Published in final edited form as:

J Mol Neurosci. 2013 November ; 51(3): . doi:10.1007/s12031-013-0086-5.

α -Synuclein overexpression represses 14-3-3 σ transcription

Huiping Ding¹, Naomi S. Fineberg², Michelle Gray¹, and Talene A. Yacoubian¹

¹Department of Neurology, Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham

²Department of Biostatistics, School of Public Health, University of Alabama at Birmingham

Abstract

Previous gene microarray studies have shown that expression of 14-3-3 σ is significantly decreased in an α -synuclein transgenic mouse model. In this study, we tested whether α -synuclein can regulate 14-3-3 σ transcription. We demonstrate that 14-3-3 σ mRNA level is decreased in SH-SY5Y cells overexpressing α -synuclein. Luciferase activity under control of the 14-3-3 σ promoter is reduced both in stable SH-SY5Y cells constitutively overexpressing α -synuclein and in doxycycline-inducible SH-SY5Y cells upon α -synuclein induction, suggesting that the regulation of 14-3-3 σ by α -synuclein occurs at the transcriptional level. Knockdown of α -synuclein by RNA interference does not increase the 14-3-3 σ mRNA level. These findings suggest that α -synuclein represses 14-3-3 σ transcription under pathologic conditions but that regulation of 14-3-3 σ expression is not a function of endogenous α -synuclein at baseline.

Keywords

α -synuclein; 14-3-3s; transcription regulation; histone deacetylation

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder marked by loss of dopaminergic neurons in the substantia nigra. Mutations in several genes including α -synuclein (α -syn) have been linked to genetic forms of PD (Polymeropoulos et al. 1997; Kruger et al. 1998; Athanassiadou et al. 1999; Dawson and Dawson 2003). In sporadic PD, α -syn is a main component of Lewy bodies, a pathological hallmark of PD (Spillantini et al. 1997), and increased levels of α -syn are hypothesized to promote neurodegeneration in PD (Singleton et al. 2003). However, the underlying mechanisms for α -syn toxicity in PD are still poorly understood. In an effort to examine these mechanisms, we performed gene microarray studies of an α -syn transgenic mouse model to examine potential changes in gene expression (Yacoubian et al. 2008). Among those altered genes were several members of the 14-3-3 protein family (Yacoubian et al. 2008).

14-3-3s are a highly conserved and ubiquitously expressed protein family, comprising about 1% of total brain soluble protein (Berg et al. 2003b). There are seven 14-3-3 isoforms in mammals (σ , η , τ , ζ , ϵ , μ , and θ). 14-3-3 proteins play important roles in various cellular

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.

Conflict of Interest

Huiping Ding, Naomi Fineberg, and Michelle Gray have no conflict of interest to declare. Talene Yacoubian declares that she has a US Patent # 7,919,262 on the use of 14-3-3s in neurodegeneration.

processes including intracellular trafficking, signal transduction, and cell growth and death (van Hemert et al. 2001). They have been recently implicated in several neurodegenerative disorders, including PD. 14-3-3s have been found in Lewy bodies (Kawamoto et al. 2002; Berg et al. 2003a). They form protein complexes with α -syn in human brains, and the levels of these protein complexes are elevated in PD brains (Ostrerova et al. 1999; Xu et al. 2002; Sato et al. 2006). We have previously found that expression of several 14-3-3 isoforms is decreased in an α -syn transgenic mouse model with 14-3-3 being the most significantly downregulated (Yacoubian et al. 2008; Yacoubian et al. 2010).

Functional genomic analysis of our previous gene array study showed that transcription is the predominant biological function altered in the α -syn transgenic mice, suggesting that α -syn overexpression is associated with transcriptional dysregulation (Yacoubian et al. 2008). Therefore, in this study we examined whether α -syn can regulate 14-3-3s at a transcriptional level. We first assessed whether α -syn overexpression in cell lines affects 14-3-3 expression in a similar manner as in the α -syn transgenic mouse model. We then tested whether α -syn regulates 14-3-3 expression at a transcriptional level using luciferase reporter assays. Our results suggest that increases in α -syn downregulate 14-3-3 transcription. Given 14-3-3s' crucial role as apoptosis inhibitors, dysregulation of 14-3-3 expression by α -syn may represent a potential mechanism of α -syn toxicity.

Materials and Methods

Cell lines and chemicals

SH-SY5Y cells were cultured in 1:1 mixture of Eagle's Minimum Essential Medium and F12K Medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS), 100 unit/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY), at 37 °C and 5% CO₂. Stable SH-SY5Y cells overexpressing α -syn and empty control cells were obtained from Dr. Janusz Tucholski from UAB and maintained in the presence of 400 μ g/ml Geneticin (Life Technologies, Grand Island, NY). SH-SY5Y cells expressing inducible α -syn were created by transducing SH-SY5Y cells with doxycycline-inducible α -syn pSLIK lentivirus followed by selection with 400 μ g/ml Geneticin. The α -syn pSLIK construct was created by the UAB Neuroscience Core Center by first cloning α -syn into the pEN_TTmcs vector (ATCC, Manassas, VA), followed by recombination with the Geneticin-selectable pSLIK lentiviral construct (Shin et al. 2006). Rotenone, sodium butyrate and trichostatin A were purchased from Sigma (St. Louis, MO). MS-275 was from Cayman Chemical (Ann Arbor, MI).

