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Increased 14-3-3 phosphorylation observed in Parkinson's disease reduces neuroprotective potential of 14-3-3 proteins

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Abstract

14-3-3 proteins are key regulators of cell survival. We have previously demonstrated that 14-3-3 levels are decreased in an alpha-synuclein (α syn) mouse model of Parkinson's disease (PD), and that overexpression of certain 14-3-3 isoforms is protective in several PD models. Here we examine whether changes in 14-3-3 phosphorylation may contribute to the neurodegenerative process in PD. We examine three key 14-3-3 phosphorylation sites that normally regulate 14-3-3 function, including serine 58 (S58), serine 184 (S184), and serine/threonine 232 (S/T232), in several models of PD and in human PD brain. We observed that an increase in S232 phosphorylation is observed in rotenone-treated neuroblastoma cells, in cells overexpressing α syn, and in human PD brains. Alterations in S58 phosphorylation were less consistent in these models, and we did not observe any phosphorylation changes at S184. Phosphorylation at S232 induced by rotenone is reduced by casein kinase inhibitors, and is not dependent on α syn. Mutation of the S232 site affected 14-3-3 θ 's neuroprotective effects against rotenone and 1-methyl-4-phenylpyridinium (MPP⁺), with the S232D mutant lacking any protective effect compared to wildtype or S232A 14-3-3 θ . The S232D mutant partially reduced the ability of 14-3-3 θ to inhibit Bax activation in response to rotenone. Based on these findings, we propose that phosphorylation of 14-3-3s at serine 232 contributes to the neurodegenerative process in PD.

Introduction

Disruption of 14-3-3 protein expression and function has been recently implicated in Parkinson's disease (PD) pathogenesis. The 14-3-3 proteins are a highly conserved family of proteins found throughout the evolutionary scale and are implicated in many cellular functions, including transcription, metabolism, and apoptosis (1, 2). This protein family,

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Conflict of Interest Disclosure

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

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which includes seven isoforms in mammals, are key regulators of cell death and act to promote cell survival through inhibition of many known pro-apoptotic factors (3, 4). 14-3-3s have been shown to interact with several key proteins implicated in PD, including alpha-synuclein (α syn), parkin, and leucine-rich repeat kinase 2 (LRRK2) (5–10). 14-3-3s are a key hub of dysregulated proteins in a transcriptional analysis of PD patients (11). 14-3-3s show homology to α syn and coimmunoprecipitate with α syn in normal brain (8, 10). Coimmunoprecipitation of 14-3-3s with α syn is increased in the substantia nigra (SN) of PD brains (9, 10), a predominant region involved in PD, and 14-3-3s colocalize with α syn in Lewy Bodies (12, 13). Four isoforms have been shown to colocalize with α syn in Lewy Bodies in human PD, including 14-3-3 ϵ , γ , θ , and ζ (12). We have previously shown that expression of several 14-3-3 isoforms is decreased with overexpression of wildtype human α syn in neuroblastoma cells or transgenic mice (14–16). Changes in 14-3-3 θ and other isoforms are observed at the mRNA level in both the substantia nigra and cortex of an α syn mouse model (14, 15). 14-3-3s are also key interactors of wildtype LRRK2, and several PD-associated LRRK2 mutants have been shown to be unable to bind 14-3-3s (5–7).

Because of 14-3-3s' anti-apoptotic role, we have previously hypothesized that disruption of 14-3-3s in PD could lead to the activation of cell death pathways that are normally inhibited by 14-3-3s. In support of this hypothesis, we have shown that overexpression of 14-3-3 θ , ϵ , or γ reduced cell loss in response to the Parkinsonian toxins rotenone and 1-methyl-4-phenylpyridinium (MPP⁺) in dopaminergic cell culture, while other isoforms showed variable effects (15). Human 14-3-3 θ and the *C. elegans* 14-3-3 homologue *ftt-2* also reduced cell loss in transgenic *C. elegans* that overexpress α syn (15). The neuroprotective effect of 14-3-3 θ against rotenone toxicity is dependent on the inhibition of the pro-apoptotic factor Bax (17).

In this study, we evaluate whether altered phosphorylation of 14-3-3s may contribute to the dysfunction of 14-3-3s in PD. A well-recognized mechanism for regulating 14-3-3 function is phosphorylation of 14-3-3s at three conserved phosphorylation sites: serine 58 (S58), serine 184 (S184), and serine/threonine 232 (S/T232) (18, 19). S58 phosphorylation, found in all isoforms except 14-3-3 σ and θ , has been shown to regulate dimerization (20, 21). Phosphorylation at S184, found in 14-3-3 β , ϵ , σ , and ζ , regulates ligand interactions (22–24). Phosphorylation at both S58 and S184 has been linked to the release of pro-apoptotic factors and cell death (22–25). Least understood is phosphorylation at S/T232, found in 14-3-3 θ and ζ (26, 27). It may regulate ligand binding as the C-terminal loop can fold back into the peptide-binding pocket (28).

Kulanthingal *et al.* have previously demonstrated in a proteomics study that alterations in 14-3-3 phosphorylation are observed in neuroblastoma cells overexpressing α syn (29). Which phosphorylation sites and which isoforms are involved have not been fully examined, nor the consequences of such phosphorylation changes in PD models. In this study, we examine which phosphorylation sites are altered and the consequences of such changes. Specifically, we test whether any of the three key phosphorylation sites are altered in cells treated with rotenone, in cells that conditionally overexpress α syn, and in human PD brains. We observe that changes in phosphorylation are observed at two of these phosphorylation sites, S58 and S232, and that phosphorylation at S232 reduces the neuroprotective effect of

14-3-3 θ . These findings suggest that increased 14-3-3 phosphorylation observed in PD may promote neurodegeneration in PD.

Material and Methods

Materials

Rotenone and MPP⁺ were purchased from Sigma (St. Louis, MO). 5,6-dichloro-1- β -D-ribofuranosyl-1H-benzimidazole (DRB) was purchased from Enzo Life Sciences (Farmingdale, NY) and 4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl)-1H-imidazol-2-yl) benzamide (D4476) was obtained from EMD Millipore/Biosciences (Billerica, MA). Primary antibodies that were used include mouse monoclonal antibody against 14-3-3 θ (Abcam, Cambridge, MA), rabbit polyclonal antibody against pan 14-3-3 isoforms (Abcam), rabbit polyclonal antibody against serine 232 14-3-3 θ (Abcam), rabbit polyclonal antibody against serine 58 14-3-3 ζ (Abcam), sheep polyclonal antibody against serine 184 14-3-3 (Enzo Life Sciences), mouse monoclonal antibody against V5 (Life Technologies, Grand Island, NY), mouse monoclonal 6a7 antibody against activated Bax (Sigma), rabbit polyclonal antibody against Bax (Cell Signaling, Danvers, MA), mouse monoclonal against cyclophilin D (EMD Biosciences), rabbit polyclonal antibody against cleaved caspase 3 (Cell Signaling), or mouse monoclonal antibody against α -tubulin (Sigma). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Cell culture

SK-N-BE(2)-M17 (M17) cells (ATCC, Manassas, VA) and SH-SY5Y cells (ATCC) were grown in Eagles minimal essential media (MEM) and F12K media (ATCC) at 1:1 ratio supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS) and 1% penicillin/streptomycin (Life Technologies) at 37°C with 5% CO₂ in a humidified incubator.

