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14-3-3 inhibition promotes dopaminergic neuron loss and 14-3-3 θ overexpression promotes recovery in the MPTP mouse model of Parkinson's disease

Huiping Ding*, Rachel Underwood, Nicholas Lavalley, and Talene A. Yacoubian

Department of Neurology, Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham

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 14-3-3 θ 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 14-3-3 θ overexpression mediated by adeno-associated virus offers neuroprotection against MPTP-induced toxicity. 14-3-3 θ overexpression using this approach did not reduce MPTP-induced dopaminergic cell loss in the substantia nigra nor the depletion of dopamine and its metabolites in the striatum at three weeks after MPTP administration. However, 14-3-3 θ -overexpressing mice showed a later partial recovery in striatal dopamine metabolites at eight weeks after MPTP administration compared to controls, suggesting that 14-3-3 θ 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 14-3-3 θ overexpression promotes recovery of dopamine metabolites whereas 14-3-3 inhibition exacerbates neuron loss in the MPTP mouse model of PD.

Keywords

14-3-3s; MPTP; dopamine; Parkinson's disease; adeno-associated virus; neurodegeneration

Corresponding author: Talene A. Yacoubian, M.D., Ph.D., Department of Neurology, Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Civitan International Research Center 560D, 1719 6th Avenue South, Birmingham, AL 35294, USA, Phone: 205-996-7543, Fax: 205-996-6580, tyacoub@uab.edu.

*Current address: Department of Physiology & Pharmacology, Wake Forest University School of Medicine Medical Center Boulevard, Winston-Salem, NC 27157

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Potential Conflict of Interest Disclosures

Talene Yacoubian declares that she has a US Patent #7,919,262 on the use of 14-3-3s in neurodegeneration.

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 (α syn), 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 α syn in Lewy bodies in human PD (Kawamoto et al., 2002, Berg et al., 2003), and regulate α syn 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 14-3-3 θ , γ , and ϵ are downregulated in cell lines overexpressing α syn and in α syn transgenic mice, with 14-3-3 θ being the most significantly downregulated isoform (Yacoubian et al., 2008, Yacoubian et al., 2010, Ding et al., 2013). Overexpression of 14-3-3 θ , 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 14-3-3 θ or the *C. elegans* 14-3-3 homologue mitigates α syn-induced toxicity in a *C. elegans* model (Yacoubian et al., 2010). On the other hand, we found that 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 MPTP mouse model. We tested whether viral vector-mediated overexpression of 14-3-3 θ 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 suggests that other 14-3-3 isoforms can compensate for knockdown of 14-3-3 θ (Yacoubian et al., 2010), we tested the effect of disruption of all 14-3-3 isoforms in place of inhibition of only 14-3-3 θ .

Experimental Procedures

Animal studies

Eight-week-old male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). 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 AAV-green fluorescent protein (GFP) vector has previously been described (St Martin et al., 2007). To create the AAV-14-3-3 θ vector, the DNA sequence corresponding to wildtype human 14-3-3 θ with a V5-His epitope tag at the C-terminal end was subcloned from the pcDNA3.1-14-3-3 θ vector into the multiple cloning site of the AAV-GFP vector. The integrity of the AAV-14-3-3 θ construct was verified by DNA sequencing analysis. Both AAV-GFP and AAV-14-3-3 θ 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 bregma; mediolateral, -1.2 mm from midline; and dorsoventral, -4.6 mm below surface of the dura. 2 μ l AAV-GFP (3.97×10^{10} genome copy/ml) or AAV-14-3-3 θ (2.83×10^{11} genome copy/ml) was injected into the right substantia nigra pars compacta (SNpc) at the rate of 0.2 μ l/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-14-3-3 θ 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 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 hours in 4%

paraformaldehyde at 4°C, and then placed into a 30% sucrose solution in PBS for 48 hours. 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-14-3-3 θ 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 minutes and then incubated overnight with primary mouse anti-tyrosine hydroxylase (TH) antibody (1:1000, Sigma, St. Louis, MO) in combination with rabbit anti-GFP antibody (1:1000, Abcam, Cambridge, MA) 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) and Alexa-488-conjugated goat anti-rabbit antibody (1:500 Invitrogen, Carlsbad, CA) were used. For AAV-14-3-3 θ 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.

