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Destabilization of Survival Factor MEF2D mRNA by Neurotoxin in Models of Parkinson's Disease

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Abstract

Progressive loss of dopaminergic (DA) neurons in the substantial nigra pars compacta (SNc) is an important pathological feature in Parkinson's disease (PD). Loss of transcription factor myocyte enhancer factor 2D (MEF2D), a key neuronal survival factor, has been shown to underlie the loss of DA neurons in SNc and the pathogenic process of PD. It is known that PD-associated neurotoxins reduce the level of MEF2D protein to trigger neuronal death. Although neurotoxins clearly destabilize MEF2D

by posttranslational mechanisms, it is not known whether regulation of MEF2D mRNA contributes to neurotoxin-induced decrease in MEF2D protein. In this work, we showed that MPP+, the toxic metabolite of MPTP, caused a significant decrease in the half-life and total level of MEF2D mRNA in a DA neuronal cell line, SN4741 cells. Quantitative PCR analysis of the SNc DA neurons captured by immune-laser capture microdissection showed that exposure to MPTP led to a marked reduction of the level of MEF2D mRNA in SNc DA neurons compared to controls. Down regulation of MEF2D mRNA alone reduced the viability of SN4741 cells and sensitized the cells to MPP+-induced toxicity. These results suggest that destabilization and reduction of MEF2D mRNA is in part responsible for neurotoxin-induced decrease in MEF2D protein and neuronal viability.

Keywords: Parkinson's disease, MPTP/MPP+, transcription factor myocyte enhancer factor 2D, mRNA.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. The disease is characterized pathologically by the loss of

pigmented dopaminergic (DA) neurons in the substantial nigra pars compacta (SNc) (de Lau & Breteler 2006, Tanner & Goldman 1996, Tatton & Kish 1997). Although the precise reasons for the selective loss of SNc DA neurons are not entirely clear, exposure to neurotoxins is considered to be one of the important etiological factors either triggering or facilitating the pathogenic process (Nagatsu 1997, Dauer & Przedborski 2003, Snyder & D'Amato 1986, Yan *et al.* 2012). Previous studies have shown that alteration in gene expression accompanies the pathogenic process of PD (Zetterström *et al.* 1997, Smits *et al.* 2003, Albéri *et al.* 2004, Salih & Brunet 2008, Maxwell *et al.* 2005). One of the mechanisms that accounts for the alteration in gene expression is changes of transcription factors in level, function and localization.

Transcription factor myocyte enhancer factor 2D (MEF2D), one of the four isoforms of MEF2s firstly identified in mammalian cells, has been shown to play a vital role in neuronal survival in several experimental paradigms by many studies (Mao *et al.* 1999, Liu *et al.* 2003, Heidenreich & Linseman 2004, Mount *et al.* 2013). In 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, it has been shown that loss of MEF2D protein function underlies MPTP-induced toxicity. Enhancing MEF2D activity protects DA neurons in SNc from

toxin-induced death (She *et al.* 2011, Smith *et al.* 2006, Mount *et al.* 2013). These findings suggest that dysregulation of MEF2D protein plays a critical role in PD pathogenic process. MEF2D protein level is controlled by multiple pathways in different survival and death signals (Gong *et al.* 2003, Wang *et al.* 2009, Li *et al.* 2001). Our recent studies showed that non-functional MEF2D is degraded by chaperone-mediated autophagy (CMA) (Yang *et al.* 2009, Thomas *et al.* 2011). Aggregation of α-synuclein can block this process, which undermines neuronal viability in degradation of non-functional MEF2D and blocks MEF2D activity in experimental and idiopathic PD (Yang et al. 2009, Thomas et al. 2011). Together, these studies reveal that one of the key mechanisms by which toxic signals reduce MEF2D protein involves post-translational modifications.

mRNA stability influences gene expression, and has an effect on protein level and function (Schwanhäusser *et al.* 2011). However, whether neurotoxins can preferentially regulate the level and stability of MEF2D mRNA remains unknown. Here we examined the level and stability of MEF2D mRNA *in vitro* and *in vivo* under neurotoxin MPTP/MPP+-induced oxidative stress. Our study showed that neurotoxins significantly affected the level of MEF2D mRNA and

markedly shortened its half-life compared to control mRNAs encoding for other transcription factors, revealing that destabilization of MEF2D mRNA underlies in part MPTP/MPP+-induced neurotoxicity.

Materials and Methods

Antibodies

The following antibodies used in this study were obtained commercially: anti-TH antibody, anti-β-actin antibody (Sigma, St Louis, MO, USA); anti-MEF2D antibody (BD Bioscience, San Jose, CA, USA); biotinylated "Universal" secondary antibody (Dako Cytomation Copenhagen, Denmark); anti-cleaved caspase-3 antibody (Cell Signaling).

