in PD, ADHD, ASD, and ID is decreased, consistent with the effect of CPF on Syp and PSD-95 in this study. These results might explain the mechanism through which CPF is associated with these neurological disorders.

Wnt proteins play a crucial role in synaptic physiology, including the modulation of the synaptic vesicle cycle, trafficking of neurotransmitter receptors, and regulation of their interaction with scaffold proteins [77], regulating synaptic NMDARs and synaptic plasticity [78]. The Wnt/β-catenin pathway is referred to as the classical Wnt signalling pathway and can control hippocampal plasticity, regulating synaptic transmission and neurogenesis [79,80]. The addition of Wnt7a to the hippocampus increases both the synapse number and large mossy fibre terminals [81,82]. β-Catenin proteins are present at high levels during synaptogenesis and localise both pre- and postsynaptically [81]. Wnt signalling pathways are implicated in a variety of human pathologies, including developmental defects [43,83] and neurodegenerative disorders [44,45]. Our study suggests that low-level CPF exposure decreases the expression of Wnt7a and β-catenin, consistent with our previous conclusion that CPF exposure might affect synaptic plasticity. However, when we overexpressed β-catenin, Syp and PSD-95 expression was not increased. This indicates that the decline in NMDAR1, GluR1, PSD-95, and Syp caused by CPF is related to Wnt/β-catenin signalling in an indirect manner. Many factors could thus be involved in regulating NMDAR1, GluR1, PSD-95, and Syp expression, and the molecular mechanisms involved require further investigation.

Conclusion

In summary, we found that exposure of primary hippocampal neurons to low-level CPF down-regulated the expression of a presynaptic protein, a postsynaptic protein, and iGluRs and impacted the Wnt7a/β-catenin pathway. Further, rat hippocampus exposure to CPF down-regulated the expression of a presynaptic protein. This affected the synaptic plasticity of primary hippocampal neurons, which is associated with numerous neurodegenerative diseases and neurodevelopmental disorders. In summary, our research provides novel information regarding the mechanism of low-level CPF-induced neurotoxicity. Further studies are needed to examine the relationship between the effects of low-level CPF on hippocampal synaptic plasticity in animals and the severity of neurobehavioral changes, which might lead to treatments for neurodevelopmental and neurodegenerative disorders.

Declarations

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Conflict of interest

All authors declare that there are no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available upon request from the corresponding author.

Code availability: Not applicable

Authors' contributions: WZ and LZ designed this research. WZ, CZ, PW, and GW performed the experiments. WZ, YD, HD, QX, JT, and MZ analysed the data. WZ and LZ wrote the manuscript. All authors read and approved the final manuscript.