For stereology analysis, brain sections were immunostained for TH to identify dopaminergic neurons in the SNpc as follows. Free-floating substantia nigra sections were washed with PBS and treated with 3% hydrogen peroxide to quench endogenous peroxidases. Sections were then blocked with 10% NGS in PBS containing 0.1% Triton X-100 for 30 minutes, and incubated overnight at 4°C with primary rabbit anti-TH antibody (1:1000, Pelfreez Biologicals, Rogers, AR) in 2% NGS. Sections were washed with PBS and incubated with secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (1:500, Jackson ImmunoResearch) in 2% NGS for two hours at room temperature. Chromogen staining was displayed with the use of diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Stained brain sections were then mounted onto slides, dehydrated in gradient ethanol, cleared in xylene, and coverslipped with permount solution (Vector Laboratories).

HPLC analysis

Animals (total n=100) were sacrificed three or eight weeks after the last MPTP injection. A 1mm thick coronal section through the striatum centered around 0.38mm from bregma was dissected out using a brain matrix and immediately placed on dry ice. These samples were shipped on dry ice to the Neurochemistry Core Lab at Vanderbilt University Medical Center for HPLC analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in the striatum.

Stereological analysis

Stereological estimates of TH-positive neuronal numbers were performed using the optical fractionator method of the StereoInvestigator 7.0 software from MBF Biosciences (Microbrightfield Inc, Williston, VT). For each animal, SNpc regions of every fourth section based on systematic random sampling were outlined according to published mouse atlas. A grid was placed randomly over the outlined region for counting. At each counting frame (50µm × 50µm) of the grid predetermined by the software setup, neurons with visible nuclei

were counted within three-dimensional optical dissectors set to 20µm with a 60X oil immersion objective using an Olympus BX51 Microscope. A 1µm guard distance from the top and bottom of the section surface was excluded from each dissector. Section thickness was measured at every tenth counting frame on each section to obtain the actual thickness after tissue processing. The total number of neurons (N_{total}) was calculated using the equation: $N_{\text{total}} = N_{\text{counted}} \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{hsf}$, where N_{counted} is the number of neurons counted, ssf is the section sampling fraction, asf is the area sampling fraction and hsf is the height sampling fraction. Coefficient of error (Gundersen, $m=1$) was set to < 0.1 . Stereology estimates were done with the investigator blind to the experimental condition. For AAV-injected mice, nigral counts were made of the injected side only. For transgenic mice, nigral counts were made of both nigra combined for each animal.

Quantification of striatal TH immunostaining

Striatal TH densitometric analysis was performed as previously described (Gibrat et al., 2009, Lu et al., 2009). Free floating brain sections were incubated overnight with primary rabbit anti-TH antibody followed by secondary Cy3-conjugated goat anti-rabbit antibody, as described above. Images of the entire striatum on one side were acquired at 4× magnification using an Olympus BX51 Microscope. Intensity of TH staining of striatal terminals was analyzed using ImageJ software, version 1.49 (National Institutes of Health; <http://imagej.nih.gov/ij>) (Schneider et al., 2012). The average intensity of TH staining in the dorsolateral striatum was measured from three adjacent sections per animal at the level of the anterior commissure (+0.14 mm to +0.62 mm from bregma). Background intensity of the non-stained corpus callosum was subtracted from the average intensity for the striatum. Striatal TH intensity measurements were done with the investigators blind to the experimental condition. For the AAV-injected mice, TH intensity was quantified for the striatum ipsilateral to the injection site. For difopein mice, TH intensity was quantified for both left and right striatum and then averaged for each animal.

Statistical analysis

All statistical analysis of experiments was performed with GraphPad Prism6 (GraphPad Inc., LaJolla, CA). HPLC data, stereology data, and striatal TH immunostaining quantification were analyzed using one-way or two-way ANOVA with a post-hoc Tukey's multiple comparison test.