Animals

Wildtype C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). BAC wildtype and G2019S LRRK2 hemizygous transgenic mice (30) obtained from Heather Melrose were backcrossed on a C57BL/6 background and were bred with wildtype C57BL/6 mice from Jackson labs (Bar Harbor, ME). The use of mice was supervised by the University of Alabama Animal Resources Program in accordance with the PHS policy on Humane Care and Use of Laboratory Animals. Mice were euthanized by CO₂ inhalation.

Western Blot

Cells were spun down at 1500g for 5 minutes, washed in PBS, and then sonicated for 10, seconds on ice in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), phosphatase inhibitor cocktail (Roche)), followed by centrifugation at 16000g for 10 minutes. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Samples were boiled for 5 min in 4 $\times$ DTT sample loading buffer (0.25 M Tris-HCl (pH 6.8), 8% SDS, 200 mM mM DTT, 30% glycerol, Bromophenol Blue),

resolved on 15 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Blots were blocked in 5% non-fat dry milk in TBST (25 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20) for one hour, and then incubated overnight with primary mouse monoclonal antibody against 14-3-3 θ (1:1000), rabbit polyclonal antibody against pan 14-3-3 isoforms (1:1000), rabbit polyclonal antibody against serine 232 14-3-3 θ (1:1000), rabbit polyclonal antibody against serine 58 14-3-3 ζ (1:1000), sheep polyclonal antibody against serine 184 14-3-3 (1:1000), mouse monoclonal antibody against V5 (1:5000), rabbit polyclonal against cleaved caspase 3 (1:1000), polyclonal or mouse monoclonal antibody against α -tubulin (1:5000). After three washes in TBST, blots were incubated with HRP-conjugated goat anti-mouse, anti-sheep, or anti-rabbit secondary antibody (Jackson ImmunoResearch) for 2 hours and then washed in TBST six times for 10 minutes each. Blots were developed with enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ, USA).

Hippocampi from mouse brains were homogenized in lysis buffer (175mM NaCl, 50mM Tris HCl, pH7.4, 5mM EDTA with protease inhibitor and phosphatase inhibitor cocktails) and sonicated for 10 seconds. After the addition of 1% Triton X ice for 30 minutes and spun at 15000g for one hour at 4C. The supernatant samples were resolved on SDS-polyacrylamide gels and analyzed by Western blotting as described above.

Bax oligomerization assay

After rotenone treatment, cells were lysed by nitrogen cavitation and enriched for mitochondria as previously described (17). The mitochondrial-enriched pellet was solubilized in 2% CHAPS buffer and then crosslinked with 1 mM ethylene-glycol-bis(succinic acid N-hydroxy-succinimide ester) (Sigma) as previously described (17). Protein samples were analyzed for Bax monomers and oligomers by Western blotting using a polyclonal antibody against Bax (1:1000).

Creation of doxycycline-inducible α syn and GFP cell lines

Green fluorescent protein (GFP) or human wildtype α syn was cloned into the tetracycline-inducible lentiviral construct pSLIK (31). M17 cells were infected with the α syn virus in the presence of 6 μ g/ml polybrene (Sigma). At 72 hours after infection, infected cells were selected for in the presence of G418 (Life Technologies). To induce GFP or α syn expression, cells were treated with doxycycline (EMD Millipore/Biosciences) at 2 μ g/ml.

Quantitative PCR

RNA was extracted from mouse substantia nigra cells that were dissected using laser capture microdissection with the Arcturus Veritas system (Mountain View, CA), as previously described (14). The extracted RNA was reverse transcribed into first-strand cDNA using the SuperScriptTM II reverse transcriptase kit (Life Technologies). For real-time quantitative PCR (QPCR), first strand cDNA created from extracted RNA was incubated with appropriate forward and reverse primers and SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA) Applied in a 96-well plate. QPCR was performed using an iQ5Cycler (BioRad, Hercules, CA), as previously described (14, 15). The quantity of each PCR sample was calculated using the Ct method (32, 33).

Creation of 14-3-3 θ lines

14-3-3 θ and 14-3-3 ζ isoforms were subcloned into the mammalian expression vector pcDNA3.1/V5-His (Life Technologies) as previously described (15). Serine 232 of 14-3-3 θ was mutated to alanine or to aspartate by site-directed mutagenesis using Quik Lightning Kit per manufacturer's directions (Agilent Technologies, Santa Clara, CA). Forward primer for S232A mutation was 5'-taacactttggacatcagacgctgcaggagaagaatgtgatg-3'; reverse primer for S232A mutation was 5'-catcacattcttctcctgcagcgtctgatgtccaaagtgtta-3'. Forward primer for S232D mutation was 5'-ctaacactttggacatcagacgatgcaggagaagaatgtgatgc-3'; reverse primer for S232D mutation was 5'-gcacacattcttctcctgcacgtctgatgtccaaagtgttag-3'. Similarly, serine 58 of 14-3-3 ζ was mutated to alanine or to aspartate by site-directed mutagenesis. Forward primer for S58A mutation was 5'-gagcccgtaggtcagcttgagggtcgtc-3'; reverse primer for S58A mutation was 5'-gacgacctccaagctgacctacgggtc-3'. Forward primer for S58D mutation was 5'-ggagcccgtaggtcagattggagggtcgtc-3'; reverse primer for S58D mutation was 5'-gagagacctccaatctgacctacgggtcc-3'. M17 cells (ATCC) were transfected with these constructs using Superfect (Qiagen, Germantown, MD), and stably-transfected cells were selected for in the presence of G418 (500 μ g/ml; Life Technologies). Experimental controls included M17 cells transfected with the empty pcDNA3.1/V5-His vector and selected for stable transfection in the presence of G418.

LDH assay

Cells were grown in pyruvate-free DMEM for a few days prior to plating in 24-well collagen-treated plates. Cells were treated with rotenone in serum-free DMEM for 48 hours or with MPP⁺ in serum-free DMEM for 24 hours. Toxicity was assayed by lactate dehydrogenase (LDH) release into media using the LDH cytotoxicity kit (Roche). LDH release into media was normalized to maximal LDH release for each well.

Immunocytochemistry

Cells were treated with rotenone for 16 hours and then fixed in 2% paraformaldehyde for 15 minutes. Immunostaining for activated Bax with the monoclonal antibody 6a7 was performed as previously described (17). Cells were imaged using a Nikon Eclipse E800 epifluorescence microscope. Twenty high power (40X) fields per well were randomly selected for quantification, and the number of 6A7-positive cells and the total number of cells stained by Hoechst 33342 were counted per high power field with the rater blind to experimental conditions.

