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Alteration of NRSF expression exacerbating 1-methyl-4-phenyl-pyridinium ion-induced cell death of SH-SY5Y cells

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ABSTRACT

Neuron-restrictive silencer factor (NRSF)/neuronal repressor element-1 silencing transcription factor (REST) and its neuron-specific truncated form REST4 participates in the pathological processes of nervous system diseases, such as global ischemia, epilepsy, Huntington disease and so on. In this paper, we investigated the changes of NRSF and REST4 in a cellular model of Parkinson's disease (PD). Our results showed that neurotoxin 1-methyl-4-phenyl-pyridinium ion (MPP^+) treatment triggered the mRNA and protein expression of NRSF and REST4, and caused both NRSF and REST4 proteins relocalized between the nucleus and cytoplasm in human dopaminergic SH-SY5Y cells. Redistribution of NRSF and REST4 derepressed the expression of target genes at late time points. Furthermore, alteration of NRSF and REST4 expression by overexpression or RNAi techniques elicited deleterious effects on cell viability of SH-SY5Y treated with toxic MPP^+.

1. Introduction

Transcriptional abnormality of neuronal genes plays an important role in the process of nervous system diseases. NRSF/REST (neuron-restrictive silencer factor or repressor element-1 silencing transcription factor), an essential zinc-finger transcription factor, restricts the expression of a group of neuron-specific genes in non-neuronal cells through histone deacetylation, chromatin remodeling, methylation and other mechanisms (Ballas and Mandel, 2005; Lunyak et al., 2002; Palm et al., 1998). Examples of such neuron-specific genes are mu-opioid receptor (Andria and Simon, 2001), AMPA receptor (AMPAR) subunit GluR2 (Calderone et al., 2003), synapsin I (Nishimura et al., 1996; Schoenherr and Anderson, 1995) and *BDNF* (Zuccato et al., 2007), Superior Cervical Ganglia 10 (*SCG10*) (Eggen and Mandel, 1997), neuron-glia cell adhesion molecule (*Ng-CAM*) (Eggen and Mandel, 1997) and neuronal β−III tubulin. Recently, lines of researches indicated that NRSF participated in the pathogenesis of nervous system diseases, such as global ischemia, epilepsy and degenerative Huntington disease (HD). That changes in the expression or localization of NRSF consequently affect the expression of downstream genes may be an important mechanism of neuronal death (Formisano et al., 2007; Spencer et al., 2006; Zuccato et al., 2003). Parkinson's disease (PD) is a common neurodegenerative disease in the central nervous system, and featured by the progressive loss of dopaminergic neurons in the substantia nigra (SN). Tyrosine hydroxylase (TH) is the rate-limiting enzyme of dopamine synthesis. In the promoter region of human TH gene, there exists a neuron-restrictive silencer element (NRSE), which could bind NRSF. The outcome of such a combination is silencing TH expression (Kim et al., 2008). Moreover, PD-related gene ubiquitin carboxyl-terminal hydroxylase L1 (*UCH-L1*) has been confirmed to be one of NRSF target genes (Barrachina et al., 2007). Therefore, we speculated NRSF might be relevant to PD. REST4, a neuron-specific truncated form of NRSF, could resist the silencing function of NRSF (Shimojo et al., 1999); however, the function of REST4 is controversial (Magin et al., 2002).

1-methyl-4-phenyl-pyridinium ion (MPP^+) is a conventional neurotoxin inducing dopaminergic cell death. By treating SH-SY5Y cells with MPP^+, we found both the expression and cellular localization of NRSF and REST4 were changed, which in turn caused expression changes of downstream genes. Alteration of NRSF and REST4 expression by overexpression or RNAi techniques decreased viability of SH-SY5Y cells under the treatment of MPP^+.

2. Materials and methods

2.1. Materials

1-methyl-4-phenyl-pyridinium ion (MPP^+) was a product of Sigma (D048, St. Louis, USA.).

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2.2. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from SH-SY5Y cells using Trizol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was carried out using random primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA). Real-time PCR was performed for quantification of *NRSF*, *REST4*, brain derived neurotrophic factor (*BDNF*), *TH*, synapsin I, *UCH-L1*, *Atp1b3* and β-actin mRNA on Rotor-Gene 3000 (Corbett Research, Sydney, Australia) based on published methods (Bian et al., 2008; Kawakami et al., 2009). For plotting a standard curve, serially diluted copies of *NRSF*, *REST4*, *BDNF*, and *TH*, synapsin I, *UCH-L1*, *Atp1b3* and β-actin DNA fragments were used in each experiment. Expression of *NRSF*, *REST4*, *BDNF*, and *TH*, synapsin I, *UCH-L1*, *Atp1b3* and β-actin was quantified to the standard curve respectively, and the relative expression value was calculated. The primers used in the real-time PCR were: 5′-GTGAGCGAGTATCACTGGAGG-3′(*NRSF*-1 or *REST4*-1), 5′-CCCATTGTGAACCTGTCTTGC-3′(*NRSF*-2); 5′-CCATCTAGATCACACTCTGAATG-3′(*REST4*-2); 5′-CAAACATCCGAGGACAAGGTGG-3′(*BDNF*-1), 5′-CTCATGGACATGTTTGCAGCATCT-3′(*BDNF*-2); 5′-AGTGCACCCAGTATATCCGCC-3′(*TH*-1), 5′-GACACGAAGTAGACTGACTGGTACGTC-3′(*TH*-2); 5′-CGTGCGTGTCCAGAAGATTG-3′(synapsinI-1), 5′-TGTGATCCCTTCCGTCCTTG-3′(synapsinI-2); 5′-CTGGGATTTGAGGATGGATCAG-3′ (*UCH-L1-1*), 5′-ACCTTGGCAGCGTCCTTCAGCAG-3′ (*UCH-L1-2*); 5′-GCCGAGTGGAAGCTCTTCATC-3′(*Atp1b3*-1), 5′-AACCATGAGTCCTGGGCTAGG-3′(*Atp1b3*-2); 5′-ATGAGGTAGTCAGTCAGGT-3′(β-actin-1), 5′-ATGGATGATGATATCGCC-3′ (β-actin-2).