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Figures

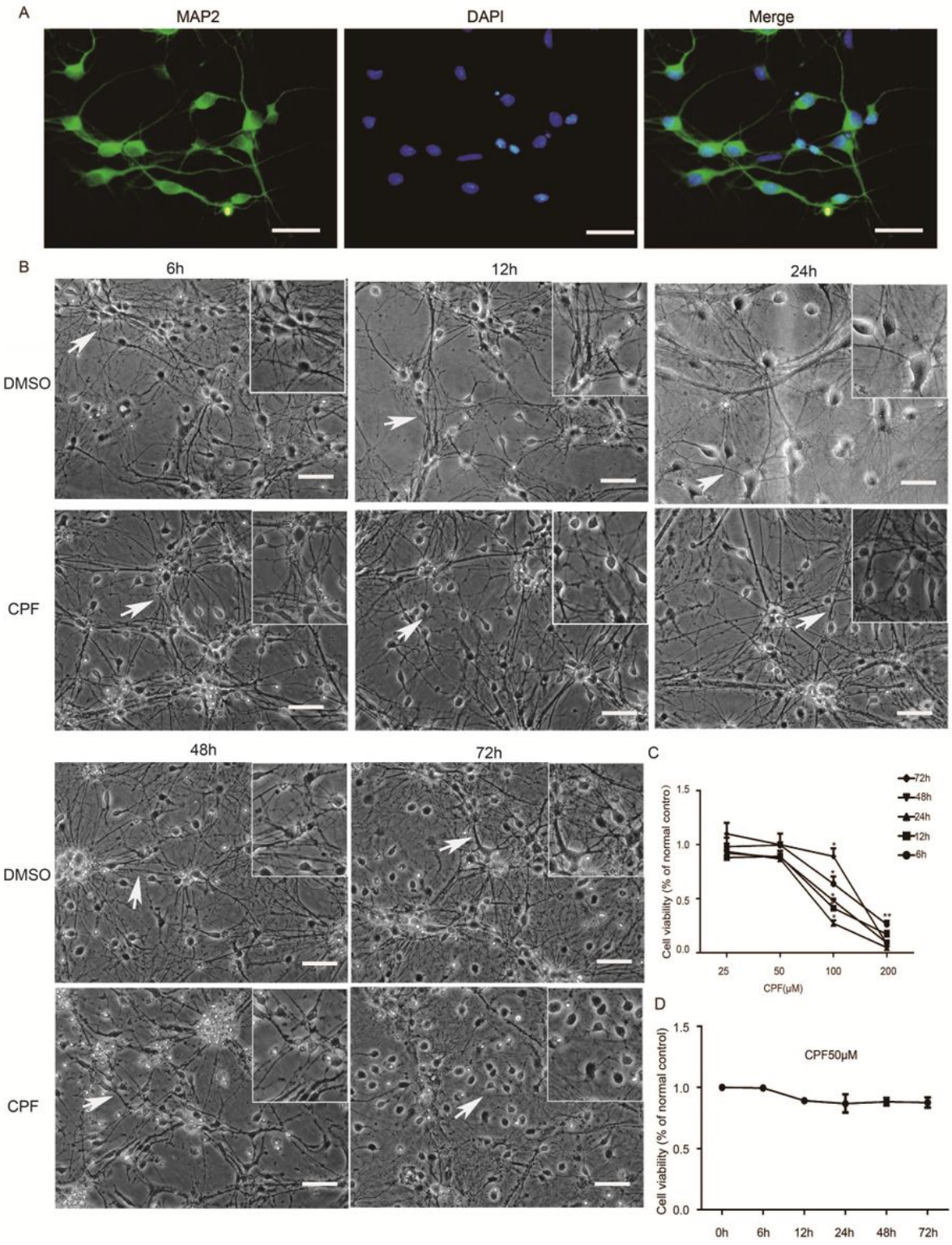

Figure 1

CPF affects the morphology, and survival of primary hippocampal neurons. A: Positive expression of MAP2 was used to identify primary hippocampal neurons (green: MAP2; Blue: DAPI, Scale bar: 25 μm). B: Representative bright-field images of primary hippocampal neurons as treated with CPF (50μM) in different time (6, 12, 24, 48, and 72h), Scale bar: 50μm.Boxed areas on the top right corner represented a magnification indicated by an arrow in the respective image. C: CCK-8 quantification of the survival of Primary hippocampal neuronsas treated withdifferent concentration of CPF (25, 50, 100, and 200μM) in each time group (6, 12, 24, 48, and 72h). D: After treated by CPF (50μM) in different time (6, 12, 24, 48, and 72h), cell viability was determined by CCK-8 assay (*p < 0.05. **p< 0.01).