Human brain tissue analysis

Fresh-frozen tissue from temporal cortices of age and gender-matched control and Parkinson's disease brains were obtained from Banner Sun Health Research Institute Brain and Body Donation Program. PD brains were neuropathologically diagnosed as Lewy Body Stage IV. Tissue was homogenized in lysis buffer (Tris/HCl 50mM pH7.4, NaCl 175mM, EDTA 5mM, protease inhibitor and phosphatase inhibitor cocktails (Roche Diagnostics)) and sonicated for 10 seconds. Cell lysates were then incubated on ice for 30 minutes after the addition of 1% Triton X-100 and then spun at 15000g for one hour at 4C. The supernatant was saved as the Triton X-100 soluble fraction. The pellet was resuspended in

lysis buffer with 2% SDS and sonicated for 10 seconds. This was designated as the Triton X-100 insoluble fraction. Samples were resolved on 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were probed for phospho-S58, phospho-S232, total 14-3-3 θ , and pan 14-3-3 as described above. Total 14-3-3 θ and pan 14-3-3 levels were normalized to total protein loading as determined by Coomassie staining.

Statistical analysis

GraphPad Prism 5 (La Jolla, CA) was used for statistical analysis of experiments. Western blot studies were analyzed by 1-way ANOVA, followed by Dunnett's or Newman-Keuls multiple comparison test. LDH assay experiments were analyzed by 2-way ANOVA, followed by post-hoc Bonferroni's multiple comparison test. Human brain sample Western blots were analyzed by unpaired Student's t-test with Welch's correction for unequal variances.

Results

Increased 14-3-3 phosphorylation in the rotenone model

Rotenone is a pesticide that induces dopaminergic neuron loss in the substantia nigra of rodents and induces α syn aggregation (34–36), and we have previously shown that overexpression of certain 14-3-3 isoforms reduces rotenone toxicity in neuroblastoma cells (15). Here we tested whether treatment of dopamine-producing M17 neuroblastoma cells with rotenone causes any changes in phosphorylation of the three key phosphorylation sites: S58, S184, and S232. M17 cells were treated with 5 μ M rotenone for 0–48 hours, and then cell lysates were examined for 14-3-3 phosphorylation using phospho-specific antibodies directed against S58, S184, and S232. With regard to the S232 site, we observed a biphasic phosphorylation response to rotenone treatment in M17 cells using an antibody against phospho-S232 14-3-3 θ . At 24 hours after rotenone treatment (5 μ M), we observed a nearly two-fold increase in 14-3-3 θ phosphorylation at S232 (Fig. 1a). However, this initial increase was followed by a reduction to near basal phosphorylation levels by 36 hours and phosphorylation at this site was nearly eliminated by 48 hours (Fig. 1a). This effect on rotenone was dose-dependent: increasing doses of rotenone caused higher levels of S232 phosphorylation when cells were treated for 18 hours (Fig. 1b).

Both 14-3-3 θ and ζ can be phosphorylated at S/T232, and these two isoforms are highly conserved around this phosphorylation site. Therefore, it is possible that the S232 phospho-14-3-3 θ antibody we used for Western blotting may possibly cross react with T232-phosphorylated 14-3-3 ζ . The increase in endogenous 14-3-3 phosphorylation detected by this antibody could thus reflect phosphorylation of either 14-3-3 θ or ζ , as both of these isoforms run at the same molecular weight. To evaluate the possibility that the S232 phospho-14-3-3 θ antibody we used for Western blotting may possibly cross react with T232-phosphorylated 14-3-3 ζ , we took advantage of stable M17 lines overexpressing individual V5-tagged 14-3-3 isoforms that we have previously created and characterized (15). Because of the V5 epitope tag, the overexpressed 14-3-3 isoform runs slightly higher than the endogenous 14-3-3s and can allow us to determine if the phospho-S232 antibody detects phosphorylation of either 14-3-3 θ and ζ upon rotenone treatment. We treated M17

cells stably transfected with empty vector, V5-tagged 14-3-3 θ , or V5-tagged 14-3-3 ζ with or without 5 μ M rotenone for 18 hours. We found that the V5-tagged 14-3-3 θ band showed a similar increase in S232 phosphorylation as the endogenous 14-3-3 band while no phosphorylation of the V5-tagged 14-3-3 ζ band was detected with the antibody (Fig. 1c). Since the level of overexpression of V5-14-3-3 θ is higher than that of V5-14-3-3 ζ in our stable cell lines, it is possible that we were unable to detect any T232 phosphorylation of V5-tagged 14-3-3 ζ due to its lower expression levels. Therefore, we also loaded excess V5-14-3-3 ζ protein lysates to account for this difference in expression. Even with a 2.5-fold excess of V5-tagged 14-3-3 ζ lysates, we still were unable to detect any T232 phosphorylation of V5-tagged 14-3-3 ζ (Fig. 1c). This suggests that either the antibody we used detects only S232 phosphorylation of 14-3-3 θ and/or that 14-3-3 θ is the main endogenous isoform that is phosphorylated in response to rotenone in M17 cells.

We next examined whether rotenone altered 14-3-3 phosphorylation at S58. M17 cells were treated with 5 μ M rotenone for 0–48 hours, and cell lysates were tested for S58 phosphorylation by Western blot using an antibody against phospho-S58 14-3-3 ζ . We found that rotenone caused a 2.4-fold increase in phosphorylation at S58 at 12 hours. Increased phosphorylation was observed as early as 6 hours and was maximal at 12 hours, but phosphorylation changes returned to baseline by 48 hours (Fig. 2a). This effect was dose-dependent, with higher doses of rotenone causing increased levels of S58 phosphorylation at 24 hours (Fig. 2b). Since several isoforms can be phosphorylated at S58 and it is possible the phospho-S58 14-3-3 ζ antibody could cross react with other S58-phosphorylated isoforms due to conserved homology around S58, we tested which isoforms may be phosphorylated in response to rotenone using stable M17 lines overexpressing individual V5-tagged 14-3-3 isoforms that run slightly higher than the endogenous 14-3-3s, similar to our approach with S232 phosphorylation. M17 cells stably-transfected with 14-3-3 β , ϵ , γ , η , or ζ were treated with 5 μ M rotenone for 12 hours and then lysates were probed for phospho-S58 14-3-3. Compared to our studies with S232, we did not see any dramatic S58 phosphorylation of the V5-tagged isoforms. There was some faint phosphorylation noted of V5-tagged 14-3-3 γ , η , and ζ isoforms (Fig. 2c). It is possible that the V5 epitope tag may interfere with phosphorylation at S58 or may reduce the detection of the epitope by the antibody used. Rotenone did not clearly increase S58 phosphorylation of the V5-tagged isoforms, unlike the endogenous 14-3-3s in each stable cell line which showed increased S58 phosphorylation with rotenone treatment (Fig. 2c). Based on the finding that other tagged isoforms are detected by the antibody, other endogenous isoforms along with 14-3-3 ζ could be phosphorylated in response to rotenone.

We next examined whether any changes in S184 were observed upon rotenone treatment. At baseline, we detected no phosphorylation of 14-3-3s at S184, but were able to detect basal S184 phosphorylation in mouse cortex (Fig. 2d, e). Treatment with increasing doses of rotenone for 24 hours did not induce any detectable S184 phosphorylation in M17 cells (Fig. 2d). We also tested if phosphorylation was observed at different time points at S184, but no S184 phosphorylation was noted following 5 μ M rotenone treatment up to 48 hours (Fig. 2e).