Animal and tissue preparations

At least 8 weeks old C57bl/6 male mice (25~35g), purchased from the Experimental Animal Center of the Fourth Military Medical University, were used in accordance with the Guidelines for Animal Care and Use of the Fourth Military Medical University (Xi'an, People's Republic of China). All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult C57bl/6 mice received 30 mg/kg free base MPTP (Sigma, St Louis, MO, USA) intraperitoneally (i.p.) once daily

for consecutive 5 days in subacute manner (Jackson-Lewis & Przedborski 2007, Thomas *et al.* 2012). Control cohorts of mice received equivalent volumes of 0.9% saline at the same frequency as MPTP used in this study. Mice were sacrificed 3 days or 5 days following the first MPTP injection. Mice were perfused transcardially with 0.9% NaCl solution for 2 min followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 35 min. The brains were then removed and processed as described in full detail (L'Episcopo *et al.* 2011, Morale *et al.* 2004). Serial coronal sections (30µm thick) containing the SNc were collected for further analysis.

Cell culture

SN4741, a mouse embryonic substantial nigra derived cell line (Son *et al.* 1999), were cultured at 33ºC with 5% CO_2 in RF medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% D-glucose, 1% penicillin-streptomycin and 140mM L-glutamine). When cells reached 80% confluence (usually 3 days), they were split into three plates. Experiments were usually done when cells reached 50-60% confluence.

Immunofluorescence

Cells or brain sections were fixed with 4% formaldehyde solution, incubated with 0.1% Triton X-100 for 30min, blocked with 5% goat serum in Phosphate Buffer Solution (PBS) for 30min. Afterwards samples were incubated with a primary antibody at 4ºC overnight, and then they were washed 3 times with PBS, incubated with a secondary antibody in room temperature for 2h, washed 3 times with PBS. Samples were counterstained with DAPI for 5 minutes and photographed using a confocal microscope (Nikon, C2 Si, Japan).

Immunoblotting

The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corporation, Bedford, MA, USA). After blocking with 5% skimmed milk in TBS-Tween (TBS-T) (50mM Tris (pH 7.6), 150mM NaCl, 0.1% Tween-20), membranes were incubated with a primary antibody with gentle shaking at 4ºC, and then they were washed three times with TBS-T and incubated with a peroxidase-conjugated secondary antibody. Protein bands were visualized by chemiluminescence detection.

Semiquantitative RT-PCR and real-time qRT-PCR

Total RNA was extracted according to the manufacturer's protocol for TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA (1μg) was reverse transcribed using Brilliant II SYBR green single-step quantitative RT-PCR master mix (Stratagene). The products (1μl of cDNA) were used as the template to amplify the specific DNA, using 25-cycle regular PCR to give a semiquantitative determination of the original RNA amount. The PCR products were separated in 1.2% agarose gels in 1× TBE buffer at 100V for 30 min and visualized under LED light. The abundance of each specific mRNA was normalized to β-actin mRNA. Real-time quantitative RT-PCR (qRT-PCR) was performed using an Mx3005 or Mx4000 multiplex quantitative PCR system (Stratagene). Quantization of mRNA was performed using Brilliant II SYBR green single-step quantitative RT-PCR master mix (Stratagene) with 10ng of total RNA in 25μl reaction mixtures. The thermal profile was 50ºC for 30min, 95ºC for 15min, and then 40 cycles of 95ºC for 30s and 55ºC for 30s. Fluorescence was measured at the end of the 55ºC step during every cycle. Primer sequences as follow: MEF2D forward, 5′-ATGGCAACAGCCTAAACAAAGT-3′; MEF2D reverse, 5′-GTGGTGAGCGAGTGGGTAGA-3′; En1 forward, 5′-CGCCTGGGTCTACTGCACA-3′; En1 reverse, 5′-TCTTCTTTAGCTTCCTGGTGCG-3′; En2 forward,

5′-GACCGGCCTTCTTCAGGTC-3′; En2 reverse,

5′-GGCCGCTTGTCCTCTTTGT-3′; Nurr1 forward,

5′-GTGCCTAGCTGTTGGGATGG-3′; Nurr1 reverse,

5′-GTAAACGACCTCTCCGGCC-3′; Pitx3 forward,

5′-GACGCAGGCACTCCACACC-3′; Pitx3 reverse,

5′-TTCTCCGAGTCACTGTGCTC-3′; FoxO3 forward,

5′-ACGGCTCACTTTGTCCCAGAT-3′; FoxO3 reverse,

5′-TTGTGCCGGATGGAGTTCTT-3′; β-actin forward,

5′-AAGGACTCCTATAGTGGGTGACGA-3′; β-actin reverse,

5′-ATCTTCTCCATGTCGTCCCAGTTG-3′.