Results

AAV-mediated 14-3-3θ overexpression reduces MPTP-induced striatal dopamine metabolite loss but not the neuronal loss

To test whether 14-3-3θ could be protective in an *in vivo* neurotoxin model of PD, the MPTP mouse model, AAV-GFP and AAV-14-3-3θ viruses were constructed and then stereotactically injected into the right SNpc of eight-week-old male mice. Expression of GFP and 14-3-3θ in TH-positive nigral neurons was verified by immunohistochemistry (Fig. 1). Four weeks following AAV injection, mice were injected intraperitoneally with 30 mg/kg MPTP once a day for 5 days and nigral dopaminergic cell counts of the injected side was estimated by stereology at 3 weeks following the last MPTP injection. Previous studies

have shown that dopaminergic neuronal loss stabilizes by 21 days after MPTP treatment in the subacute MPTP model (Seniuk et al., 1990, Tatton and Kish, 1997, Jackson-Lewis and Przedborski, 2007). There was no statistically significant difference between AAV-GFP and AAV-14-3-3 θ mice that were treated with saline, suggesting 14-3-3 θ did not affect TH-positive cell counts at baseline (Fig. 2B). MPTP treatment caused a statistically significant reduction of 39% in TH-positive cell counts in the SNpc compared to saline injection in AAV-GFP mice at 3 weeks after treatment (Fig. 2B). MPTP caused a non-significant reduction of 20% in TH-positive nigral counts in AAV-14-3-3 θ mice compared to those AAV-14-3-3 θ mice treated with saline. While the loss of TH-positive counts was less in the AAV-14-3-3 θ mice, there was no statistically significant difference between AAV-GFP and AAV-14-3-3 θ treated with MPTP (Fig. 2B).

We also did a semi-quantitative analysis of the TH-positive terminals in the striatum by measuring TH-immunostaining in the striatum (Fig. 2C). MPTP treatment caused significant reduction in striatal TH-immunostaining in both AAV-GFP mice and AAV-14-3-3 θ mice compared to those mice treated with saline (Fig. 2C). There was no statistically significant difference in striatal TH staining between AAV-GFP and AAV-14-3-3 θ treated with MPTP (Fig. 2C).

We next examined mouse brains by HPLC for striatal dopamine metabolites at three weeks after MPTP treatment. There was no statistical difference in striatal HVA level between AAV-GFP and AAV-14-3-3 θ mice that were treated with saline, but striatal DA and DOPAC levels were slightly lower in AAV-14-3-3 θ mice compared to AAV-GFP mice treated with saline (Fig. 3A). In AAV-GFP mice, treatment with MPTP caused a reduction of 74%, 62%, 49% in DA, DOPAC and HVA striatal levels, respectively, when compared to mice treated with saline. Similarly, MPTP treatment caused a reduction of 73%, 55%, 42% in DA, DOPAC and HVA striatal levels, respectively, in AAV-14-3-3 θ mice. There was no statistical difference in the levels of striatal dopamine metabolites between AAV-GFP and AAV-14-3-3 θ mice that were treated with MPTP at three weeks after treatment (Fig. 3A).

To test whether 14-3-3 θ may aid in the recovery of dopaminergic neurons that survive, we measured striatal dopamine metabolite levels in a second set of AAV-GFP and AAV-14-3-3 θ mice at a later time point. Eight weeks following the last MPTP injection, AAV-14-3-3 θ mice treated with MPTP showed increased striatal DOPAC and HVA levels compared to MPTP-treated AAV-GFP mice (Fig. 3B). AAV-14-3-3 θ mice treated with MPTP also showed a non-significant trend of increased DA striatal levels compared to AAV-GFP mice treated with MPTP (Fig. 3B).