2.3. Protein extraction and Western blot analysis

The method of protein extraction and Western blot analysis has been described elsewhere (Bian et al., 2008). Briefly, Cells were lysed in RIPA buffer (50 mM Tris-HCl, PH7.5; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate) containing complete protease inhibitor cocktail (Calbiochem, San Diego, USA). Nuclear and cytosolic extracts were prepared according to the manufacturer's instruction (Beyotime, Haimen, China). Protein samples were separated on 8% or 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked by 5% non-fat dried milk in TBS-T [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween] at room temperature for 2 h and incubated with the following primary antibodies at room temperature for 2 h: rabbit anti-NRSF (SC-25398X, Santa Cruz, CA, USA.) (1:1000 dilution), rabbit anti-histone H1 (SC-10806, Santa Cruz, CA, USA.) (1:1000 dilution), mouse anti-FLAG (F7425, Sigma, St. Louis, USA.) (1:1000 dilution) and mouse anti-actin (sigma, St. Louis, USA.) (1:6000 dilution). Subsequently, the membranes were washed with TBS-T and incubated for 1 h with goat peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (1:10,000 dilutions in TBS-T) at room temperature. They were then washed three times with TBS-T and detected with a chemiluminescence detection system (sc-2048; Santa Cruz, CA, USA). The protein levels were quantified by densitometry analysis using Quantity One 4.5.2 software (Bio-Rad, Hercules, USA).

2.4. Plasmid constructs and Lentivirus production

NRSF-FLAG or REST4-FLAG fragments were cloned into the mammalian expression vector pcDNA3.1 (named pcDNA3.1-NRSF-FLAG or pcDNA3.1-REST4-FLAG) and sequenced. Additionally, we constructed three lentiviral vectors backed upon pLL3.7 (a CMV-EGFP expression cassette locates downstream of the U6 promoter): Lenti-NRSF-FLAG, Lenti-REST4-FLAG and Lenti-NRSF shRNA. DNA fragments containing CMV promoter and coding sequences of NRSF-FLAG or REST4-FLAG in plasmid pcDNA3.1-NRSF-FLAG or pcDNA3.1-REST4-FLAG were cut out by restriction enzymes Sal I (blunted with Klenow) and Xho I, and inserted into Lentiviral vector pLL3.7 digested with Xba I (blunted with Klenow) and Xho I (in this way, U6 promoter was removed). For Lenti-NRSF shRNA construct, synthesized DNA fragment targeting 19 nucleotides of human NRSF mRNA sequence (position 1012–1030, NM_005612.3) was cloned downstream to U6 promoter of pLL3.7. The top strand is: 5′ **T**GCTACAATACTAATCGATAttcaagagaTATCGATTAGTATTGTAGCttttt**C** 3′ and the bottom strand is: 5′ **TCGAG**aaaaaaGCTACAATACTAATCGATAtctcttgaaTATCGATTAGTATTGTAGC**A** 3′. The targeting sequence for the NRSF is underlined. All three final constructs were proved by DNA sequencing. The expression of NRSF, REST4 or the efficiency of shRNA were confirmed by Western blotting after transfecting SH-SY5Y cells with these lentiviral vectors using transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, USA.).

Lentivirus was produced by transfection of the lentiviral vector together with packing plasmid pCMVΔ8.9 and VSV-G into HEK293T cells. Culture supernatants were harvested by 48 h, and viral particles were concentrated by ultracentrifugation. Lentiviral particles were added onto SHSY-5Y cells, and 48 h later, the infection efficiency was determined by analyzing GFP expression using flow cytometry.

2.5. Intracellular localization of NRSF or REST4 assessed by immunofluorescence confocal imaging

The SH-SY5Y cells were cultured in DMEM with 10% fetal calf serum at 37 °C in 5% CO_2. For transfection, cells were plated in 6-well plates and transfected the next day according to the manufacturer's instruction (Lipofectamine 2000, Invitrogen). The transfected cells were grown for 24 h, digested and plated on circular glass coverslips (13 mm) in 24-well plates. After 24 h, cells were administrated with MPP^+ (final concentration, 1 mM) for 3, 8, and 20 h. Cells were washed with Dulbecco's Phosphate Buffered Saline (PBS), fixed for 10 min with 4% paraformaldehyde in PBS, washed three times, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked with 10% normal goat serum for 30 min at 37 °C. Fixed cells were then incubated overnight in primary antibodies at 4 °C, washed with PBS, incubated with secondary antibodies for 1 h at 37 °C, washed again, followed by counterstaining with Hoechst 33258 (Beyotime, Haimen, China) for 5 min and then washed with PBS. Coverslips were mounted in glass slides. Fluorescence was observed using confocal laser scanning microscopy (TCS SP2, Leica, Germany).

2.6. WST-1 cell viability assay

Cell viability was measured by modified MTT assay. Briefly, 2×10^3 cells were cultured in 96 well plate of flat bottom in a final volume of 100 μl/well culture medium. After 24 h, the cells were administrated with 1 mM MPP^+ or PBS for 48 h. 10 μl WST-1 (Beyotime, Haimen, China) was added to each well and cells were cultured for additional 4 h in a humidified atmosphere. Then, the plate was shaked thoroughly for 1 min. The absorbance of samples was measured under a wavelength of 450 nm using a microtiter plate reader (Sunrise Remote/Touch screen, Columbusplus, Austria).