Casein kinases mediate S232 phosphorylation changes in response to rotenone

Casein kinases 1 and 2 can both phosphorylate 14-3-3 proteins at S232 (27, 37, 38). To test whether casein kinases are required for phosphorylation of 14-3-3s at S232 induced by rotenone, we treated M17 cells with rotenone in the presence of the CK1 inhibitor D4476 or the CK2 inhibitor DRB. M17 cells were treated with and without 5 μ M rotenone at different concentrations of D4476 (25 or 50 μ M) or DRB (25 or 50 μ M) for 18 hours, and then lysates were probed by Western blot for phospho-S232. D4476 diminished the increase in S232 phosphorylation in response to rotenone, while it did not have much of an effect on basal S232 phosphorylation in M17 cells (Fig. 3a). Similarly, the CK2 inhibitor DRB also reduced rotenone's induction of S232 phosphorylation with no significant effect on basal S232 phosphorylation (Fig. 3b). These results suggest that casein kinase 1 and/or 2 are involved in the increase in S232 phosphorylation in response to rotenone.

Alteration in S232 phosphorylation in α syn model

Increased phosphorylation in response to α syn overexpression has been described in a proteomics study (29), but the site of phosphorylation is not known. We created a tetracycline-inducible M17 stable cell line that expresses wildtype α syn upon doxycycline treatment. Cells were treated with 2 μ g/ml doxycycline for 0–48 hours and lysates probed for α syn expression and for S58, S184, and S232 phosphorylation. α Syn expression increased more than 30-fold at 24 and 48 hours after 2 μ g/ml doxycycline induction (Fig. 4a). α Syn induction was associated with a nearly 3-fold increase in phosphorylation at S232 at 24 hours (Fig. 4a). Doxycycline treatment alone of M17 cells did not induce any significant changes in S232 phosphorylation (Fig. 4b). Similarly, induction of a control line that expresses GFP upon doxycycline treatment did not cause any change in S232 phosphorylation (Fig. 4c). α Syn levels in M17 cells and the inducible GFP line with and without doxycycline were comparable to that of endogenous α syn levels in the inducible α syn cell line in the absence of doxycycline (Fig. 4d). No statistically significant change in S58 phosphorylation was observed upon α syn induction with doxycycline (Fig 4a). No S184 phosphorylation was detected at baseline or upon induction with 2 μ M doxycycline up to 48 hours (Fig. 4a).

We next examined whether knockdown of α syn affected basal 14-3-3 phosphorylation levels. M17 cells were infected with a control plko.1 lentivirus or a lentiviral shRNA targeting α syn that caused mild reduction in α syn protein levels. M17 cells in which α syn was knocked down showed similar phosphorylation at S58 and S232 compared to control (data not shown). Because of the limited amount of α syn knockdown in M17 cells, we then looked at the effect of α syn knockdown in SH-SY5Y cells which show a higher reduction in α syn protein levels with the α syn shRNA. α Syn levels in α syn knockdown SH-SY5Y cells were 46% of that in cells transduced with plko.1 (Fig. 4e). No change in S58 or S232 phosphorylation was noted in SH-SY5Y cells infected with α syn shRNA compared to control shRNA cells, at least with the level of α syn reduction that was achieved with the α syn shRNA (Fig. 4e).

α Syn does not mediate S232 phosphorylation in response to rotenone

We have found that both rotenone and α syn overexpression cause phosphorylation at S232 in M17 cells. Because rotenone can increase α syn levels in M17 cells (34–36), we next asked whether α syn is required in order for rotenone to cause S232 phosphorylation. We treated control and α syn shRNA SH-SY5Y cells with 5 μ M rotenone for 21 hours, and then cell lysates were examined for S232 phosphorylation by Western blot. We observed that induction of S232 phosphorylation by rotenone was similar in α syn shRNA cells compared to control cells (Fig. 5). Therefore, α syn is unlikely to mediate rotenone's effect on S232 phosphorylation.

LRRK2 does not induce 14-3-3 phosphorylation changes

Since 14-3-3s have been shown to interact with LRRK2 (5–7), we next assessed whether overexpression of wildtype or mutant LRRK2 could affect 14-3-3 phosphorylation at S58 or S232. HEK293T cells were transfected with hemagglutinin (HA)-tagged wildtype or mutant G2019S LRRK2, and lysates were then analyzed for phosphorylation of endogenous 14-3-3s at S58 and S232 by Western blot. No changes in phosphorylation at either serine site were noted with either wildtype or G2019S overexpression when compared to untransfected control cells (Fig. 6a). We then tested for changes in 14-3-3 phosphorylation in a BAC G2019S transgenic mouse, in which highest G2019S expression is found in the hippocampus (30). Hippocampal lysates from 5 week old G2019S mice showed similar levels of 14-3-3 phosphorylation at S58 and S232 compared to nontransgenic littermates (Fig. 6b).

Increased S232 phosphorylation blocks 14-3-3s' neuroprotective effects against rotenone and MPP⁺

We next examined the functional consequence of increased phosphorylation at S232 on the neuroprotective effects of 14-3-3s against neurotoxins. We first focused on 14-3-3 θ , as we have previously shown that overexpression of this isoform is protective in multiple PD models, compared to other isoforms which show less broad protective effects (15). 14-3-3 θ colocalizes with α syn in Lewy Bodies in human PD (12), and we have demonstrated that this isoform is expressed in brain regions affected by PD. We have detected mRNA expression of 14-3-3 θ and other 14-3-3 isoforms in the substantia nigra and cortex of 3-month-old mice (Supp. Table 1; (14, 15)). By Western blotting with an isoform specific antibody against the 14-3-3 θ isoform, we have also detected protein expression of this isoform in both ventral midbrain and cortex of adult mice (Supp. Fig. 1).

To test the effect of S232 phosphorylation on cell survival, we created 14-3-3 phosphorylation mutants of 14-3-3 θ at S232 and created stable lines that overexpressed these phosphorylation mutants at comparable levels to our wildtype 14-3-3 θ stable lines. Upon treatment with rotenone for 48 hours, cells expressing the S232A 14-3-3 θ mutant showed more resistance to rotenone toxicity compared to 14-3-3 θ cells, while in contrast the S232D mutant showed no protection against rotenone (Fig. 7a). Expression levels of wildtype 14-3-3 θ and the two S232 mutants were comparable (Fig. 7d). We confirmed this finding in other stable clones of these mutants. This finding suggests that increased phosphorylation at S232 reduces 14-3-3 θ 's protective effect against rotenone. We saw a

similar protective effect of the S232A mutant in cells treated with MPP⁺ for 24 hours, while the S232D mutant showed no protection against MPP⁺, comparable to control cells (Fig. 7b).

We also examined whether mutation of S58 affected the protective effect of 14-3-3 ζ against rotenone. Overexpression of wildtype 14-3-3 ζ was protective against rotenone treatment for 48 hours, as we have previously observed (15). The S58A 14-3-3 ζ mutant showed a trend of protection against rotenone, yet this was not statistically significant (Fig. 7c). The S58D mutant of 14-3-3 ζ was not protective; however, it should be noted that the level of overexpression of this mutant was lower than wildtype 14-3-3 ζ and the S58A mutant (Fig. 7d).