Disruption of 14-3-3 functions by difopein exacerbates MPTP-induced neuron loss

To test the effect of disruption of all 14-3-3 isoforms *in vivo*, we used a transgenic mouse that overexpresses difopein tagged with eYFP (Qiao et al., 2014) as a model for 14-3-3 inhibition. Difopein is a high-affinity competitive antagonist peptide that inhibits 14-3-3/ligand interactions without selectivity among the 14-3-3 isoforms (Masters and Fu, 2001). The particular difopein transgenic line used for MPTP experiments expresses difopein-eYFP in TH-positive neurons in the substantia nigra, as demonstrated by immunohistochemistry (Fig. 4). In our initial pilot study, we did not observe a significant reduction in TH counts

when wildtype mice from this transgenic line were treated with 30 mg/kg/day MPTP for five days, while 45 mg/kg/day for five days was too toxic to mice (data not shown). Therefore, we treated wildtype and difopein transgenic littermates with 37.5 mg/kg/day dose of MPTP for five days for our stereology and HPLC analysis. MPTP has been shown to induce variable levels of dopamine loss between different mouse strains and even in the same mouse strain from different vendors (Heikkila, 1985, Sonsalla and Heikkila, 1986, Jackson-Lewis and Przedborski, 2007).

In both wildtype and difopein transgenic mice, MPTP treatment at 37.5 mg/kg/day for five days caused significant reductions in the levels of striatal DA, DOPAC, and HVA when compared to saline treatment (Fig. 5). Difopein mice treated with saline showed slightly reduced levels of striatal DOPAC and HVA but not DA compared to wildtype mice treated with saline (Fig. 5). There was no significant difference in the levels of all three dopamine metabolites between wildtype and difopein transgenic mice treated with MPTP (Fig. 5). Consistent with the HPLC data, we did not observe differences in TH immunostaining in the striatum between difopein and wildtype mice when treated with MPTP (Fig. 6c)

We next estimated nigral dopaminergic cell counts of both SN by stereology. There was no difference between wildtype and difopein transgenic mice that were treated with saline, suggesting difopein expression did not affect TH-positive cell counts at baseline (Fig. 6A,B). However, difopein transgenic mice showed a more significant reduction in TH-positive cells (41%) when treated with MPTP compared to wildtype mice (30%), indicating that 14-3-3 inhibition exacerbated MPTP-induced dopaminergic cell loss (Fig. 6A,B).

Discussion

Previous studies have shown a protective role of 14-3-3s in cellular and worm models of PD (Yacoubian et al., 2010). In this report, we investigated if targeted 14-3-3 θ overexpression was protective and, conversely, if disruption of 14-3-3 function was detrimental in the MPTP mouse model of PD. Our results showed that 14-3-3 θ overexpression alone did not reduce dopaminergic cell loss by MPTP but did ameliorate striatal dopamine metabolite loss at later time points. Conversely, transgenic expression of 14-3-3 pan-inhibitor difopein exacerbated MPTP-induced dopaminergic cell loss in the substantia nigra.

Using stereotactic technique we targeted AAV-mediated 14-3-3 θ overexpression in the SNpc. Our results here indicate that targeted 14-3-3 θ overexpression by AAV did not clearly prevent dopaminergic cell loss in the SNpc following MPTP treatment. Similarly, we did not observe an effect of 14-3-3 θ overexpression on striatal DA metabolites at three weeks after MPTP treatment. However, when we assessed striatal DA metabolites at eight weeks after MPTP injection, we observed a recovery of these metabolites in the AAV-14-3-3 θ mice compared to AAV-GFP mice. This finding suggests that, while 14-3-3 θ overexpression may not be able to prevent dopamine cell loss in the nigra, it may aid in the functional recovery of those dopamine neurons that survive. 14-3-3s have been shown to regulate tyrosine hydroxylase activity (Itagaki et al., 1999, Wang et al., 2009), and it is perhaps this function of 14-3-3s that helps to promote this recovery in striatal dopamine metabolites at eight weeks. However, other enzymes, including monoamine oxidase and catechol-o-

methyltransferase, can regulate dopamine levels and thus play a potential role in 14-3-3 θ 's effect on dopamine metabolites. Another possibility is that 14-3-3 θ could promote regeneration of dopaminergic axonal terminals in the striatum. Primates treated with MPTP show partial functional recovery in DA and HVA levels over time due to sprouting of terminals by surviving nigral neurons (Elsworth et al., 2000). As 14-3-3s have been shown to regulate axonal growth in other systems (Kajiwar et al., 2009, Yoon et al., 2012, Marzinke et al., 2013), 14-3-3 θ overexpression here could cause partial dopamine metabolite recovery by promoting axonal terminal growth by surviving dopamine neurons at later time points.