2.7. Data analysis

Experiments were repeated at least three times. Data were analyzed using SPSS software (version 11.5; SPSS, Chicago, USA). For comparison of statistical significance between groups,

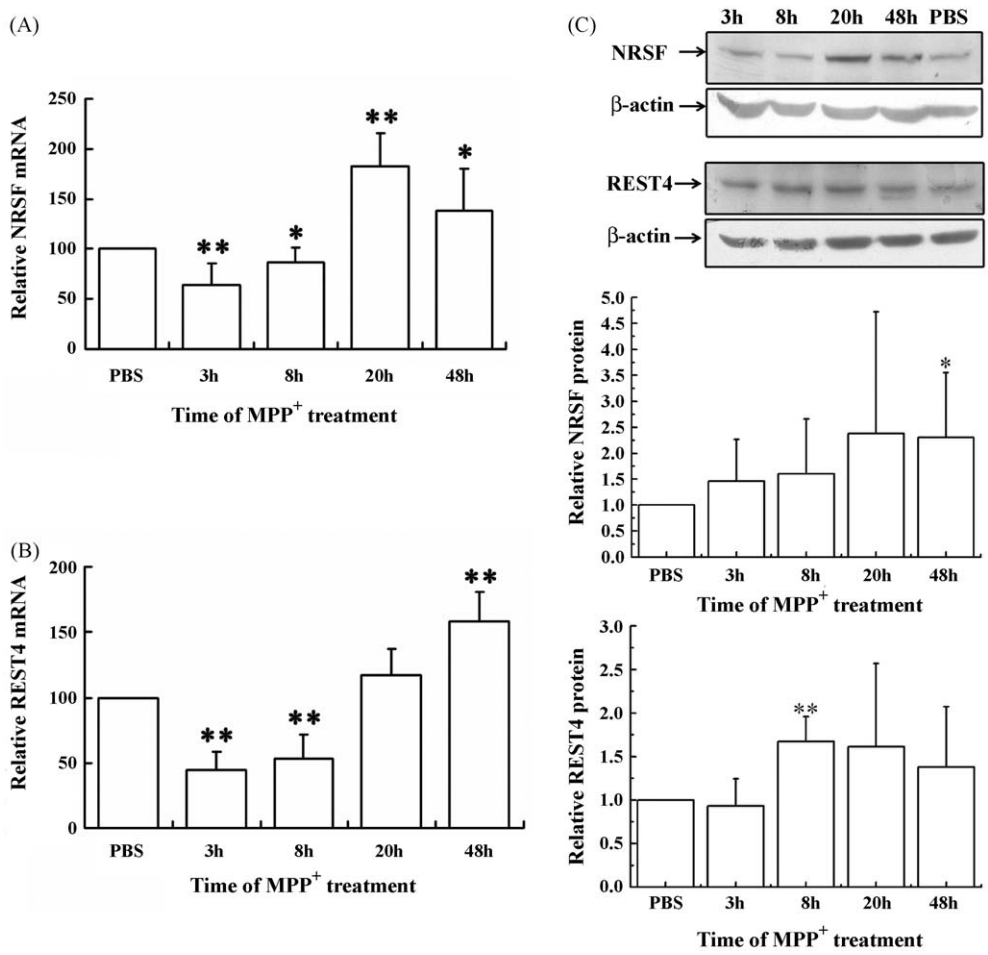

Fig. 1. Effects of MPP^+ treatment on the expression of NRSF and REST4 in SH-SY5Y cells. Cells were treated with 1 mM MPP^+ for 3, 8, 20, 48 h or PBS (served as control). Data are presented as mean ± SD ($^*P < 0.05$ or $^{**}P < 0.01$, $n = 6$); Real-time RT-PCR analysis of NRSF (A) and REST4 (B) mRNA. (C) Western blot analysis for NRSF and REST4, β-acting served as a loading control; Bars show relative NRSF and REST4 protein abundance.

Student's *t*-test was used. *P* value <0.05 was considered as significant.

3. Results

3.1. MPP^+ treatment increased the expression of NRSF and REST4 in SH-SY5Y cells

To investigate the effect of MPP^+ on the expression of NRSF and REST4 in human dopaminergic SH-SY5Y cells, real-time PCR was performed to detected the mRNA expression levels after treatment with 1 mM MPP^+ for 3, 8, 20 or 48 h. At early time points, 3 and 8 h after exposure to MPP^+, the transcriptional expression of *NRSF* and *REST4* decreased dramatically compared to PBS controls ($P < 0.05$). However, their mRNA expression recovered or exceeded the basal levels at 20 and 48 h after the MPP^+ administration. Expression of *NRSF* increased significantly at 20 h ($P < 0.01$) and 48 h post stimulation ($P < 0.05$), while *REST4* mRNA expression increased at 48 h after the MPP^+ exposure ($P < 0.01$) (Fig. 1A and B).

We also investigated the changes of NRSF and REST4 proteins after MPP^+ treatment in SH-SY5Y cells. The results indicated that the protein level of NRSF significantly increased at 48 h ($P < 0.01$) and REST4 increased dramatically at 8 h ($P < 0.01$) post stimulation compared to controls by Western blotting (Fig. 1C).

3.2. Effect of MPP^+ treatment on cellular localization of NRSF and REST4 in SH-SY5Y cells

NRSF as a transcription silencer executes its functions in the nucleus. Abnormal changes in its localization also cause nervous disorders (Schoenherr and Anderson, 1995). It is of interest to examine whether MPP^+ could affect the cellular distribution of NRSF and REST4. To this end, we analyzed nuclear and cytosolic extracts of SH-SY5Y cells after administrating MPP^+ for 20 h by Western blotting. The results showed that compared with controls, the relative content of NRSF and REST4 protein significantly increased in the cytoplasm after 20 h MPP^+ treatment ($P < 0.05$). Meanwhile, the relative content of NRSF and REST4 protein in the nuclei was dramatically decreased ($P < 0.05$). This result suggested that MPP^+ stimuli caused a relocation of both NRSF and REST4 in SH-SY5Y cells (Fig. 2A).