Immune-laser capture microdissection

Eight-micrometer sections on membrane-mounted slide (Microdissect GmbH, Germany) were collected in the cryostat (Leica CM 1900, Germany) and immediately fixed in prechilled 100% ethanol for 20 min; the sections were incubated in 0.1% Triton X-100 with PBS for 5 min and then incubated with anti-TH primary antibody in the PBS solution for 30 min in a humid chamber, and washed twice for 3 min each in PBS. Afterwards, all sections were incubated with biotinylated “Universal” secondary antibody for 20 min at room temperature. After being washed twice, all the sections were developed with diaminobenzidine (Sigma, St Louis, MO, USA) for 1 to 2 min and counterstained with

hematoxylin (Sigma, St Louis, MO, USA) and the sections were ready for LCM. A Varitas™ Arcturus LCM, combined with TR/UV system (Arcturus Molecular Devices, CA, USA), supply a narrow ultraviolet laser beam to "draw around" and cut out the interested cells. Laser cut parameters were: spot size = 4μm, power = 80 mw, pulse duration = 15 ms. Time between pulses = 400 ms.

Lentivirus infection

Recombinant lentivirus vector for MEF2D gene (shRNA-MEF2D) was obtained commercially from NeuronBiotech, Shanghai, China. SN4741 cells were seeded in six-well plate with antibiotic-free medium. Cells were infected with lentivirus for 72h. Afterwards, the cells were harvested for further analysis.

Measurement of apoptosis by flow cytometry

Cells preinfected with lentivirus were plated in six-well plates. MPP+ (50μM, Sigma, St Louis, MO, USA) were added to the culture. After treatment for 24 h, apoptosis was measured by ApopNexin™ fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (APT750, Millipore, Temecula, CA). Fluorescence due to FITC and PI staining was measured in a flow cytometer (Cytomics FC 500, Beckman Coulter, Brea, CA).

MTT assay

The assay was performed according to the specification of the manufacturer. Briefly, 10 µl of 5 mg/ml MTT labeling reagent were added to SN4741 cells cultured in 96-well plate in 100 µl of medium, and the plate was incubated for 4h in a humidified incubator at 37ºC. After the incubation, the absorbance of the samples was measured at a wavelength of 570 nm with 655 nm as a reference wavelength.

Statistical analyses

Data were expressed as mean ± SEM from at least three independent experiments. Statistical significance was determined using Student t-test. Statistical analyses were carried out using SPSS version 19.0. A value of $p<0.05$ was considered statistically significant.

Results

Analysis of gene expression profiles of transcription factors after MPP+ treatment in SN4741

Regulation of transcription factors plays an important role in neuronal survival and death under stress. To test whether neurotoxins may modulate transcription factors on gene expression, we examined the

mRNA levels of several key factors known to be required for the maintenance and survival of DA neurons after neurotoxin treatment (Zetterström et al. 1997, Smits et al. 2003, Albéri et al. 2004, Salih & Brunet 2008, Maxwell et al. 2005). We chose to use a mouse midbrain dopaminergic neuronal cell line, SN4741, because it has been widely used to study the neuronal response induced by PD related stress signals (Son et al. 1999, She et al. 2011). MPP+/MPTP have been used to study neuronal injury in PD. Following treatment with 50 μM MPP+, the total mRNA was extracted and analyzed for mRNA levels by quantitative real-time PCR. The short treatment with MPP+ led to a clear reduction of mRNA levels for MEF2D, Nurr1, En1/2 and Pitx3 while prolonger treatment only reduced mRNA levels of MEF2D and En1 (Fig. 1a-f). Compared to other transcription factors, the MEF2D mRNA is more sensitive to MPP+-induced stress. Since our previous studies have shown that MEF2D activity is required for the survival of SNc DA neurons and regulated by neurotoxins, we chose to focus on MEF2D mRNA to study gene expression by neurotoxic stress.