As discussed above, AAV-mediated 14-3-3 θ overexpression in the SNpc does not protect against MPTP-induced cell loss, at least in mice. There are three possible explanations for this outcome. First, expression of the 14-3-3 θ isoform alone was not enough to offer protection, and overexpression of other 14-3-3 isoforms may be also required to promote neuroprotection. Second, the level of 14-3-3 θ expression produced by stereotactic AAV injection did not reach the therapeutic threshold required for 14-3-3 θ 's potential protective response *in vivo*. Third, overexpression in a limited region around the SNpc may not be sufficient but broader 14-3-3 overexpression may be required for any potential 14-3-3 θ 's effects. Pan neuronal overexpression of 14-3-3 θ using a transgenic line may be explored as an alternative approach.

We also investigated the effects of disrupting 14-3-3s in the MPTP mouse model of PD. There are two ways to disrupt 14-3-3s: 1) knockdown of 14-3-3 expression, and 2) inhibition of 14-3-3s with competitive inhibitors, such as difopein or dominant negative 14-3-3s (Masters and Fu, 2001, Zhou et al., 2003). Attempts to create 14-3-3 θ knockout mice have not been successful because 14-3-3 θ knockout is embryonic lethal. Similarly, virtually all homozygous 14-3-3 ϵ null mice died at birth (Toyo-oka et al., 2003). In addition, we previously found that knockdown of 14-3-3 θ expression alone did not promote neurotoxin-induced toxicity, whereas inhibition of all 14-3-3 isoforms promoted toxicity in response to rotenone (Yacoubian et al., 2010), suggesting that other 14-3-3 isoforms can compensate for the loss of one isoform. Therefore, in this study, we chose to examine the effects of inhibition of all 14-3-3 isoforms by transgenic expression of difopein in mouse. Our results showed that pan 14-3-3 inhibition by difopein did promote dopaminergic cell loss by MPTP. At this time, it is unclear the mechanisms by which difopein promotes neuronal loss, but one possibility is that difopein prevents the inhibition of pro-apoptotic factors, such as Bax, by endogenous 14-3-3s. Bax activation has been observed in the subacute MPTP model (Vila et al., 2001, Perier et al., 2005), and we have previously shown that 14-3-3 θ overexpression reduced Bax activation in response to rotenone in culture (Slone et al., 2011). While we observed changes in neuronal counts, we did not observe changes in striatal TH immunoreactivity between difopein and wildtype mice treated with MPTP, consistent with our HPLC data. We used a semi-quantitative measurement of TH-positive striatal terminals by quantifying TH immunoreactivity instead of doing stereological counts of TH terminals, and this method may lack sensitivity to detect small differences in TH-positive terminal counts. An alternative explanation for the discrepancy between nigral TH counts and striatal terminal staining is that 14-3-3 inhibition may have promoted anterograde degeneration

starting at the soma where difopein is predominantly expressed (in contrast to the usual retrograde degeneration caused by MPTP); in this scenario, it is possible that complete degeneration of striatal terminals of difopein-expressing nigral neurons may not have fully occurred by 21 days at which point TH neuronal counts were already decreased in response to difopein expression.

