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14-3-3 INHIBITION PROMOTES DOPAMINERGIC NEURON LOSS AND 14-3-3θ OVEREXPRESSION PROMOTES RECOVERY IN THE MPTP MOUSE MODEL OF PARKINSON'S DISEASE

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Abstract—14-3-3s are a highly conserved protein family that plays important roles in cell survival and interact with several proteins implicated in Parkinson's disease (PD). Disruption of 14-3-3 expression and function has been implicated in the pathogenesis of PD. We have previously shown that increasing the expression level of 14-3-3θ is protective against rotenone and 1-methyl-4-phenylpyridinium (MPP^+) in cultured cells. Here, we extend our studies to examine the effects of 14-3-3s in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. We first investigated whether targeted nigral 14-3-3θ overexpression mediated by adeno-associated virus offers neuroprotection against MPTP-induced toxicity. 14-3-3θ overexpression using this approach did not reduce MPTP-induced dopaminergic cell loss in the substantia nigra nor the depletion of dopamine (DA) and its metabolites in the striatum at three weeks after MPTP administration. However, 14-3-3θ-overexpressing mice showed a later partial recovery in striatal DA metabolites at eight weeks after MPTP administration compared to controls, suggesting that 14-3-3θ overexpression may help in the functional recovery of those dopaminergic neurons that survive. Conversely, we investigated whether disrupting 14-3-3 function in transgenic mice expressing the pan 14-3-3 inhibitor difopein exacerbates MPTP-induced toxicity. We found that difopein expression promoted dopaminergic cell loss in response to MPTP treatment. Together, these findings suggest that 14-3-3θ overexpression promotes recovery of DA metabolites whereas 14-3-3 inhibition exacerbates neuron loss in the MPTP mouse model of PD.

Key words: 14-3-3s, MPTP, dopamine, Parkinson's disease, adeno-associated virus, neurodegeneration.

INTRODUCTION

Disruption of 14-3-3 expression and function has been implicated in the pathogenesis of Parkinson's disease (PD). 14-3-3s are a highly conserved and ubiquitously expressed protein family that includes seven isoforms, comprising about 1% of total brain soluble protein (Dougherty and Morrison, 2004). 14-3-3 proteins mediate a wide variety of protein–protein interactions and play crucial roles in intracellular protein trafficking, signal transduction, and cell survival (Mackintosh, 2004; Porter et al., 2006). By inhibiting pro-apoptotic factors, 14-3-3s protect cells against apoptosis (Masters and Fu, 2001; Porter et al., 2006). Recently, 14-3-3s have been demonstrated to interact with several key proteins implicated in PD, including alpha-synuclein (αsyn), parkin, and LRRK2 (Ostrerova et al., 1999; Sato et al., 2006; Dzamko et al., 2010; Nichols et al., 2010; Li et al., 2011). 14-3-3s colocalize with αsyn in Lewy bodies in human PD (Kawamoto et al., 2002; Berg et al., 2003), and regulate αsyn aggregation (Yacoubian et al., 2010; Plotegher et al., 2014). 14-3-3s are a key hub of dysregulated proteins in a transcriptional analysis of PD patients (Ulitsky et al., 2010). We have recently shown that an increase in 14-3-3 phosphorylation is observed in human PD brains, and that this increase in 14-3-3 phosphorylation disrupts 14-3-3s' pro-survival function (Slone et al., 2015).

Previous studies from our lab have found that 14-3-3θ, γ, and ε are downregulated in cell lines overexpressing αsyn and in αsyn transgenic mice, with 14-3-3θ being the most significantly downregulated isoform (Yacoubian et al., 2008, 2010; Ding et al., 2013). Overexpression of 14-3-3θ, γ, or ε reduces susceptibility to the neurotoxins rotenone and 1-methyl-4-phenylpyridinium (MPP^+) in cultured cells, whereas the other 14-3-3 isoforms have no or mild protective effects (Yacoubian et al., 2010; Slone et al., 2011). In addition, expression of human 14-3-3θ or the *Caenorhabditis elegans* 14-3-3 homolog mitigates αsyn-induced toxicity in a *C. elegans* model (Yacoubian et al., 2010). On the other hand, we found that

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Abbreviations: AAV, adeno-associated virus; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; eYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; HPLC, high performance liquid chromatography; HVA, homovanillic acid; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NGS, normal goat serum; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; αsyn, alpha-synuclein.

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inhibition of 14-3-3 proteins with the pan 14-3-3 inhibitor difopein (dimeric fourteen-three-three peptide inhibitor) promoted toxicity in response to rotenone (Yacoubian et al., 2010).

Based on our previous data that 14-3-3s can regulate cell death by MPP^+ in culture (Yacoubian et al., 2010), we extend our previous studies to examine whether alterations in 14-3-3s can regulate dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP) mouse model. We tested whether viral vector-mediated overexpression of 14-3-3θ in the substantia nigra reduces neurotoxicity of MPTP *in vivo*. Conversely we tested whether MPTP increases toxicity in a transgenic mouse line that expresses difopein, a competitive inhibitor that interferes with all 14-3-3 isoforms. Since our previous data suggest that other 14-3-3 isoforms can compensate for knockdown of 14-3-3θ (Yacoubian et al., 2010), we tested the effect of disruption of all 14-3-3 isoforms in place of inhibition of only 14-3-3θ.