By exploring immunofluorescence and confocal imaging, the effects of MPP^+ treatment on cellular localization of NRSF and REST4 in SH-SY5Y cells were further elucidated. First, to validate the constructed plasmids, pcDNA3.1-NRSF-FLAG and pcDNA3.1-REST4-FLAG were transfected into SH-SY5Y cells respectively. In SDS-polyacrylamide gel electrophoresis, NRSF-FLAG migrated at 160 kD and exhibited an additional band of about 200 kD, and REST4-FLAG migrated at approximately 50 kD, no band could be observed in the extracts from non-transfected SH-SY5Y cells (vehicle) as shown (Fig. 2B). Similar results with multiple

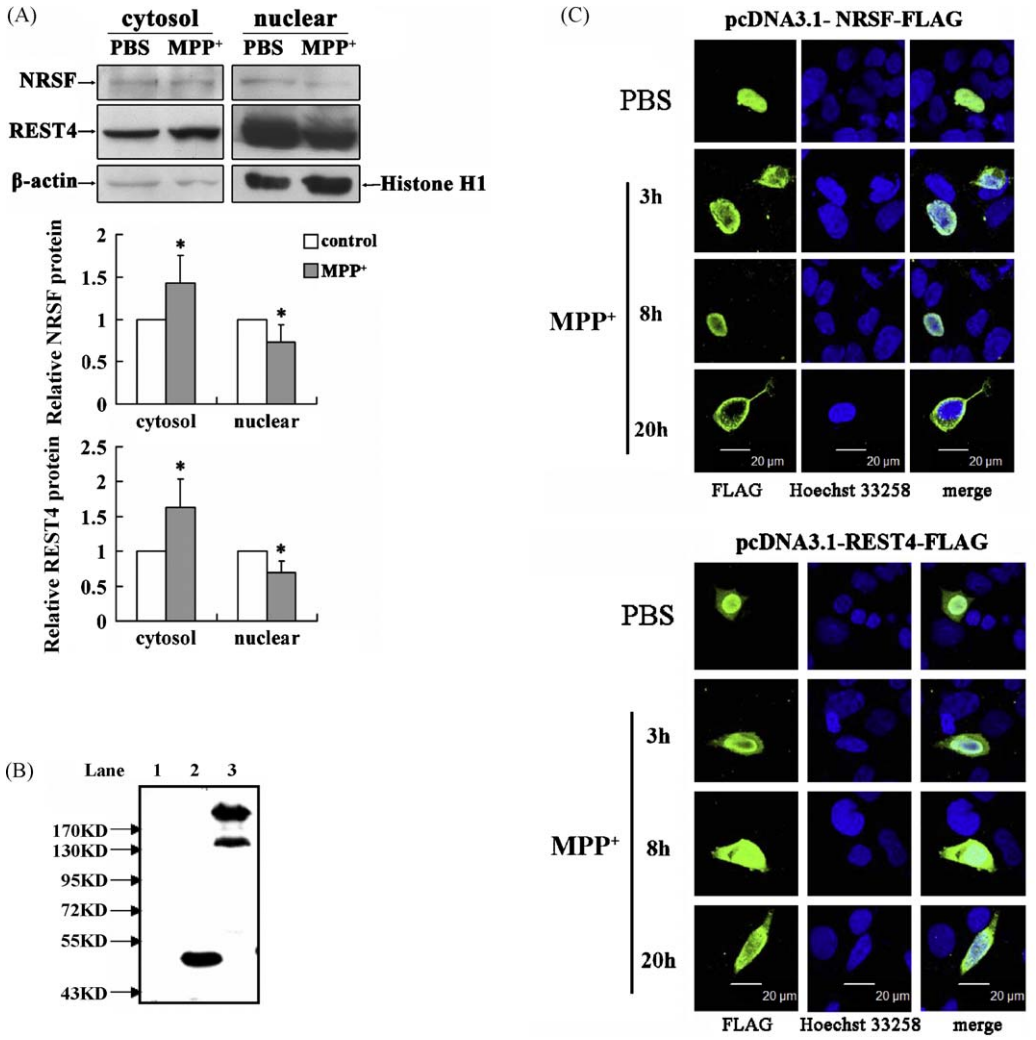

Fig. 2. Cytoplasmic and nuclear localization of NRSF or REST4 protein in SH-SY5Y cells after the treatment of MPP+ or PBS (served as control) for 20 h. Data are presented as mean ± SD (*$P < 0.05$, $n = 6$). (A) Western blot analysis for endogenous NRSF and REST4, β-acting and Histone H1 served as a loading control for cytoplasmic and nuclear protein respectively; Bars show relative NRSF and REST4 protein abundance. (B) Western blot analysis for the expression of pcDNA3.1-NRSF-FLAG and pcDNA3.1-REST4-FLAG in SH-SY5Y cells by anti-FLAG antibody. Lane 1: pcDNA3.1, Lane 2: pcDNA3.1-REST4-FLAG, Lane 3: pcDNA3.1-NRSF-FLAG. Immunoflurescence confocal analysis for the intracellular localization of NRSF (C) or REST4 (D) in SH-SY5Y cells after MPP+ treatment for 3h, 8 h and 20 h. Cells were stained with anti-FLAG antibody (green) as well as Hoechst 33258 (blue). Also shown are merged images (right) of FLAG (left) and Hoechst (middle) staining (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

immunoreactive bands have been reported previously, suggesting post-translational modifications of NRSF (Lee et al., 2000; Shimojo et al., 2001). By immunofluorescence staining with the anti-FLAG antibody, we demonstrated that both NRSF and REST4 were predominantly located in the nuclei of SH-SY5Y cells under the normal culture conditions. They mainly localized around the nuclear membrane region at 3 h after MPP+ treatment. NRSF still distributed around the nuclear membrane region, and REST4 distributed evenly in the nuclei and cytosol at 8 h after MPP+ treatment. Both NRSF and REST4 were primarily in cytosol at 20 h after exposure to MPP+ (Fig. 2C and D). These data indicated both NRSF and REST4 delocalized from the nucleus to the cytosol after MPP+ intoxication. Worthy of that not in all cells NRSF and REST4 had a coherent relocalization, so NRSF and REST4 still could be detected in nuclear extracts after MPP+ stimulation.