Increased S232 phosphorylation partially reduces 14-3-3 θ 's effects on Bax activation

We have previously shown that the protective effect of 14-3-3 θ overexpression against rotenone is dependent on inhibition of Bax activation by 14-3-3 θ (17). Therefore, we next examined whether phosphorylation of 14-3-3 θ impacted 14-3-3 θ 's ability to inhibit Bax activation in response to rotenone treatment. In response to rotenone, Bax undergoes conformational changes, translocates to the mitochondrial outer member, and undergoes oligomerization (39, 40). We first examined whether mutation at S232 impacted 14-3-3 θ 's ability to reduce Bax conformational changes in response to rotenone. We did immunocytochemistry against activated Bax using the monoclonal antibody 6A7 that detects an N-terminal Bax epitope that is exposed upon Bax activation (41, 42). Vector control, wildtype 14-3-3 θ , mutant S232A 14-3-3 θ , and mutant S232D 14-3-3 θ stable cells were incubated in the absence or presence of 5 μ M rotenone for 16 hours, and then cells were fixed and stained with the 6A7 antibody. As expected, the number of 6A7-positive cells increased with rotenone treatment in vector control cells, and this increase was significantly attenuated in wildtype 14-3-3 θ cells (Fig. 8a). The number of 6A7-positive cells upon treatment with rotenone was not statistically different in S232A or S232D mutant-expressing cells compared to wildtype 14-3-3 θ cells (Fig. 8a), although there was a slight trend of increased 6A7-positive cells in the S232D group.

We then tested whether the formation of Bax oligomers is affected by 14-3-3 θ phosphorylation at S232. Vector control, wildtype 14-3-3 θ , mutant S232A 14-3-3 θ , and mutant S232D 14-3-3 θ stable cells were treated with 5 μ M rotenone for 18 hours, and then mitochondrial-enriched cell lysates were crosslinked with 1 mM ethylene-glycol-bis(succinic acid N-hydroxy-succinimide ester), as previously described (17, 43). After crosslinking, lysates were run on a gel and immunoblotted with an antibody against Bax to detect monomers and oligomers of Bax. Rotenone induced oligomerization of Bax in vector control cells, while wildtype 14-3-3 θ cells showed a 64% reduction in Bax dimers in response to rotenone treatment (Fig. 8b), as we have previously shown. Bax oligomerization was reduced to an even greater extent in S232A mutant cells (87% reduction), while the levels of Bax oligomers in cells expressing S232D 14-3-3 θ was between that for wildtype 14-3-3 θ cells and vector control (Fig. 8b). This finding suggests that inhibition of Bax oligomerization by 14-3-3 θ is at least partially impaired by phosphorylation at the S232 site.

We finally looked at signaling downstream of Bax activation in the rotenone model. Upon activation and oligomerization, Bax causes permeabilization of the outer mitochondrial membrane that results in cytochrome C release and caspase 3 activation. As we have previously demonstrated (17), rotenone-mediated caspase 3 activation at 24 hours was reduced to near untreated levels in 14-3-3 θ cells compared to vector control cells (Fig. 8c). S232A cells showed complete elimination of cleaved caspase 3 activation upon rotenone treatment, while S232D mutant-expressing cells showed no reduction in cleaved caspase 3 activation upon rotenone treatment compared to control (Fig. 8c). Therefore, we concluded that S232 phosphorylation prevented 14-3-3 θ from inhibiting signaling downstream of Bax activation.

Alterations in 14-3-3 phosphorylation in human PD

We examined phosphorylation of 14-3-3s in human control and PD samples. Pathology in human PD is noted in many brain regions besides the substantia nigra. By the time of clinical diagnosis, most patients with PD have extensive nigral dopaminergic loss such that examination of the substantia nigra in postmortem tissue may not necessarily reflect the pathophysiological changes occurring within dopamine cells, a key neuronal population affected in PD. Thus, we examined samples from the temporal cortex, another brain region affected in PD but at later stages, for changes in 14-3-3 phosphorylation. Samples were obtained from patients at a disease stage at which α syn pathology is evident but significant cell loss has not occurred in the temporal cortex. Fresh frozen temporal cortices from age and gender-matched control and idiopathic PD brains were obtained from the Banner Sun Health Research Institute Brain and Body Donation Program. Temporal cortex from these PD patients showed high density of α syn pathology without significant neuronal loss, and these brains were pathologically diagnosed as Lewy Body Stage IV. Triton X-100-soluble and insoluble fractions were examined by Western blot for S58 and S232 phosphorylation, in addition to total 14-3-3 θ and pan-14-3-3 levels. In the Triton X100-soluble fractions, we observed more than a twofold increase in S232 phosphorylation in PD samples compared to control samples (Fig. 9a). The Triton X-100 insoluble fractions showed a similar trend but did not reach statistical significance (Fig. 9a). In contrast, S58 phosphorylation was decreased significantly in the Triton X-100 insoluble fraction in PD brains compared to control, with a similar but non-statistically significant trend in the soluble fraction (Fig. 9b). No significant differences were noted in total 14-3-3 θ or pan 14-3-3 levels between control and PD brains (Fig. 9c,d).

Discussion

In this study, we examined the potential changes in phosphorylation of the 14-3-3 proteins in several PD models and in human PD brains. We found that both rotenone and α syn overexpression caused an initial increase in S232 phosphorylation in neuroblastoma cells, and a similar increase in S232 phosphorylation was observed in the temporal cortex of PD patients. Inhibition of casein kinases 1 or 2 blocked the increase in S232 phosphorylation by rotenone, and knockdown of α syn did not reduce rotenone's induction of S232 phosphorylation. Phosphorylation at S232 was associated with a reduction in 14-3-3 θ 's neuroprotective effects, as the phosphomimetic S232D 14-3-3 θ mutant failed to protect

against rotenone or MPP⁺ toxicity compared to wildtype or the S232A mutant. The S232D mutant also showed impairment in its ability to inhibit Bax oligomerization and downstream caspase 3 cleavage. Based on these findings, we propose that the increase in 14-3-3 θ phosphorylation at S232 observed in human PD brains is pathogenic and contributes to the neurodegenerative process in PD.

Because of the consistent increase in S232 phosphorylation in toxin and α syn models and in human PD brain, we conclude that the change in this phosphorylation site is likely to play a pathogenic role. Only two 14-3-3 isoforms have this phosphorylation site: 1) 14-3-3 θ and 2) 14-3-3 ζ . To test the effect of phosphorylation at this site, we focused on the 14-3-3 θ isoform, as this isoform is clearly phosphorylated in response to rotenone, based on our evaluation of tagged isoforms (Fig. 1c). This 14-3-3 θ isoform has been found to be colocalized with α syn in Lewy Bodies (12), and we have previously show that this isoform demonstrates the broadest protection in several PD models (15). We have shown that this isoform is expressed in areas affected in PD, including the cortex and SN (Supp. Table 1; Supp. Fig. 1), and other groups have demonstrated expression of 14-3-3 θ in rat or human cortex, striatum, or forebrain (44–47). We did demonstrate an elimination of protection against rotenone toxicity when 14-3-3 θ was mutated to a phosphomimetic aspartate at S232.