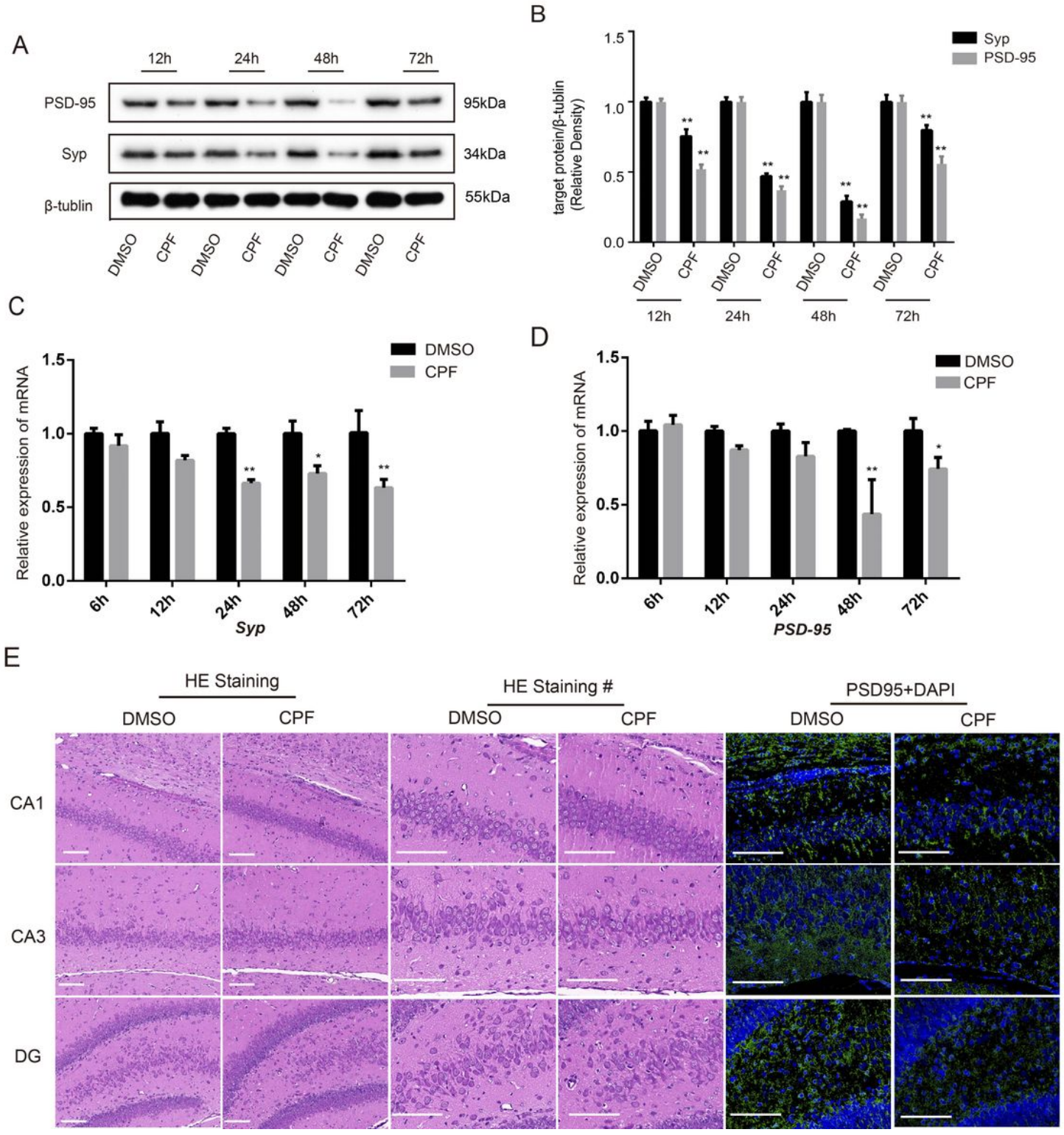

Figure 2

CPF affects the expression of Syp and PSD-95 in primary hippocampal neurons and the expression of PSD-95 in the rat hippocampus. A: Western blot analysis demonstrates levels of Syp and PSD-95 in Primary hippocampal neurons of each groups. β-tubulin was used as an internal gel loading control. B: Densitometry of the Syp and PSD-95 bands are correlated with the β-tubulin band (n = 3/group; ∗∗p< 0.01). C: Quantitative PCR analysis of Syp mRNA levels in primary hippocampal neurons of each groups

(n = 3/group; ∗p< 0.05; ∗∗p< 0.01). D: Quantitative PCR analysis of PSD-95 mRNA levels in primary hippocampal neurons of each groups (n = 3/group; ∗p< 0.05; ∗∗p< 0.01). E: Representative images of HE staining (different magnifications), PSD-95 (green), and DAPI (blue) immunofluorescence staining in CA1, CA3, and DG regions of the rat hippocampus in the two groups (scar bar: 100 μm).