The level of MEF2D mRNA and protein was significantly reduced by MPP+ in SN4741

Previous studies have shown that exposure to neurotoxic stress reduces the level of MEF2D proteins in models of PD (Smith et al. 2006, Tang *et*

al. 2005). To establish this model, we treated SN4741 cells with 50μM MPP+. At different times following this treatment, we measured the levels of MEF2D protein by immunocytochemistry (Fig. 2a) and immunoblot (Fig. 2b). MPP+ treatment led to a time-dependent gradual decline in the level of MEF2D protein. To test whether neurotoxin associated with PD may affect MEF2D mRNA, we employed the same treatment paradigm and determined the MEF2D mRNA level by semi-quantitative PCR and quantitative real-time PCR. These data showed that the levels of MEF2D mRNA were markedly reduced in a similar time-dependent manner as MEF2D protein (Fig. 2c and 2d). Thus, neurotoxin MPP+ clearly decreases the level of MEF2D mRNA and the reduction of MEF2D mRNA correlated closely with the decrease in its protein level.

The mRNA level of MEF2D was significantly decreased in the SNc DA neurons in MPTP-PD mouse model

To confirm the above finding *in vivo*, we used the MPTP mouse model of PD. For this, we injected mice intraperitoneally with MPTP following a sub-acute regimen and prepared the midbrain region at 3 and 5 days after the first injection (Day3, Day5) for subsequent analysis. Immunofluorescence analysis of the midbrain sections from the MPTP

treated mice revealed a significant decline in the level of MEF2D protein in the SNc TH positive neurons (Day3, Day5) compared to the control group (Fig. 3a). To determine the levels of MEF2D mRNA in TH positive neurons at the SNc region, we used immunohistochemistry to identify TH positive neurons in the SNc and applied laser capture microdissection (LCM) to isolate TH positive neurons (Fig. 3b). We then extracted total RNA from the dissected neurons and measured MEF2D mRNA by quantitative real-time PCR. This analysis showed that MPTP greatly reduced the level of MEF2D mRNA in the SNc DA neurons, which correlated with the decrease of MEF2D protein *in vivo* ($p<0.01$) (Fig. 3c).

Neurotoxin MPP+ destabilized MEF2D mRNA in DA neuronal SN4741

mRNA stability influences gene expression in almost all organisms. Although the level of mRNA is affected by its rate of synthesis, the half-lives of mRNA play an important role in determination of mRNA abundance (Ross 1995, Wang *et al.* 2002, Guhaniyogi & Brewer 2001). Actinomycin D is one of the most frequently used transcription inhibitors and it can provide an efficient and rapid way to block gene transcription (Dreyfuss *et al.* 1984). To determine whether MPP+ decreased the levels of MEF2D mRNA by increasing its degradation, we determined the half life of MEF2D mRNA. We treated SN4741 cells with transcription

inhibitor actinomycin D with or without MPP+ treatment and then measured the levels of MEF2D mRNA over time by real-time quantitative PCR. Actinomycin D treatment alone revealed that MEF2D mRNA decays with a half-life of about 1.5 h. Addition of actinmoycin D and MPP+ simultaneously to SN4741 cells significantly accelerated the decrease in MEF2D mRNA level (Fig. 4a and 4b). The estimated half-life of MEF2D mRNA was shortened to 0.7 h, suggesting that MPP+ destabilizes MEF2D mRNA in SN4741 cells.

Down-regulation of MEF2D mRNA level reduces the survival of DA neuronal line SN4741 cells and enhances MPP+-induced toxicity

Our above results showed that neurotoxin MPTP/MPP+ reduced the stability of MEF2D mRNA level in PD models. Study has shown that shRNA could guide the degradation of target mRNA (Yamanaka *et al.* 2012). To test whether down-regulation of MEF2D mRNA affects neuronal viability, we incubated SN4741 cells with lentivirus expressing shRNA to MEF2D for 72h. Semi-quantitative RT-PCR and Real-time quantitative PCR showed that the obvious down-regulation of MEF2D mRNA level in shMEF2D group, which confirmed the effect of lentivirus-shMEF2D (Fig. 5a). Analysis of cell viability by flow cytometry showed that decrease in MEF2D mRNA level caused a marked increase

in cell death (14%) compared to the control groups (0.4%) and the FUGW group (0.5%) (Fig. 5b). These data suggest down-regulated MEF2D mRNA level is sufficient to reduce neuronal viability.

To test the effects of down-regulation of MEF2D mRNA level on the cell sensitivity to neurotoxin, we preinfected SN4741 cells with lentivirus expressing shRNA to MEF2D as described above and then treated cells with MPP+. Cell viability was determined by western blot for cleaved caspase-3. Analysis of the level of cleaved caspase-3 indicated that interfering MEF2D mRNA alone was sufficient to activate caspase-3. Pre-inhibition of MEF2D mRNA function also markedly increased the level of activated caspase-3 following MPP+ treatment (Fig. 5c). Consistent with these findings, pre-inhibition of MEF2D mRNA function also sensitized SN4741 cells to MPP+-induced toxicity as determined by MTT assay (Fig. 5d top). The ratio of surviving cell (%) before and after MPP+ treatment in shMEF2D group was significantly lower than in the Control and FUGW groups (Fig. 5d bottom).