To our knowledge, this is the first demonstration that this phosphorylation site can play a role in cell survival. Since Bax inhibition is required for the protective effect of 14-3-3 θ (17), we hypothesized that S232 phosphorylation may prevent 14-3-3 θ from inhibition of Bax, allowing it to translocate to the mitochondria and activate pro-apoptotic signaling cascades. In support of this hypothesis, we observed that inhibition of Bax activation (as determined by Bax oligomerization) by 14-3-3 θ overexpression was impaired by the phosphomimetic 14-3-3 θ mutant S232D but not by the non-phosphorylated mutant S232A. The S232D mutant also failed to inhibit cleaved caspase 3 activation compared to wildtype 14-3-3 θ and the S232A mutant.

While S232 phosphorylation impacts the ability of 14-3-3 θ to inhibit Bax activation, it is likely that the ability of 14-3-3 θ to inhibit other apoptotic factors could also be affected by phosphorylation at this site. The C-terminal has been shown to loop back into the binding domain of 14-3-3s, and phosphorylation at S232 reduces binding of phosphopeptides into the binding pocket (28). Thus, it is possible that phosphorylation changes at S232 in the C terminal region may impact the binding of other ligands in the binding pocket of 14-3-3 dimers. Indeed, we found that, while the S232D mutant partially reduced Bax activation compared to control, it completely failed to inhibit caspase 3 cleavage. This suggests that this phosphorylation impacts other apoptotic factors that can also lead to cleavage of caspase 3.

Interestingly, we saw a biphasic response in neuroblastoma cells treated with rotenone, with an initial increase in S232 phosphorylation followed by a reduction in this phosphorylation. To test whether an increase or decrease in phosphorylation is detrimental to the prosurvival function of 14-3-3 θ , we mutated S232 to the nonphosphorylatable S232A mutant or to the phosphomimetic S232D mutant. Because the S232A mutant is protective while S232D lacks protection, we conclude that the initial increase is what contributes to cell death in these

cells, and that the subsequent reduction in phosphorylation may reflect a compensatory response of the dying cells to try to minimize cell death. It is possible that the subsequent decrease in S232 phosphorylation is an artifact of significant cell death noted at these later time points. At the early time points when we observe an increase in S232 phosphorylation, there is minimal cell death.

We also examined the two other conserved phosphorylation sites of 14-3-3 proteins – S58 and S184. No changes were observed in S184 phosphorylation with treatment with the Parkinsonian toxin rotenone or with α syn overexpression; in fact, no basal S184 phosphorylation was noted in the neuroblastoma cells used. We did observe changes in S58 phosphorylation with rotenone treatment and in human PD brains but not with α syn overexpression. In the rotenone model, S58 phosphorylation was increased transiently, while such phosphorylation was reduced in human PD temporal cortex. No significant changes were noted with α syn overexpression, but there was a trend of decreased S58 phosphorylation. This discrepancy with regard to S58 phosphorylation between the rotenone model and human brain could be possibly explained by the fact that the changes observed in the human brain samples may reflect compensatory changes or late stage changes compared to the rotenone model which may be more reflective of early changes in disease. Alternatively, rotenone treatment may not be predictive of all the changes observed in human disease.

Whether changes in S58 phosphorylation may promote pathogenesis in PD is less clear. While increased S232 phosphorylation was observed in several PD models and in human PD brain, the alterations in S58 were less consistent in the observed models as discussed above. Phosphorylation at S58 has been demonstrated in other cellular systems to promote cell death. For example, S58 phosphorylation of 14-3-3 ζ causes release of the pro-apoptotic protein ASK1 and cell death in response to oxidative stress in 3T3 cells (25), and increased S58 phosphorylation is associated with neuronal loss in mice treated with kainic acid to induce seizures (48). We did test the effect of mutations at this phosphorylation site on the protective effect of 14-3-3 ζ against rotenone. As compared to the S232 mutants, we did not observe as dramatic an effect on 14-3-3 ζ neuroprotection. The S58D mutant failed to show any protection compared to control cells, yet with this S58D mutant, we were not able to induce as much expression of this mutant compared to wildtype or the S58A mutant; thus, it is possible that a lack of protection by this phosphomimetic could be due to lower expression levels. The nonphosphorylatable S58A mutant showed a trend of protection against rotenone, yet this did not reach statistical significance. It is also possible that phosphorylation of S58 of a different isoform may be more relevant to PD, and mutations of S58 in 14-3-3 β , ϵ , γ , η should be considered in future studies. Our current data suggests that S58 phosphorylation could contribute to toxicity and that decreased S58 phosphorylation observed in human PD may reflect a compensatory response.

Our study shows some consistency with the findings of Kulanthinal *et al.* who have demonstrated by proteomics alterations in 14-3-3 phosphorylation in tet-off BE-2-M17D neuroblastoma cell line that conditionally overexpresses α syn (29). They observed increased phosphorylation of 14-3-3 ϵ at 14 days after α syn induction and increased phosphorylation of 14-3-3 ζ at 28 days after α syn induction (29). In our study, we focused on early time points

after induction, such that a direct comparison of their results with ours is not possible. In their study, they did not determine which phosphorylation sites were involved. They did test for changes in three PD nigral samples and they observed a trend of increased phosphorylation but which phosphorylation site showed the increase was not examined (29). We evaluated the temporal cortex to assess for 14-3-3 phosphorylation changes instead of the SN and observed an increase in S232 phosphorylation in the temporal cortex. Dopamine nigral neurons in patients with clinically diagnosed PD are massively depleted, such that any changes noted in tissue from nigral tissue may not reflect pathogenic changes in the dopamine cells but may instead reflect changes in other nigral cell populations that are not susceptible in PD. Other brain regions besides the SN are also affected in PD, such as the temporal cortex in which neuronal loss occurs at a later stage than in the SN. The samples chosen for our human analysis were pathologically diagnosed as Lewy Body Stage IV, at which stage the tissue demonstrates extensive α syn pathology without significant neuronal loss. Therefore, the changes we observed in the temporal cortex are likely to reflect pathogenic changes, especially when assessed in conjunction with our mutagenesis data. While these brains were obtained from patients clinically and pathologically diagnosed with Parkinson's disease, some of the brain samples used did demonstrate Alzheimer's pathology with amyloid plaques. Therefore, it is possible that the Alzheimer's pathology we observed in our PD samples could have contributed to the 14-3-3 phosphorylation changes. However, the consistency of the S232 phosphorylation changes in several PD models suggests that S232 phosphorylation is important to PD pathology. Future evaluation of S232 phosphorylation in other neurodegenerative disorders is warranted. If this 14-3-3 phosphorylation is specific to PD, then this phosphorylation could serve as a potential biomarker in the diagnosis and/or progression of PD.