3.3. Effect of MPP+ treatment on NRSF target gene expression in SH-SY5Y cells

The consequent effects of relocalization of NRSF and REST4 on the expression of target genes were examined by real-time PCR. Among hundreds of NRSF target genes, *BDNF*, *TH*, synapsin I and *UCH-L1* were selected due to their relevance to PD. At 3 or 8 h after MPP+ treatment, their mRNA expression levels were significantly decreased ($P < 0.05$), which might be related to cell stress response. Afterwards, the transcriptional levels of *BDNF*, *TH*, synapsin I returned to normal levels at 20 and 48 h after MPP+ treatment. And *UCH-L1* mRNA expression significantly increased at 20 and 48 h after MPP+ stimulation ($P < 0.05$) (Fig. 3). To evaluate the specificity of these transcriptional changes, we also quantified the mRNA level of house-keeping gene *Atp1b3* (coding for the β3-subunit of Na^+/K^+ ATPase). The mRNA expression of *Atp1b3* did not vary significantly during the treatment of MPP+.

3.4. Changes of NRSF or REST4 reduced cell viability in MPP+-treated SH-SY5Y cells

Moreover, the impact of changes of NRSF or REST4 on cell viability was conducted. By Western blotting, in transfected SH-SY5Y cells, the shRNA could downregulate the expression of NRSF by 39% (Fig. 4A). In non-transfected cells, or cells transfected with

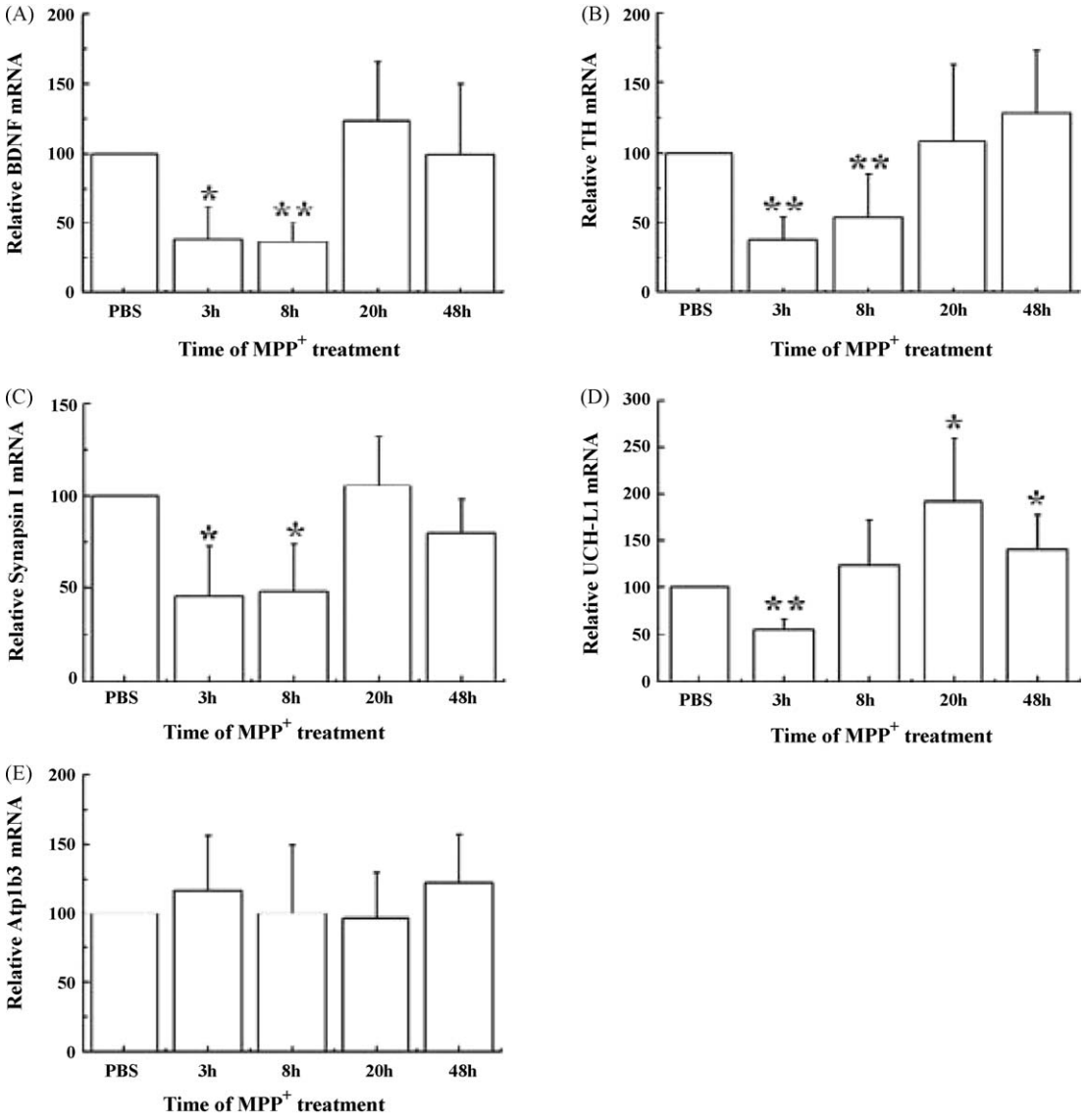

Fig. 3. Effects of MPP+ treatment on NRSF target genes expression in SH-SY5Y cells. Cells were treated with 1 mM MPP+ for 3, 8, 20, 48 h or PBS (served as control). Data are presented as mean ± SD(* $p < 0.05$ or ** $P < 0.01$, $n = 6$); Real-time RT-PCR analysis of *BDNF* (A), *TH* (B), synapsin I (C), *UCH-L1*(D) and Atp1b3 (E) mRNA.