Discussion

Oxidative stress and toxic signals known to cause age-related diseases such as neurodegenerative disorders often target key proteins by post-translational modifications to regulate their levels and functions.

For PD, previous studies have mainly focused on proteins involved in mitochondrial damage and pathways leading to neuronal death. Protein level and function are also controlled by gene expression and levels of mRNA in many physiological and pathological processes. Whether neurotoxins may target critical mRNAs or gene transcription in PD pathogenesis remains to be fully delineated.

Stability of mRNA influences gene expression in organisms. For eukaryotes, mRNA decay rate significantly contributes to the level of gene expression (Heintz *et al.* 1983). The transcription factors including En1, En2, Nurr1, MEF2D, Pitx3 and FoxO3 have all been identified as factors playing a key role in maintenance and survival of DA neurons (Zetterström et al. 1997, Smits et al. 2003, Maxwell et al. 2005, Yang et al. 2009). Our current data revealed now that the mRNA levels of these key factors display different profiles in response to oxidative stress. MPP+ reduced MEF2D mRNA in time-dependent manner via significant increase in its rate of decay. This may contribute to the overall reduction of MEF2D under stress.

Many studies have shown that transcription factor MEF2D plays critical roles in different cell processes including neuronal survival (McKinsey *et al.* 2002, Potthoff & Olson 2007, Mao et al. 1999). Stress signals inhibit MEF2D activity by reducing MEF2D level in neurons.

Several mechanisms by which stress signals reduce MEF2D protein level have been identified. For example, it has been shown that phosphorylation of MEF2D by Cdk5 following excitotoxicity stress promotes its degradation by caspases. In addition, chaperon-mediated autophagy also participates in degradation of non-functional MEF2D in models of PD (Yang et al. 2009, Gong et al. 2003, Tang et al. 2005). However, the regulation of mef2d gene expression is still undetermined. Our current studies show that neurotoxin reduces MEF2D mRNA stability to down-regulate MEF2D protein level. These findings suggest that decrease of MEF2D mRNA may contribute to the pathogenic process of PD. Previous studies on mef2 gene transcription mainly focused on single Drosophila Mef2 (Bour *et al.* 1995, Taylor *et al.* 1995) and murine mef2c (Dodou *et al.* 2004), another member of MEF2s family. Our findings now help establish regulation of mef2d mRNA as part of the mechanism contributing to stress-induced dysregulation of MEF2D.

The abundance of mRNA is regulated by a change in the mRNA half-life. The stability of short-lived (*t*=2 h or less) mRNA changes in response to internal and external stimuli, such as developmental, nutritional, pharmacological (de Nadal *et al.* 2011). A small fluctuation in half-lives of such mRNAs is known to cause significant changes in their protein abundance. Our data showed that MPP+ reduced MEF2D mRNA in a

time-dependent manner, correlating with a decrease in protein level. Based on our data, the half-life of MEF2D mRNA is about 1.5 h. Neurotoxin MPTP/MPP+ destabilized short-lived MEF2D mRNA and markedly shortened its half-lives from 1.5 h to 0.7 h. Together, these findings support destabilization of MEF2D mRNA as an important mechanism by which neurotoxin reduces MEF2D protein. Down-regulation of MEF2D mRNA alone by sh-MEF2D induced cell death and sensitized cells to MPP+-induced toxicity.

Compared to samples of whole tissues or sections, isolation of desired cellular population from a well-defined tissue region provides more relevant and specific information for the targets. This is particularly important for researches in the central nervous system where there is a great heterogeneity of neural phenotypes. Many studies have demonstrated the advantages of analyzing samples dissected by LCM (Bustin 2002, Simunovic *et al.* 2009, Finak *et al.* 2006). In this study, we combined immunohistochemistry and LCM to isolate TH positive DA neurons from SNc. This approach allowed us to significantly eliminate the contaminating signals from non-neuronal cells or TH negative neurons and to obtain a much more precise and reliable quantification of the changes of MEF2D mRNA levels in TH positive neurons in the SNc.

Our finding that MPTP/MPP+ specifically reduces the levels of MEF2D mRNA in TH positive neurons of SNc is significant since it establishes the relevance of regulating MEF2D mRNA in PD model *in vivo*.