In conclusion, we have demonstrated increased 14-3-3 phosphorylation at S232 with rotenone treatment, with α syn overexpression, and in human PD brain. Less consistent alterations are observed at S58. The S232D phosphomimetic of 14-3-3 θ shows a complete elimination of its neuroprotective effects against the neurotoxins rotenone and MPP⁺. We conclude that increased phosphorylation at S232 is likely pathogenic in nature and may serve as a target for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αsyn	alpha-synuclein
CK	casein kinase
GFP	green fluorescent protein
LDH	lactate dehydrogenase
LRRK2	leucine-rich repeat kinase 2
MPP⁺	1-methyl-4-phenylpyridinium
n.s	not significant
PD	Parkinson's disease
PBS	phosphate-buffered saline
SN	substantia nigra
M17	SK-N-BE(2)-M17

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Highlights

- 14-3-3 phosphorylation at serine 232 is increased in rotenone and α syn models.
- Changes at other 14-3-3 phosphorylation sites are inconsistent between models.
- 14-3-3 phosphorylation at serine 232 reduces protective effects of 14-3-3 θ .
- 14-3-3 phosphorylation at serine 232 is increased in human PD brains.

Figure 1. Rotenone promotes biphasic changes in serine 232 phosphorylation of 14-3-3θ

A) Rotenone causes an initial increase in S232 phosphorylation of 14-3-3θ at 24 hours but then a reduction in S232 phosphorylation below baseline at 48 hours. M17 neuroblastoma cells were treated with 5 μM rotenone for 24, 36, or 48 hours and then cell lysates were analyzed for phospho-S232 and total 14-3-3θ by Western blot. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phosphorylated 14-3-3θ to total 14-3-3θ is quantified for three independent rounds. **p<0.01 and ***p<0.001 (Newman-Keuls multiple comparison test).

B) S232 phosphorylation increases with increasing doses of rotenone at 18 hours. M17 cells were treated with 0.04, 0.2, 1, or 5 μM rotenone for 18 hours. Cell lysates were analyzed for phospho-S232 and total 14-3-3θ by Western blot. Tubulin was used as loading control. The ratio of phosphorylated 14-3-3θ to total 14-3-3θ is quantified for three independent rounds. *p<0.05 and ***p<0.001 (Dunnett's multiple comparison test)

C) Rotenone induces S232 phosphorylation of V5-tagged 14-3-3θ. Empty vector control, 14-3-3θ-overexpressing, and 14-3-3ζ-overexpressing stable M17 cells were treated with 5 μM rotenone for 18 hours, and then evaluated for S/T phosphorylation. Top arrow indicates V5-tagged 14-3-3 isoforms; bottom arrow indicates endogenous 14-3-3s. The antibody used detects an increase in phosphorylation in the V5-tagged 14-3-3θ but none with V5-tagged 14-3-3θ, even when a 2.5-fold excess of 14-3-3ζ lysate was loaded to control for potential reduction in V5-tagged 14-3-3ζ phosphorylation due to the lower expression.

Figure 2. Rotenone induces 14-3-3 phosphorylation at serine 58

A) M17 cells are treated with 5 μ M rotenone for 6, 12, 24, and 48 hours. Cell lysates were analyzed for S58 phosphorylation and for total 14-3-3 levels by Western blot.

Representative Western blot is shown. Tubulin was used as loading control. The ratio of phosphorylated 14-3-3s at S58 to pan 14-3-3 levels is quantified for three independent rounds. * $p < 0.05$ (Newman-Keuls multiple comparison test).

B) S58 phosphorylation increases with increasing doses of rotenone. M17 cells were treated with 0.04, 0.2, 1, or 5 μ M rotenone for 24 hours.

C) S58 phosphorylation of V5-tagged 14-3-3 isoforms is observed for at least for 14-3-3 γ , η , and ζ . Empty vector control and various V5-tagged 14-3-3-overexpressing stable M17 cells were treated with 5 μ M rotenone for 12 hours, and then evaluated for S58 phosphorylation. Top arrow indicates V5-tagged 14-3-3 isoforms; bottom arrow indicates endogenous 14-3-3s.

D) M17 cells do not demonstrate basal phosphorylation at S184, and rotenone does not induce any S184 phosphorylation of 14-3-3s. M17 cells were treated with 0.04, 0.2, 1, or 5 μ M rotenone for 24 hours. Cell lysates were analyzed for phospho-S184 and pan 14-3-3 by Western blot. Tubulin was used as loading control.

E) No phosphorylation at S184 is detected at several different time points after rotenone treatment. M17 cells were treated with 5 μ M rotenone up to 48 hours. Cell lysates were analyzed for phospho-S184 and pan 14-3-3 by Western blot. Tubulin was used as loading control. Mouse brain lysate was loaded as a control to verify that primary antibody was capable of detecting phosphorylated S184.

Figure 3. Casein kinase inhibitors reduce S232 phosphorylation induced by rotenone

A) M17 cells are treated with 5 μ M rotenone for 18 hours with or without the presence of the casein kinase I inhibitor D4476. Cell lysates were analyzed for phospho-S232 and total 14-3-30 by Western blot. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S232 to total 14-3-30 is quantified for three independent rounds. *p<0.05 and ***p<0.001 (Newman-Keuls multiple comparison test).

B) M17 cells are treated with 5 μ M rotenone for 18 hours with or without the presence of the casein kinase II inhibitor DRB. Cell lysates were analyzed for phospho-S232 and total 14-3-30 by Western blot. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S232 to total 14-3-30 is quantified for three independent rounds. *p<0.05 (Newman-Keuls multiple comparison test).

Figure 4. Overexpression of α syn induces 14-3-3 phosphorylation at S232

A) A tetracycline-inducible M17 cell line that overexpresses α syn upon doxycycline treatment is treated with 2 μ M doxycycline for 18, 24, or 48 hours. Cell lysates were analyzed for phosphorylation at S58, S184, and S232 and total 14-3-3s by Western blot. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S232 to pan 14-3-3s is quantified for three independent rounds. Similarly, the ratio of S58 phosphorylation to pan 14-3-3s is quantified for three independent runs. α Syn levels are also quantified for three independent runs. *** p <0.001 (Newman-Keuls multiple comparison test) for phospho-S232. n.s. for phospho-S58 (1 way ANOVA). * p <0.05, ** p <0.01, *** p <0.001 (Dunnett's multiple comparison test) for α syn.

B) Doxycycline treatment alone does not induce 14-3-3 phosphorylation. M17 cells were treated with or without 2 μ g/ml doxycycline for 18, 24, or 48 hours. Cell lysates were analyzed for phosphorylation at S58 and S232 and total 14-3-3s by Western blot. Ratio of phospho-S232 to pan 14-3-3s is quantified for three independent rounds. n.s. (1 way ANOVA).

C) Doxycycline treatment of a tetracycline-inducible GFP M17 cell line does not induce 14-3-3 phosphorylation at S232. GFP-inducible cells were treated with or without 2 μ g/ml doxycycline for 18, 24, or 48 hours. Cell lysates were analyzed for phosphorylation at S232 and total 14-3-3s by Western blot. Ratio of phospho-S232 to pan 14-3-3s is quantified for three independent rounds. n.s. (1 way ANOVA).