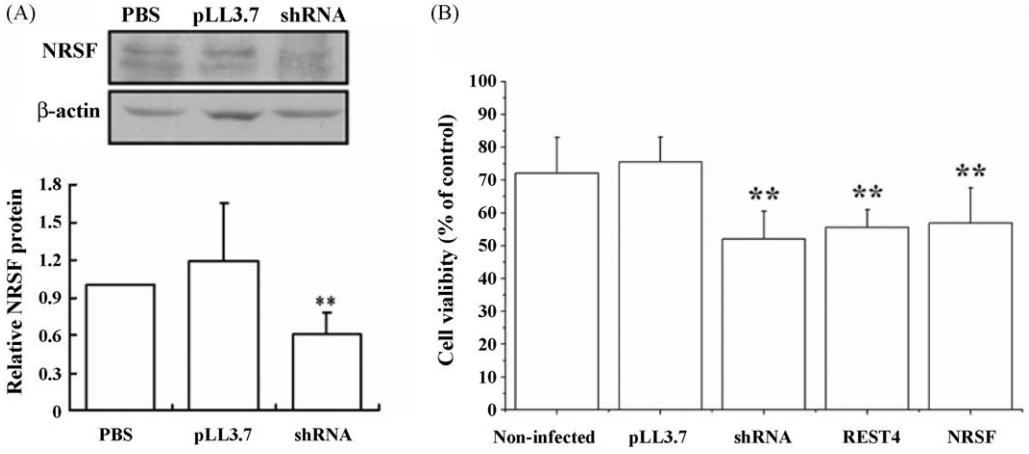

Fig. 4. The effect of NRSF and REST4 downregulation or overexpression on SH-SY5Y cell proliferation after MPP+ treatment for 48 h. Data are represented as means ± SD (** $P < 0.01$, $n = 6$). (A) Western blot revealed NRSF protein following infection with pLL3.7 or Lenti-NRSF shRNA in SH-SY5Y cells, non-infected cells served as control, and β-acting served as a loading control; Bars show relative NRSF protein abundance. (B) The cells proliferations were analyzed by WST-1 assay.

pLL3.7, NRSF proteins expression had no significant difference. The validation of lentiviral vectors Lenti-NRSF-FLAG and Lenti-REST4-FLAG were confirmed by Western blotting using FLAG antibody in transfected SH-SY5Y cells (data not shown). For the longer expressions and avoiding the toxic effect of transfection reagent, SH-SY5Y cells were infected with lentiviral particles, expressing NRSF-FLAG, REST4-FLAG, NRSF shRNA or the control respectively. We detected the viability of infected cells after 48 h MPP^+ treatment by modified MTT assay. The viability rate of SH-SY5Y cell after MPP^+ treatment decreased 27.93%, which confirmed the toxic effects of MPP^+. And pLL3.7 lentiviral infection didn't alter cell viability. The relative cell viabilities of SH-SY5Y cells infected with Lenti-NRSF shRNA, Lenti-NRSF-FLAG or Lenti-REST4-FLAG viral particles were 52.02%, 55.59% and 56.86%, respectively, and they were significantly lower than the control group ($P < 0.01$) (Fig. 4B). These results suggest that neither overexpression nor downregulation of NRSF or REST4 was conducive to cell proliferation.

The transcriptional levels of the downstream gene *BDNF*, *TH*, *synapsin I* and *UCH-L1* in the NRSF or REST4 overexpressed as well as knock down cells with or without MPP^+ treatment were further examined by real-time PCR analysis. Our results showed that BDNF, synapsin I and UCH-L1 mRNA expression increased dramatically compared to PBS controls at 20 or 48 h after exposure to MPP^+ in Lenti-shRNA infected cells ($P < 0.05$) (Fig. 5A). BDNF, synapsin I and UCH-L1 mRNA expression decreased significantly after 3, 8 or 48 h MPP^+ treatment in Lenti-NRSF-FLAG infected cells ($P < 0.05$) (Fig. 5B), while TH, Synapsin I and UCH-L1 mRNA expression decreased dramatically after 3, 8 or 48 h MPP^+ treatment in Lenti-REST4-FLAG infected cells ($P < 0.05$) (Fig. 5C). The changing patterns of BDNF, TH, synapsin I and UCH-L1 mRNA expression in the NRSF or REST4 overexpressed cells were to some extent similar with non-infected SH-SY5Y cells after MPP^+ treatment.

4. Discussion

The expression and localization of NRSF and REST4 are well-regulated in cells (Calderone et al., 2003; Formisano et al., 2007; Spencer et al., 2006; Zuccato et al., 2003). Changes of NRSF or REST4 play important roles in the pathological process of nervous system diseases. For examples, global ischemia triggers NRSF expression, which suppresses GluR2 promoter activity and gene expression in neurons destined to die (Calderone et al., 2003). In the epilepsy model, the interplay between NRSF and REST4 alters the expression of pro-convulsant genes, and may therefore regulate the progression of epileptogenesis (Spencer et al., 2006). Moreover, mutant huntingtin fails to tether NRSF in the cytoplasm, which causes silencing of neuronal genes in Huntington disease (Zuccato et al., 2003).

Here, our present study showed that NRSF and REST4 expression increased in SH-SY5Y cells after MPP^+ exposure. That