Our data showing increased dopamine cell loss with 14-3-3 inhibition in the difopein mouse suggest that reduced 14-3-3 expression observed in PD models (Miller et al., 2004, Yacoubian et al., 2008, Storvik et al., 2010, Yacoubian et al., 2010, Ding et al., 2013) could contribute to the loss of neurons in PD. Reduction of several 14-3-3 isoforms has been noted in human PD brain in several microarray studies (Zhang et al., 2005, Simunovic et al., 2009). While loss of 14-3-3s is not likely the initial cause of neurodegeneration, it could accelerate the neurodegenerative process once initiated. Our previous data shows that α syn overexpression causes reduced 14-3-3 expression (Yacoubian et al., 2010, Ding et al., 2013), suggesting that the loss of 14-3-3 levels may serve as one mechanism mediating α syn toxicity in disease. We have previously shown that 14-3-3 θ overexpression decreases dopaminergic neuron loss in an α syn *C. elegans* model, and are currently investigating the effects of 14-3-3s on α syn propagation. We are also investigating the effects of 14-3-3s on LRRK2, which has been shown to interact with 14-3-3s (Dzamko et al., 2010, Nichols et al., 2010, Li et al., 2011). If 14-3-3s show significant protection in multiple models of PD, boosting 14-3-3 expression could serve as a potential therapeutic target. Several commercially available drugs can induce 14-3-3 expression – suggesting the feasibility of small molecule treatments to enhance 14-3-3 expression (Ferguson et al., 2000, Parker et al., 2003, Liou et al., 2006, Brunelli et al., 2007, Wu et al., 2009)

In conclusion, we found that inhibition of all 14-3-3 isoforms exacerbated dopaminergic neuron loss, while 14-3-3 θ overexpression promoted recovery of striatal DA metabolites in the MPTP model. Whether inhibition of one particular isoform alone is sufficient to promote MPTP toxicity *in vivo* is not known. Expression of dominant-negative mutant of 14-3-3 θ in transgenic mice or creation of a conditional 14-3-3 θ knockout mouse could be alternative approaches used to study the isoform-specific effects in neurodegeneration.

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Abbreviations

α syn	alpha-synuclein
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
eYFP	enhanced yellow fluorescent protein

GFP	green fluorescent protein
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

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Highlights

- Inhibition of 14-3-3 proteins exacerbates dopamine neuron loss by MPTP in mice
- 14-3-3 θ overexpression does not prevent dopamine cell loss by MPTP in mice
- 14-3-3 θ promotes late partial recovery of dopamine metabolites after MPTP treatment

Figure 1. Viral vector mediated expression of 14-3-3θ or GFP in dopaminergic neurons in the substantia nigra

Targeted overexpression of either GFP or 14-3-3θ in the SNpc of mouse brain at eight weeks after stereotaxic AAV injection. **(A)** Brain sections through the SNpc from the AAV-14-3-3θ mice were double immunostained for 14-3-3θ using a primary antibody against the V5 tag at the C-terminal end of 14-3-3θ and a Cy3-conjugated secondary antibody (red panel) and for TH using a primary antibody against TH and an Alexa488-conjugated secondary antibody (green panel). **(B)** Brain sections through the SNpc from the AAV-GFP mice were double immunostained for GFP using a primary antibody against GFP and an Alexa488-conjugated secondary antibody (green panel) and for TH using a primary antibody against TH and a Cy3-conjugated secondary antibody (red panel). Scale bars = 100 μm.

Figure 2. AAV-mediated 14-3-3θ overexpression does not reduce MPTP-induced dopaminergic cell loss in the SNpc

(A) Timeline of experimental protocol to assess effect of AAV-14-3-3θ nigral injection against MPTP toxicity in mice. (B) Stereology estimates of TH-positive neuronal counts in the injected SNpc of AAV-GFP and AAV-14-3-3θ mice at three weeks after the last injection of saline or MPTP at 30 mg/kg/day for five days. MPTP induced significant reductions in TH-positive neurons as compared to saline. There was no statistical difference in TH-positive cell counts between MPTP-treated AAV-GFP mice and MPTP-treated AAV-14-3-3θ mice. $n=10$ for all groups except $n=9$ for saline-treated AAV-14-3-3θ mice. Error bars reflect S.E.M. ** $p<0.01$, n.s. = nonsignificant (2 way ANOVA followed by Tukey's multiple comparison test). (C) Optical density measurements of TH immunostaining in the striatum of AAV-GFP and AAV-14-3-3θ mice at three weeks after the last injection of saline or MPTP. $n=10$ for all groups except $n=9$ for saline-treated AAV-14-3-3θ mice. Error bars reflect S.E.M. ** $p<0.01$, *** $p<0.001$ (2 way ANOVA followed by Tukey's multiple comparison test).