EXPERIMENTAL PROCEDURES

Animal studies

Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Transgenic mice expressing difopein-enhanced yellow fluorescent protein (eYFP) under the neuronal promoter Thy1.2 were obtained from Dr. Yi Zhou at Florida State University (Qiao et al., 2014). Difopein hemizygous mice were bred with C57BL/6 mice from Jackson Laboratories. All animal use and study protocols were approved and guided by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham (UAB).

Construction of AAV virus

The construction of the adeno-associated virus (AAV)-green fluorescent protein (GFP) vector has previously been described (St Martin et al., 2007). To create the AAV-14-3-3θ vector, the DNA sequence corresponding to wildtype human 14-3-3θθ with a V5-His epitope tag at the C-terminal end was subcloned from the pcDNA3.1-14-3-3θ vector into the multiple cloning site of the AAV-GFP vector. The integrity of the AAV-14-3-3θ construct was verified by DNA sequencing analysis. Both AAV-GFP and AAV-14-3-3θ viral vectors were packaged at the Vector Core of Massachusetts General Hospital.

Stereotactic injection

Eight-week-old male C57BL/6 mice were anesthetized with 3% isoflurane and then placed into a stereotactic frame. Anesthesia was maintained during the procedure using 1.5–2% isoflurane mixed in oxygen through a nose tip built into the stereotactic frame. Burr holes were drilled for single unilateral injection at the following stereotactic coordinates: anterior-posterior, −3.2 mm from the bregma; mediolateral, −1.2 mm from midline; and dorsoventral, −4.6 mm below surface of the dura. 2-μl AAV-GFP (3.97×10^{10} genome copy/ml) or AAV-14-3-3θ (2.83×10^{11} genome copy/ml) was injected into the right substantia nigra pars compacta (SNpc) at the rate of 0.2 μl/min. Following a four-minute waiting period after the injection, the needle was slowly retracted to allow proper diffusion of virus. Animals were allowed to recover on a heated pad before returning to cages and were monitored closely for signs of pain.

MPTP administration

MPTP handling and safety measures were in accordance with the UAB's IACUC guidelines. All mice (total $n = 245$) were injected intraperitoneally with saline (total $n = 116$) as control or MPTP (total $n = 129$) daily for five consecutive days. For AAV-GFP or AAV-14-3-3θ injected mice, at four weeks after stereotactic viral injection, saline or MPTP at 30 mg/kg/day for 5 days was administered for subsequent high performance liquid chromatography (HPLC) analysis ($n = 4$–8 per group), stereology analysis ($n = 9$–10 per group), and striatal tyrosine hydroxylase (TH) immunoreactivity analysis ($n = 9$–10 per group), as illustrated in Fig. 2A. For wildtype or difopein mice, saline or MPTP at 37.5 mg/kg/day for 5 days was administered when mice were 12–18 weeks old for both HPLC ($n = 13$–15 per group), stereology ($n = 25$–29 per group), and striatal TH immunoreactivity analysis ($n = 24$–28 per group).

Tissue processing for histology

At three weeks after the last MPTP injection, mice (total $n = 145$) were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in PBS. Brains were dissected out, postfixed for 24 h in 4% paraformaldehyde at 4 °C, and then placed into a 30% sucrose solution in PBS for 48 h. Brains were frozen in a dry ice bath of 2-methylbutane and sectioned coronally on a Leica microtome with cut thickness of 40 μm. Sections were collected serially throughout the SNpc, placed into 50% glycerol in PBS, and stored at −20 °C until further analysis.

Immunohistochemistry

To identify localization and expression of AAV-GFP and AAV-14-3-3θ in injected cells, free floating brain sections were blocked with 10% normal goat serum (NGS) in PBS containing 0.1% Triton X-100 for 30 min and then incubated overnight with primary mouse anti-tyrosine hydroxylase (TH) antibody (1:1000, Sigma, St. Louis, MO, USA) in combination with rabbit anti-GFP antibody (1:1000, Abcam, Cambridge, MA, USA) or rabbit anti-V5 antibody (1:1000, Sigma) at 4 °C. For AAV-GFP mouse tissue, secondary Cy3-conjugated goat anti-mouse antibody (1:500 Jackson ImmunoResearch, West Grove, PA, USA) and Alexa-488-conjugated goat anti-rabbit antibody (1:500 Invitrogen, Carlsbad, CA, USA) were used. For AAV-14-3-3θ mouse tissue, secondary Alexa-488-conjugated goat anti-mouse antibody (1:500 Invitrogen) and Cy3-conjugated goat anti-rabbit antibody (1:500 Jackson ImmunoResearch) were used.