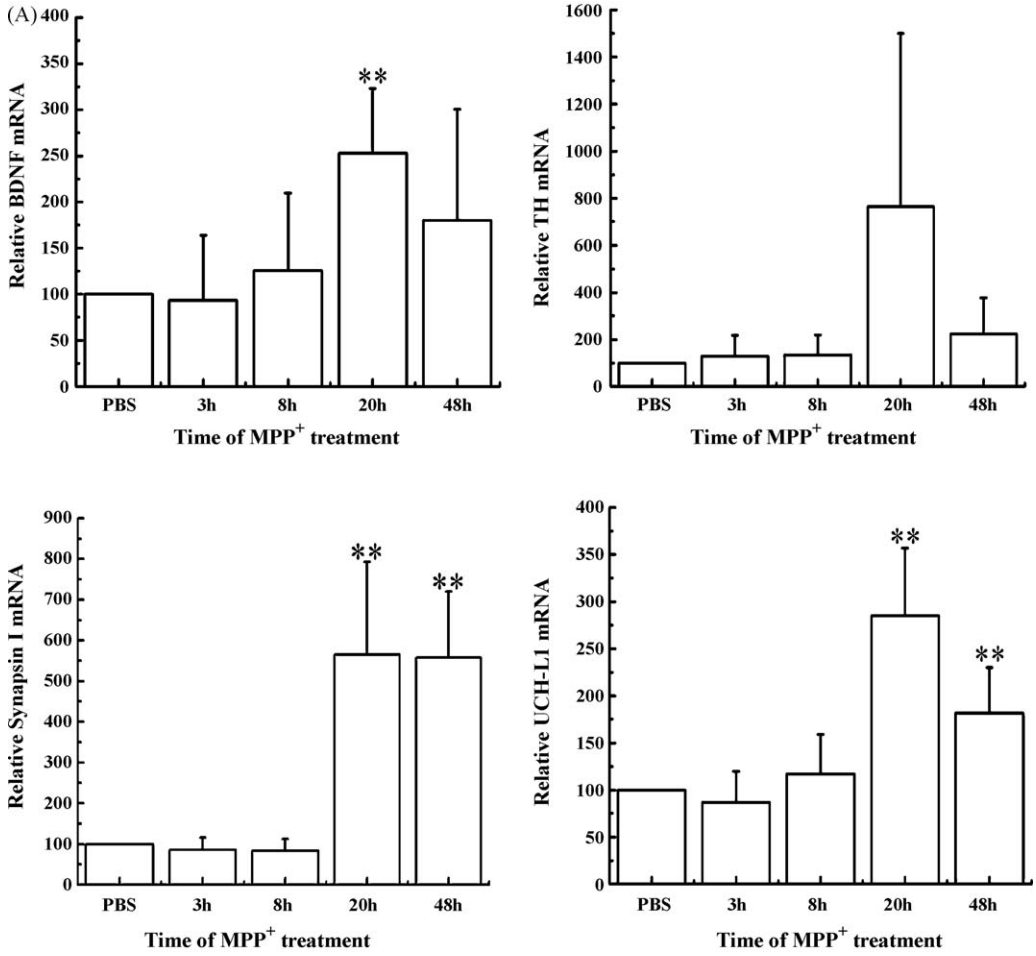

Fig. 5. Effects of MPP^+ treatment on the expression of NRSF target gene *BDNF*, *TH*, *synapsin I* and *UCH-L1* in SH-SY5Y cells infected with Lenti-NRSF shRNA (A), Lenti-NRSF-FLAG (B) or Lenti-REST4-FLAG (C). Cells were treated with 1 mM MPP^+ for 3, 8, 20, 48 h or PBS (served as control). Data are presented as mean ± SD ($^*P < 0.05$ or $^{**}P < 0.01$, $n = 6$).

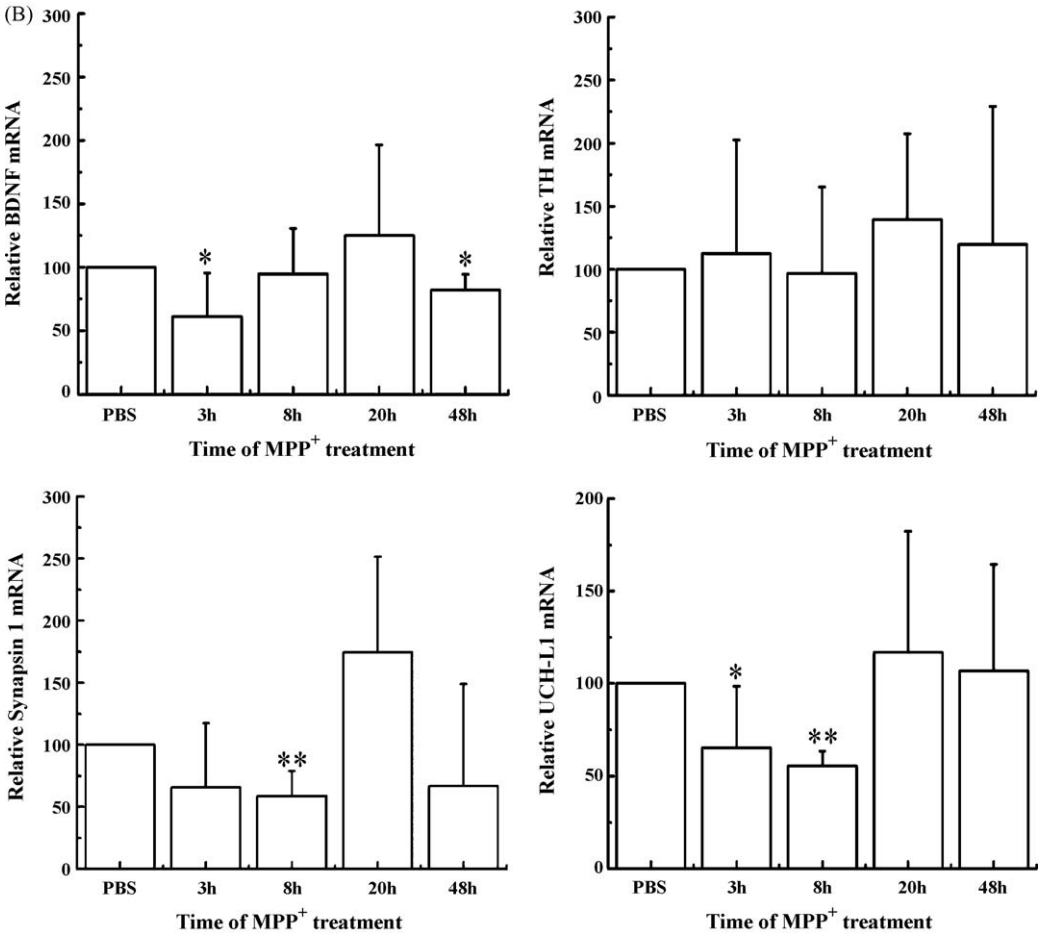

Fig. 5. (*Continued*)

the changes of NRSF and REST4 protein expression had no accordance with mRNA expression suggested additional regulatory mechanisms on NRSF and REST4 expression at the translation or post-translational levels (Calderone et al., 2003; Kim et al., 2008; Palm et al., 1998). Nuclear protein import, including that of transcription factor such as NRSF or REST4, was a key control point in regulating gene expression. The cellular compartments of NRSF and REST4 are determined by variant factors, like cell types and physiological conditions. As for REST4, there is much less controversies of its cellular locations. REST4 is mainly settled in the nuclei.