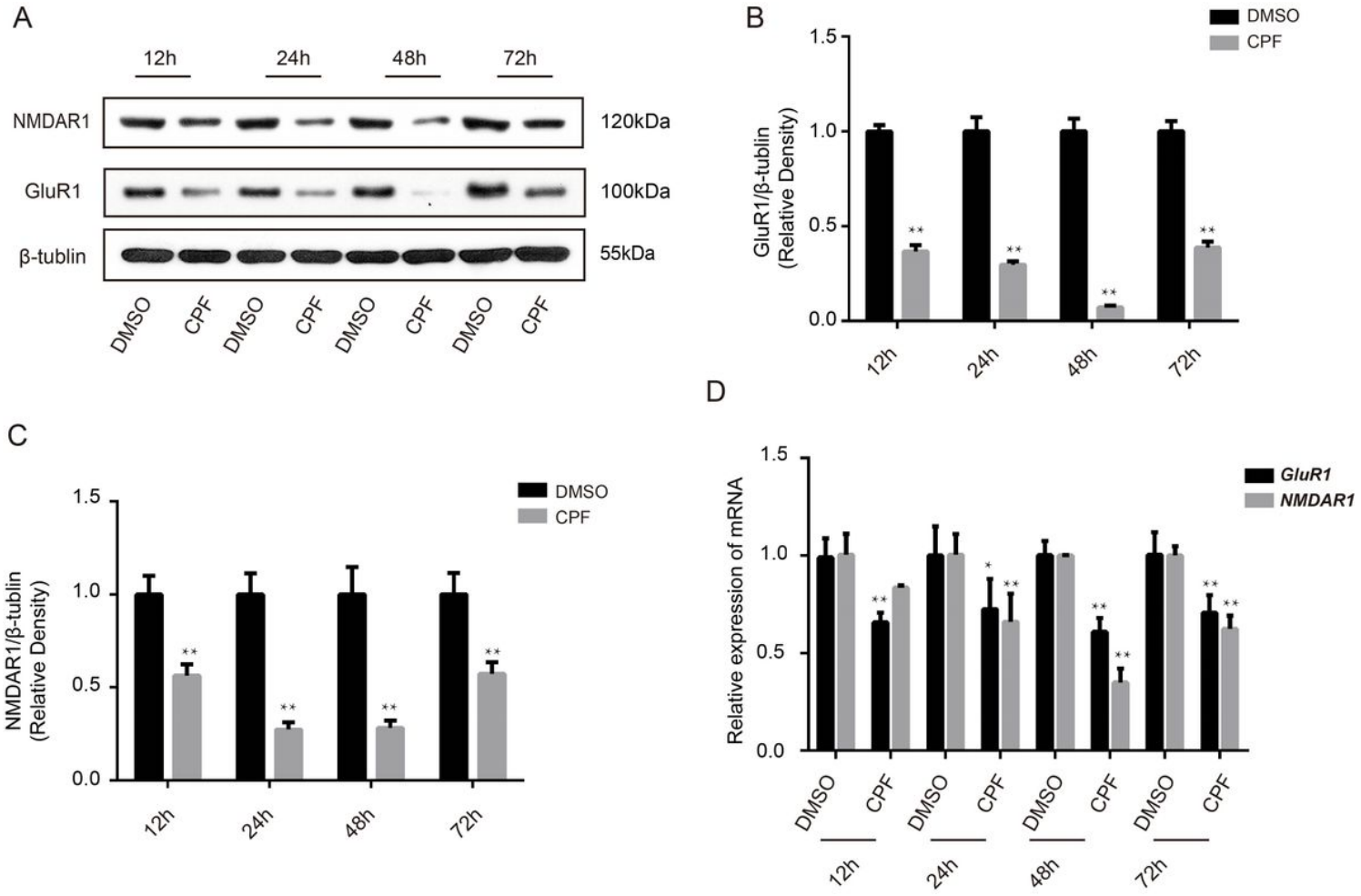

Figure 3

CPF affects the expression of GluR1 and NMDAR1 in primary hippocampal neurons. A: Western blot analysis demonstrates levels of GluR1 and NMDAR1 in Primary hippocampal neurons of each groups. β-tubulin was used as an internal gel loading control. B and C: Densitometry of the GluR1 and NMDAR1 bands are correlated with the β-tubulin band (n = 3/group;∗∗p< 0.01). D: Quantitative PCR analysis of GluR1 and NMDAR1 mRNA levels in primary hippocampal neurons of each groups (n = 3/group; ∗p< 0.05; ∗∗p< 0.01).