D) α Syn levels are dramatically increased upon doxycycline treatment in inducible α syn cells compared to control M17 or inducible GFP cells. α Syn expression is quantified for three independent runs. *** p <0.001 compared to α syn + doxy condition (Newman-Keuls multiple comparison test).

E) Knockdown of α syn by shRNA in SH-SY5Y cells did not alter basal 14-3-3 phosphorylation at S58 or S232. Cell lysates from control shRNA and α syn shRNA

transduced cells were analyzed for phospho-S58 and phospho-S232 and pan 14-3-3s by Western blot. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S58 to pan 14-3-3, the ratio of phospho-S232 to pan 14-3-3, and the ratio of α syn to tubulin are quantified for three to four independent runs. * $p < 0.05$ (Student's t-test).

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Figure 5. α Syn knockdown does not block induction of S232 phosphorylation by rotenone
 A) Control shRNA- and α syn shRNA-transduced SH-SY5Y cells were treated with 5 μ M rotenone for 21 hours, and cell lysates were analyzed for phospho-S232 and total 14-3-3 θ by Western blot. Representative Western blot is shown. Tubulin was used as loading control.
 B) The ratio of phospho-S232 to total 14-3-3 θ is quantified for five independent rounds.
 *p<0.05 and **p<0.01 (Newman-Keuls multiple comparison test).

Figure 6. Wildtype and mutant LRRK2 expression does not affect 14-3-3 phosphorylation at S58 or S232

A) HEK 293T cells were transfected with wildtype or G2019S LRRK2, and cell lysates were analyzed for phospho-S58 or phospho-S232 by Western blot. Untransfected HEK 293T cells were used as control. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S58 to pan 14-3-3 and the ratio of phospho-S232 to pan 14-3-3 are quantified for five independent runs. n.s. (1 way ANOVA). B) Hippocampal lysates from nontransgenic or G2019S littermates were analyzed for phospho-S58 or phospho-S232 by Western blot. Representative Western blot is shown. Tubulin was used as loading control. n = three mice per genotype. n.s. (Student's unpaired t-test).

Figure 7. Phosphorylation at S232 blocks 14-3-30 neuroprotective effects against rotenone and MPP⁺

A) Serine 232 of 14-3-30 was mutated to either alanine (S232A) or aspartic acid (S232D) to test the effect of phosphorylation of 14-3-30's prosurvival function. S232A overexpression reduces rotenone toxicity even more than wildtype 14-3-30, while S232D overexpression fails to protect against rotenone. M17 cells stably transfected with empty vector, wildtype 14-3-30, S232A 14-3-30, and S232D 14-3-30 were treated with increasing doses of rotenone for 48 hours, and cell death was measured by LDH release. Results reflect three independent runs with two replicates per run. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (2 way ANOVA, followed by Bonferroni's multiple comparison test).

B) S232A mutant protected more effectively against MPP⁺-induced cell death compared to wildtype 14-3-30, while the S232D mutant was more susceptible to MPP⁺. M17 cells stably transfected with empty vector, wildtype 14-3-30, S232A 14-3-30, and S232D 14-3-30 were treated with 5mM MPP⁺ for 24 hours, and cell death was measured by LDH release. Results reflect three independent runs with two replicates per run. *** $p < 0.001$ (2 way ANOVA, followed by Bonferroni's multiple comparison test).

C) S58D mutation of 14-3-30 showed reduced protection against rotenone. Control, wildtype, S58A, and S58D 14-3-30 cells were treated with increasing doses of rotenone for 48 hours, and cell death was measured by LDH release. Results reflect three independent runs with two replicates per run. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (2 way ANOVA, followed by Bonferroni's multiple comparison test).

D) Wildtype and mutant S232 14-3-30 expression levels were comparable. S58D 14-3-30 expression was lower than that for wildtype and S58A 14-3-30. Lysates from M17 cells stably transfected with wildtype or mutant 14-3-3 were analyzed for V5-tagged 14-3-3 expression by Western blot.

Figure 8. S232 phosphorylation partially reduces the ability of 14-3-38 to inhibit Bax activation in response to rotenone

A) M17 cells stably transfected with empty vector, wildtype 14-3-3θ, S232A 14-3-3θ, and S232D 14-3-3θ were treated with 5 μM rotenone for 16 hours, and cells were then stained with a monoclonal antibody that recognizes an activated conformation of Bax. Results reflect two independent runs with two to three separate wells per run. ** $p < 0.01$ (Newman-Keuls multiple comparison test).

B) S232D mutant showed reduced ability while S232A mutant was more effective in inhibiting Bax oligomerization in response to rotenone. M17 cells stably transfected with empty vector, wildtype 14-3-3θ, S232A 14-3-3θ, and S232D 14-3-3θ were treated with 5 μM rotenone for 18 hours. Mitochondrial-enriched fractions were crosslinked with ethylene-glycol-bis(succinic acid N-hydroxy-succinimide ester) and analyzed for Bax oligomerization by Western blot. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (Newman-Keuls multiple comparison test). Note that the order of samples on the blot is not the same order as on the graph.

C) S232D mutant fails to block cleavage of caspase 3 in response to rotenone treatment. M17 cells stably transfected with empty vector, wildtype 14-3-3θ, S232A 14-3-3θ, and S232D 14-3-3θ were treated with 5 μM rotenone for 24 hours. Cell lysates were analyzed for cleaved caspase 3 by Western blot. * $p < 0.05$ and ** $p < 0.01$ (Newman-Keuls multiple comparison test).

Figure 9. S232 phosphorylation is increased in human PD brain

A) Lysates of human temporal cortices from control and PD patients were fractionated in Triton X-100 soluble and insoluble fractions and then analyzed for phospho-S232 and total 14-3-3θ by Western blot. The ratio of phospho-S232 to total 14-3-3θ is quantified for 8 age-matched control and 8 PD samples. p=0.0204 (Student's t-test with Welch's correction) for Triton X-100 soluble fraction.

B) Phosphorylation of S58 is reduced in Triton X-100 insoluble fractions from PD samples. Triton X-100 soluble and insoluble fraction lysates of human temporal cortices from control and PD patients were analyzed for phospho-S58 and total 14-3-3s by Western blot. The ratio

of phospho-S58 to pan14-3-3s is quantified for 8 age-matched control and 8 PD samples. $p=0.0099$ (Student's t-test with Welch's correction) for Triton X-100 insoluble fraction.

C) Total 14-3-3 θ levels are not significantly changed in human PD brain. Triton X-100 soluble and insoluble fraction lysates of human temporal cortices from control and PD patients were analyzed for 14-3-3 θ by Western blot. 14-3-3 θ was normalized to total protein loading as determined by Coomassie staining. $n=8$ brains per group. n.s. (Student's t-test with Welch's correction).

D) Total 14-3-3s levels are not significantly changed in human PD brain. Triton X-100 soluble and insoluble fraction lysates of human temporal cortices from control and PD patients were analyzed for pan 14-3-3 by Western blot. Pan 14-3-3 was normalized to total protein loading as determined by Coomassie staining. $n=8$ brains per group. n.s. (Student's t-test with Welch's correction).