Base on all the findings in this study, we propose that neurotoxic signals related to PD repress MEF2D activity by two pathways: targeting MEF2D protein for degradation and destabilizing MEF2D mRNA (Fig. 6). It will be important to test which mRNA decay pathways are involved in regulation of MEF2D mRNA stability under PD-related stress.

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Competing interests

The authors declare that no competing interests exist.

References

Albéri, L., Sgadò, P. and Simon, H. H. (2004) Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons. *Development,* **131,** 3229-3236.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. and Nguyen, H. T. (1995) Drosophila MEF2, a transcription factor that is essential for myogenesis. *Genes & Development,* **9,** 730-741.

Bustin, S. (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of molecular endocrinology,* **29,** 23-39.

Dauer, W. and Przedborski, S. (2003) Parkinson's disease: mechanisms and models. *Neuron,* **39,** 889-909.

de Lau, L. M. and Breteler, M. (2006) Epidemiology of Parkinson's disease. *The Lancet Neurology,* **5,** 525-535.

de Nadal, E., Ammerer, G. and Posas, F. (2011) Controlling gene expression in response to stress. *Nature Reviews Genetics,* **12,** 833-845.

Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S.-M. and Black, B. L. (2004) Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development,* **131,** 3931-3942.

Dreyfuss, G., Adam, S. A. and Choi, Y. (1984) Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. *Molecular and cellular biology,* **4,** 415-423.

Finak, G., Sadekova, S., Pepin, F. et al. (2006) Gene expression signatures of morphologically normal breast tissue identify basal-like tumors. *Breast Cancer Research,* **8,** R58.

Gong, X., Tang, X., Wiedmann, M., Wang, X., Peng, J., Zheng, D., Blair, L. A., Marshall, J. and Mao, Z. (2003) Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. *Neuron,* **38,** 33-46.

Guhaniyogi, J. and Brewer, G. (2001) Regulation of mRNA stability in mammalian cells. *Gene,* **265,** 11-23.

Heidenreich, K. A. and Linseman, D. A. (2004) Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. *Molecular neurobiology,* **29,** 155-165.

Heintz, N., Sive, H. and Roeder, R. (1983) Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. *Molecular and cellular biology,* **3,** 539-550.

Jackson-Lewis, V. and Przedborski, S. (2007) Protocol for the MPTP mouse model of Parkinson's disease. *Nature protocols,* **2,** 141-151.

L'Episcopo, F., Tirolo, C., Testa, N. et al. (2011) Reactive astrocytes and Wnt/β-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine model of Parkinson's disease. *Neurobiology of disease,* **41,** 508-527.

Li, M., Linseman, D. A., Allen, M. P., Meintzer, M. K., Wang, X., Laessig, T., Wierman, M. E. and Heidenreich, K. A. (2001) Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons. *The Journal of neuroscience,* **21,** 6544-6552.

Liu, L., Cavanaugh, J. E., Wang, Y., Sakagami, H., Mao, Z. and Xia, Z. (2003) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. *Proceedings of the National Academy of Sciences,* **100,** 8532-8537.

Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M. and Greenberg, M. E. (1999) Neuronal activity-dependent cell survival mediated by transcription factor MEF2. *Science,* **286,** 785-790.

Maxwell, S. L., Ho, H.-Y., Kuehner, E., Zhao, S. and Li, M. (2005) Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Developmental biology,* **282,** 467-479.

McKinsey, T. A., Zhang, C. L. and Olson, E. N. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends in biochemical sciences,* **27,** 40-47.

Morale, M. C., Serra, P. A., Delogu, M. R. et al. (2004) Glucocorticoid receptor deficiency increases vulnerability of the nigrostriatal dopaminergic system: critical role of glial nitric oxide. *The FASEB journal,* **18,** 164-166.

Mount, M. P., Zhang, Y., Amini, M., Callaghan, S., Kulczycki, J., Mao, Z., Slack, R. S., Anisman, H. and Park, D. S. (2013) Perturbation of Transcription Factor Nur77 Expression Mediated by Myocyte Enhancer Factor 2D (MEF2D) Regulates Dopaminergic Neuron Loss in Response to 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). *Journal of Biological Chemistry,* **288,** 14362-14371.

Nagatsu, T. (1997) Isoquinoline neurotoxins in the brain and Parkinson's disease. *Neuroscience research,* **29,** 99-111.

Potthoff, M. J. and Olson, E. N. (2007) MEF2: a central regulator of diverse developmental programs. *Development,* **134,** 4131-4140.

Ross, J. (1995) mRNA stability in mammalian cells. *Microbiological reviews,* **59,** 423-450.

Salih, D. A. and Brunet, A. (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. *Current opinion in cell biology,* **20,** 126-136.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W. and Selbach, M. (2011) Global quantification of mammalian gene expression control. *Nature,* **473,** 337-342.