We demonstrated that both NRSF and REST4 relocalized between the nucleus and the cytoplasm of SH-SY5Y cells under MPP^+ treatment. Like epileptic insults causing REST4 moving into the cytoplasm and dendrites of the hippocampal cells (Spencer et al., 2006), MPP^+ induced similar effect on the localization of REST4. Unlike ischemia or epileptogenic reagents increased NRSF protein expression in nuclei of hippocampal neurons (Calderone et al., 2003; Spencer et al., 2006), toxic MPP^+ increased NRSF protein in the cytoplasm of SH-SY5Y cells. The divergence could be due to the different cell types.

In this PD cell model, there was a dramatic but transient decrease of NRSF target gene *BDNF*, *TH*, synapsin I and *UCH-L1* which could be related to cell stress responses. The recovery or increase of NRSF target genes expression might be explained by the relocation of NRSF and REST4. However, the expressions of *BDNF*, *TH*, synapsin I only restore to normal levels and didn't show an overshot phase, which suggests that the expressions of neuron-specific genes are regulated concertedly by a variety of factors. This situation is similar with previous report that patterns of derepression through mutant NRSF or a dominant-negative form of NRSF exhibited by SCG10, Ng-CAM and neuronal β III tubulin are distinct (Eggen and Mandel, 1997). The changes of *UCH-L1* gene expression in MPP^+-treated SH-SY5Y cells are similar to those under OGD conditions in human embryonal carcinoma NT2/D1 cells (Shen et al., 2006). UCH-L1 may undertake a role commonly inside cells under different insults. In the NRSF or REST4 modified SH-SY5Y cells, after exposure to MPP^+, transcriptional levels of *BDNF*, *TH*, *synapsin I* and *UCH-L1* do not show a coherent change. All the findings indicate that complex mechanisms control these genes' specific expression.

Our results show that bi-directional changes of NRSF expression are not conducive to cell viability of SH-SY5Y cells after MPP^+ intoxication. A comprehensive change of downstream genes may contribute to MPP^+-induced apoptosis. In mice, a targeted mutation of REST, the gene encoding NRSF, caused derepression of neuron-specific tubulin in a subset of non-neural tissues and embryonic lethality (Chen et al., 1998). The increased expression of NRSF or REST4 in global cerebral ischemia and epilepsy is unfavorable factors in the pathological process (Calderone et al., 2003; Spencer et al., 2006). These researches suggest the accurate regulation of NRSF or REST4 expression is required for normal cellular functions.

We first report the effects of MPP^+ on the expression and cellular localization of NRSF and REST4 in SH-SY5Y cells. These findings indicate abnormal expression of NRSF or REST4 may be involved in the pathological process of PD.

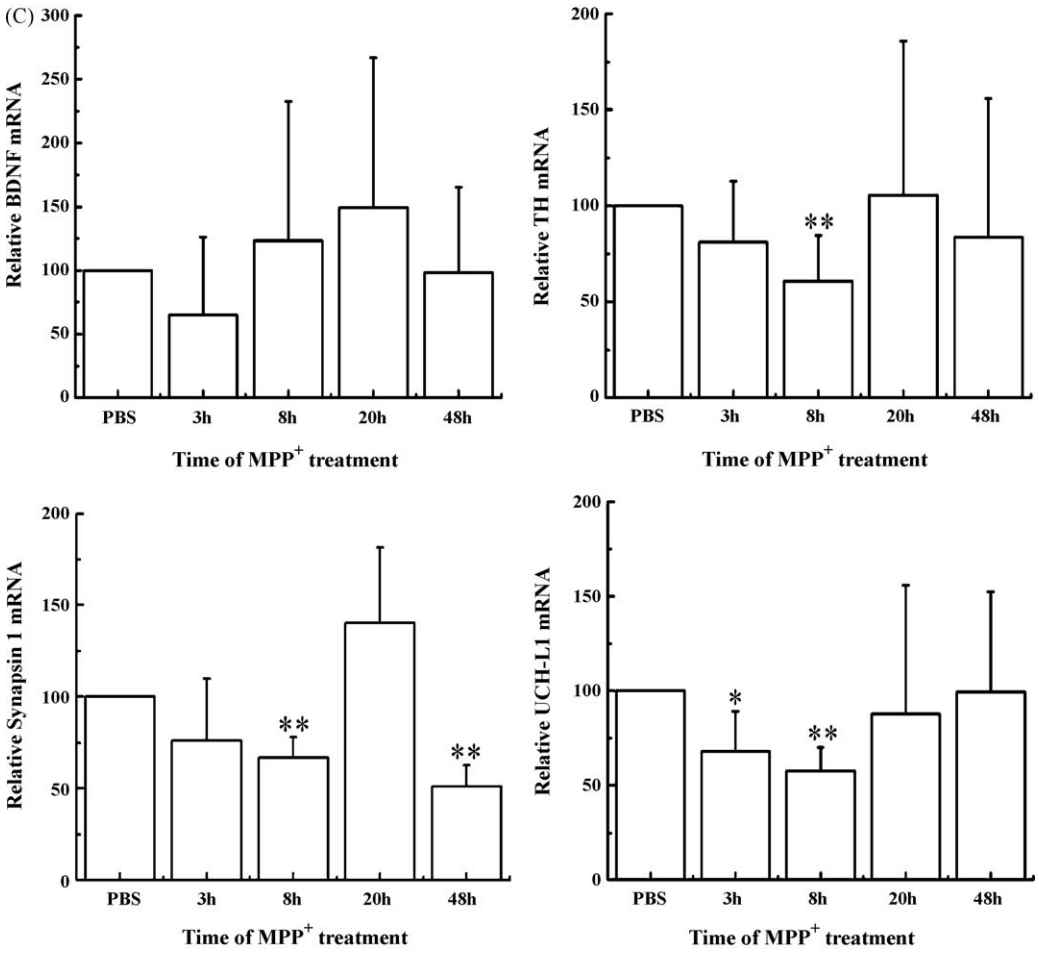

Fig. 5. (*Continued*).

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