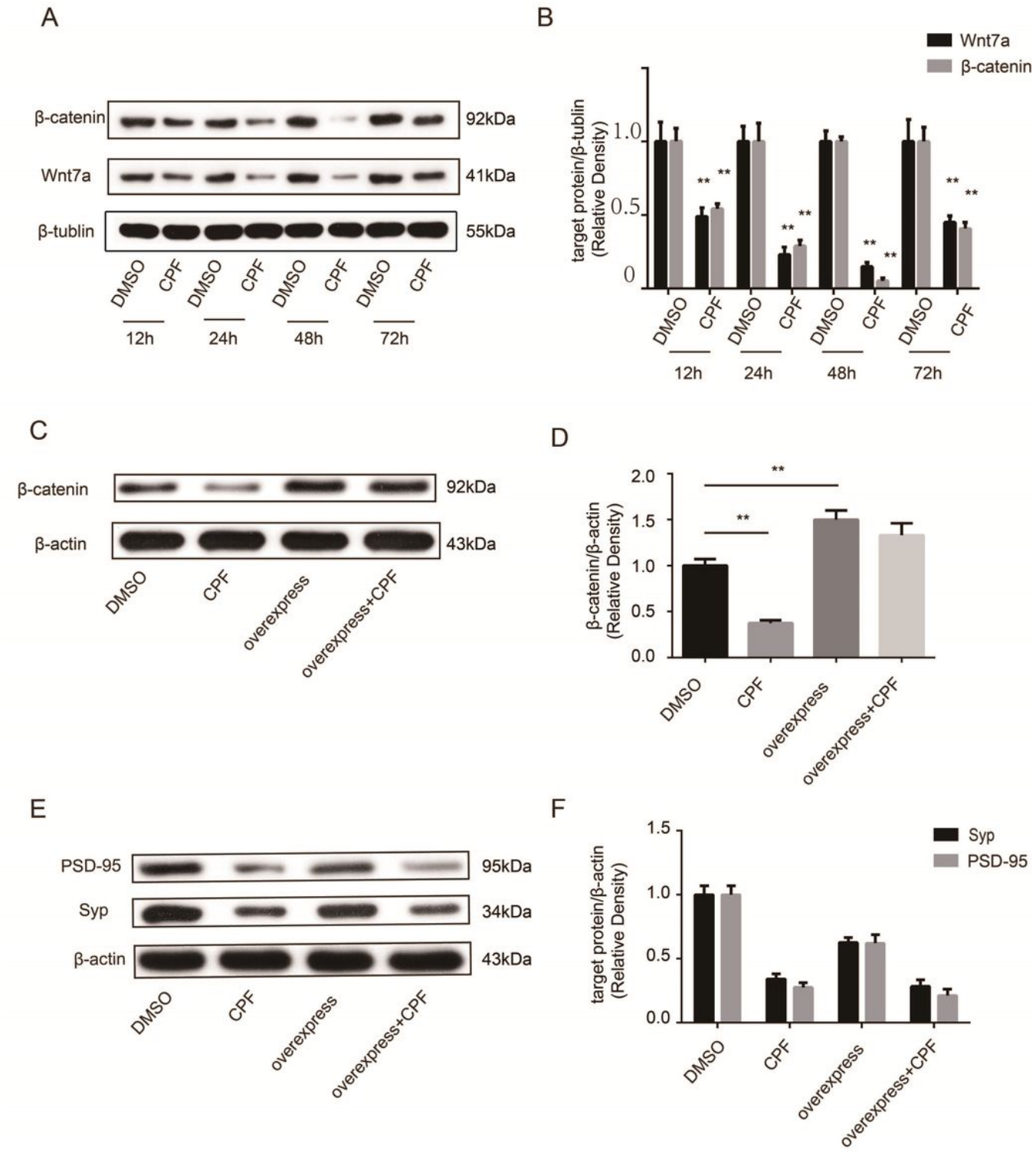

Figure 4

CPF affects the expression of Wnt7a and active-β-catenin in primary hippocampal neurons, but overexpress β-catenin did not improve the synaptophysin and PSD-95 protein levels caused by CPF. A: Western blot analysis demonstrates levels of Wnt7a and active-β-catenin in Primary hippocampal neurons of each groups. β-tubulin was used as an internal gel loading control. B: Densitometry of the Wnt7a and active-β-catenin bands are correlated with the β-tubulin band (n = 3/group; ∗∗p< 0.01). C: β-

catenin protein expression was determined by western blot in primary hippocampal neurons of each groups (CPF: 50μM, 48h overexpress: overexpress β-catenin). β-actin was used as an internal gel loading control. D: Densitometry of the β-catenin bands are correlated with the β-actin band(n = 3/group; ∗∗p< 0.01). E: Syp and PSD-95 protein expression was determined by western blot in Primary hippocampal neurons of each groups. β-actin was used as an internal gel loading control. F: Densitometry of the Syp and PSD-95 bands are correlated with the β-actin band (n = 3/group; ∗p< 0.05).