She, H., Yang, Q., Shepherd, K., Smith, Y., Miller, G., Testa, C. and Mao, Z. (2011) Direct regulation of complex I by mitochondrial MEF2D is disrupted in a mouse model of Parkinson disease and in human patients. *The Journal of clinical investigation,* **121,** 930.

Simunovic, F., Yi, M., Wang, Y. et al. (2009) Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson's disease pathology. *Brain,* **132,** 1795-1809.

Smith, P. D., Mount, M. P., Shree, R. et al. (2006) Calpain-regulated p35/cdk5 plays a central role in dopaminergic neuron death through modulation of the transcription factor myocyte enhancer factor 2. *The Journal of neuroscience,* **26,** 440-447.

Smits, S. M., Ponnio, T., Conneely, O. M., Burbach, J. P. H. and Smidt, M. P. (2003) Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. *European Journal of Neuroscience,* **18,** 1731-1738.

Snyder, S. H. and D'Amato, R. J. (1986) MPTP A neurotoxin relevant to the pathophysiology of Parkinson's disease: The 1985 George C. Cotzias Lecture. *Neurology,* **36,** 250-250.

Son, J. H., Chun, H. S., Joh, T. H., Cho, S., Conti, B. and Lee, J. W. (1999) Neuroprotection and neuronal differentiation studies using substantia nigra dopaminergic cells derived from transgenic mouse embryos. *The Journal of neuroscience,* **19,** 10-20.

Tang, X., Wang, X., Gong, X., Tong, M., Park, D., Xia, Z. and Mao, Z. (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. *The Journal of neuroscience,* **25,** 4823-4834.

Tanner, C. M. and Goldman, S. M. (1996) Epidemiology of Parkinson's disease. *Neurologic clinics,* **14,** 317-335.

Tatton, N. and Kish, S. (1997) detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange staining. *Neuroscience,* **77,** 1037-1048.

Taylor, M. V., Beatty, K. E., Hunter, H. K. and Baylies, M. K. (1995) Drosophila MEF2 is regulated by and is expressed in both the primordia and differentiated cells of the embryonic somatic, visceral and heart musculature. *Mechanisms of development,* **50,** 29-41.

Thomas, B., Banerjee, R., Starkova, N. N. et al. (2012) Mitochondrial permeability transition pore component cyclophilin D distinguishes nigrostriatal dopaminergic death paradigms in the MPTP mouse model of Parkinson's disease. *Antioxidants & redox signaling,* **16,** 855-868.

Thomas, B., Mandir, A. S., West, N., Liu, Y., Andrabi, S. A., Stirling, W., Dawson, V. L., Dawson, T. M. and Lee, M. K. (2011) Resistance to MPTP-neurotoxicity in α-synuclein knockout mice is complemented by human α-synuclein and associated with increased β-synuclein and Akt activation. *PloS one,* **6,** e16706.

Wang, X., She, H. and Mao, Z. (2009) Phosphorylation of neuronal survival factor MEF2D by glycogen synthase kinase 3β in neuronal apoptosis. *Journal of Biological Chemistry,* **284,** 32619-32626.

Wang, Y., Liu, C. L., Storey, J. D., Tibshirani, R. J., Herschlag, D. and Brown, P. O. (2002) Precision and functional specificity in mRNA decay. *Proceedings of the National Academy of Sciences,* **99,** 5860-5865.

Yamanaka, S., Mehta, S., Reyes-Turcu, F. E., Zhuang, F., Fuchs, R. T., Rong, Y., Robb, G. B. and Grewal, S. I. (2012) RNAi triggered by specialized machinery silences developmental genes and retrotransposons. *Nature*.

Yan, M. H., Wang, X. and Zhu, X. (2012) Mitochondrial Defects and Oxidative Stress in Alzheimer's Disease and Parkinson's Disease. *Free Radical Biology and Medicine*.

Yang, Q., She, H., Gearing, M., Colla, E., Lee, M., Shacka, J. J. and Mao, Z. (2009) Regulation of neuronal survival factor MEF2D by chaperone-mediated autophagy. *Science,* **323,** 124-127.

Zetterström, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L. and Perlmann, T. (1997) Dopamine neuron agenesis in Nurr1-deficient mice. *Science,* **276,** 248-250.

Figure legends

Fig. 1 Analysis of gene expression profiles of transcription factors in SN4741 cells after MPP+ treatment. (a-f) SN4741 cells were exposed to 50 μM MPP+ for 12 or 24h, and the mRNA levels of MEF2D, En1, En2, Nurr1, FoxO3 and Pitx3 were quantified by qRT-PCR and normalized to β-actin mRNA level as described in Materials and Methods. (mean±SEM; n=3; * $p < 0.05$, ** $p < 0.01$).