Figure 3. AAV-mediated 14-3-3θ overexpression ameliorates striatal dopamine metabolite loss at eight weeks

(**A**) HPLC analysis of levels of striatal dopamine (DA) and its metabolites DOPAC and HVA at three weeks after the last injection of saline or MPTP. MPTP caused significant reductions in the levels of DA, DOPAC and HVA as compared to saline in both AAV-GFP and AAV-14-3-3θ mice. There was no statistical difference between MPTP-treated AAV-GFP mice and MPTP-treated AAV-14-3-3θ mice. $n=7$ for saline-treated AAV-14-3-3θ mice and for MPTP-treated AAV-GFP mice and $n=8$ for saline-treated AAV-GFP mice and for MPTP-treated AAV-14-3-3θ mice. Error bars reflect S.E.M. $*p<0.05$, $***p<0.001$ (2 way ANOVA followed by Tukey's multiple comparison test). (**B**) HPLC analysis of levels of striatal dopamine (DA) and its metabolites DOPAC and HVA at eight weeks after the last MPTP injection. AAV-14-3-3θ mice showed less reduction in dopamine metabolites compared to AAV-GFP mice when treated with MPTP. $n=5$ for saline-treated AAV-GFP mice and for MPTP-treated AAV-GFP mice and $n=4$ for MPTP-treated AAV-14-3-3θ mice. Error bars reflect S.E.M. $*p<0.05$, $***p<0.001$ (1 way ANOVA followed by Tukey's multiple comparison test).

Figure 4. Expression of difopein in dopaminergic neurons in the substantia nigra
Representative brain sections of difopein-YFP transgenic mouse were double immunostained for YFP (green) to detect expression of YFP-tagged difopein and for TH (red) to detect dopaminergic neurons. Overlay image shows colocalization of difopein and TH in the SNpc neurons. Scale bars = 100 μ m.

Figure 5. MPTP effects on striatal dopamine metabolites in difopein mice

HPLC analysis of levels of striatal dopamine (DA) and its metabolites DOPAC and HVA at three weeks after the last injection of saline or 37.5 mg/kg/day MPTP. $n=14$ for saline-treated wildtype mice, $n=13$ for saline-treated difopein mice, $n=15$ for MPTP-treated wildtype mice, and $n=14$ for MPTP-treated difopein mice. Error bars reflect S.E.M. $*p<0.05$, $***p<0.001$ (2 way ANOVA followed by Tukey's multiple comparison test).

Figure 6. Disruption of 14-3-3 functions by difopein exacerbates MPTP-induced dopaminergic cell loss in the SNpc

(A) TH staining of brain sections through the substantia nigra from wildtype and difopein transgenic mice treated with saline or MPTP at 37.5 mg/kg/day for five days. Scale bars = 100 μ m. (B) Stereology estimates of TH-positive neuronal counts in both SNpc of wildtype and difopein transgenic mice at three weeks after the last injection of saline or MPTP. When treated with MPTP, there was more reduction in TH-positive cells in difopein transgenic mice than in wildtype mice. $n=25$ for saline-treated wildtype mice, $n=25$ for saline-treated difopein mice, $n=27$ for MPTP-treated wildtype mice, and $n=29$ for MPTP-treated difopein mice. Error bars reflect S.E.M. $*p<0.05$, $***p<0.001$ (2 way ANOVA followed by Tukey's multiple comparison test). (C) Optical density measurements of TH immunoreactivity in the striatum of wildtype and difopein mice at three weeks after the last injection of saline or MPTP. $n=25$ for saline-treated wildtype mice, $n=24$ for saline-treated difopein mice, $n=27$ for MPTP-treated wildtype mice, and $n=28$ for MPTP-treated difopein mice. Error bars reflect S.E.M. $***p<0.001$ (2 way ANOVA followed by Tukey's multiple comparison test).