Fig. 2 Neurotoxin MPP+-induced decrease in MEF2D mRNA in SN4741 cells. (a) Immunofluorescence analysis of MEF2D in SN4741 cells following MPP+ treatment. SN4741 cells were treated with 50 μM MPP+ for 12 or 24 h and then analyzed by immunofluorescence (blue: DAPI; green: MEF2D). Bar = 200 μm. **(b)** Western blot analysis of MEF2D protein level after MPP+ treatment. SN4741 cells were treated as described in (a) and MEF2D protein was testified (n =3). **(c)** and **(d)** Effect of MPP+ on the levels of MEF2D mRNA in SN4741 cells. SN4741 cells were treated with MPP+ as described in (a). Total RNA was extracted from SN4741 cells and analyzed by semi-quantitative reverse-transcriptase PCR (c) and by real-time quantitative PCR (d). The values presented were corrected for β-actin controls (mean±SEM; n=3; * $p < 0.05$, ** $p < 0.01$).

Fig. 3 MPTP-induced decrease in the level of MEF2D mRNA in mouse SNc DA neurons. (a) Immunofluorescence analysis of MEF2D in the SNc region of MPTP mouse model of PD. Mice were treated with saline or MPTP. Sections of SNc region of mouse brain were analyzed for MEF2D by immunofluorscence (green: MEF2D; red: TH; blue: DAPI; Day3 or 5: three or five days after the first MPTP injection). Bar = 25 μm. **(b)** Dissection of DA neurons from mouse brain SNc region by LCM. Mouse brain sections from saline/MPTP treated mice were stained with anti-TH antibody for immunohistochemical analysis (top panel). TH^+ neurons in

the SNc were dissected by LCM. (middle panel: a representative photo for brain sections after microdissection; bottom panel: a representative photo for TH^+ neurons harvested on the caps). The data were represents from three independent experiments. Bar = 200 μm. **(c)** Quantitative analysis of MEF2D mRNA in LCM-dissected TH^+ neurons in SNc. Total RNA was extracted from the microdissected TH^+ neurons in SNc as shown in (b) and analyzed for MEF2D mRNA by real-time quantitative PCR. The values presented are corrected for β-actin control (mean±SEM; n=3; ** $p < 0.01$).

Fig. 4 Destabilization of MEF2D mRNA by MPP+ in SN4741 cells. (a) Determination of the effect of MPP+ on MEF2D mRNA stability. SN4741 cells were treated with actinomycin D (10 μg/ml) with or without 50 μM MPP+. Total RNA was isolated at the indicated time points and analyzed for MEF2D mRNA by real-time quantitative PCR. Half-life of MEF2D mRNA was determined after normalization to the level of β-actin mRNA. The experiments were repeated three times. **(b)** Quantification of changes in MEF2D mRNA half-life following MPP+ treatment (mean±SEM; n=3; p=0.002).

Fig. 5 Inhibition of SN4741 cell survival by down-regulation of MEF2D mRNA. (a) Effects of lentivirus-shMEF2D on level of MEF2D mRNA. SN4741 cells were incubated with lentivirus expressing shRNA to MEF2D

for 72h and the levels of MEF2D mRNA were determined by semi-quantitative PCR and quantitative real-time PCR. (mean±SEM; n=3; ** $p < 0.01$). **(b)** Effects of down-regulation of MEF2D mRNA level on the cell survival. SN4741 cells were incubated with lentivirus carrying empty vector (FUGW) or shMEF2D for 72h (control sample was not infected). Cell viability was determined by Annexin V-FITC/PI double staining and quantified by flow cytometry. The experiments were repeated three times. **(c)** Effects of down-regulation of MEF2D mRNA on cleaved caspase-3. SN4741 cells preinfected with lentivirus-control or shMEF2D were exposed to 50 μM MPP+ for additional 24 h. The levels of cleaved caspase-3 were determined by immunoblot. **(d)** Effects of down-regulation of MEF2D mRNA on MPP+-induced cell death. SN4741 cells were treated as described in (a). Cell viability was measured using the MTT assay (top) and the ratio of surviving cell (%) before and after MPP+ treatment (bottom) (mean±SEM; n=3; ** $p < 0.01$).

Fig. 6 Schematic diagram of MPTP/MPP+-mediated dual regulation of MEF2D. Data from current and previous studies support a dual regulatory model in which MEF2D activity is reduced by neurotoxic stress via a posttranslational and mRNA pathways.