α -Syn knockdown

The pLKO.1- α -syn shRNA lentiviral vectors were purchased from Thermo Scientific (Waltham, MA) and packaged by the UAB Neuroscience Core Center. SH-SY5Y cells were transduced with the empty vector pLKO.1 viruses or α -syn shRNA viruses in the presence of 6 μ g/ml polybrene (Sigma, St. Louis, MO). 72 hrs later, transduced cells were selected with 1 μ g/ml puromycin (Mediatech, Herndon, VA).

Real-time quantitative PCR

RNA was extracted from SH-SY5Y cells using TRI reagent (Life Technologies, Grand Island, NY), purified using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed into first-strand cDNA using the SuperScript II Reverse Transcriptase Kit (Life Technologies, Grand Island, NY). Primer sets used for 14-3-3 (NM_011739) were forward 5'-aggagtgcacagcacacttg-3' and reverse 5'-gttgctctgaaggaacctc-3'; for 14-3-3 (NM_009536) forward 5'-ttgggtgttagcttgagtg-3' and reverse 5'-gaggagtcggcaagaatgag-3'; and for 14-3-3 (NM_018871) forward 5'-tctgtgtcccgcttgactg-3' and reverse 5'-

aggcaggcacacttctcaac-3'. For real-time quantitative PCR, cDNA was incubated with primers and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 96-well plate. Quantitative PCR was performed using an iQ5Cycler (BioRad, Hercules, CA) set to the following protocol: 1 cycle of denaturation at 95 °C for 10 min; 50 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, and polymerization at 72 °C for 45 sec; and finally, 80 cycles at 0.5 °C increases in temperature (starting at 55 °C) to collect melting curve data. For each primer set, we used a standard curve with known concentrations of cDNA to calculate primer efficiency and to quantitate PCR products. The quantity of each PCR sample was calculated using the C_t method (Livak and Schmittgen 2001).

For the quantitative PCR study using BACHD mice expressing human mutant huntingtin (Gray et al. 2008), RNA was extracted from the cortex of 12 month old wildtype and BACHD mice and reverse transcribed. Quantitative PCR for 14-3-3 was performed as described above. We used hypoxanthine phosphoribosyltransferase (HPRT) (forward primer 5'-tggtgttgatgaccccttg-3'; reverse primer 5'-tgcgctcatcttaggctttg-3') to normalize PCR results.

Luciferase reporter constructs and assay

To create luciferase reporter constructs, varying lengths of the putative promoter region upstream from the translation start site for 14-3-3 gene were subcloned into the firefly luciferase reporter vector pGL4.17[luc2/Neo] (Promega, Madison, WI). The promoter regions (F2, F9, F12, F15 and F17) were amplified by polymerase chain reaction using genomic DNA isolated from human SK-N-BE(2)-M17 cells as template and the following primer sets. Forward primers were F2 (-1597/-103, 1.5 kB): 5'-ctcagatctctcgttcagcagggtgttc-3'; F9 (-2325/-103, 2.22 kB): 5'-ctcagatcttgacaatttcctctctggg-3'; F12 (-2648/-103, 2.55 kB): 5'-gtcagatcttccaacccgagttcttcag-3'; F15 (-3019/-103, 2.9 kB): 5'-cttggtaccggctggtctgtcatccatccta-3'; F17 (-4052/-103, 3.95 kB): 5'-catgttaccttggtgaaagtcaccactc-3'. Reverse primer for F2, F9, F12, and F15 was 5'-ggttaagcttgagcgagaaacaaaagcag-3' and for F17 was 5'-gatggtaccgagcgagaaacaaaagcag-3'. The nucleotide numbers are relative to the translation start site of 14-3-3. Amplified DNA was purified, digested and ligated into pGL4.17 vector. Five constructs were created and their sequences were confirmed by DNA sequencing analysis.

For the luciferase assay, stable SH-SY5Y cells constitutively overexpressing α -syn or control vector were transfected with the firefly luciferase reporter constructs using Superfect reagent (Qiagen, Valencia, CA). To normalize for transfection efficiency, a Renilla luciferase vector pRL-TK (Promega, Madison, WI) was co-transfected. 48 hrs later, luciferase activity was measured by a luminometer using a Dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activities were normalized to Renilla luciferase activities.

For the luciferase assay in SH-SY5Y cells expressing inducible α -syn, uninduced cells and cells that had been induced with 1 μ g/ml doxycycline for 1 or 4 days were transfected with firefly luciferase reporter construct F9 and Renilla luciferase vector pRL-TK. Doxycycline was maintained in induced cells after transfection. 48 hrs later, luciferase activity was measured using a Dual Luciferase Reporter Assay System.

Cell lysis and immunoblotting

Cells were collected 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)), sonicated for 10 sec on ice and centrifuged at 12,000 g for 10 min. Protein

concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Samples were boiled for 5 min in sample loading buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 200 mM DTT, 30% glycerol, bromophenol blue), separated on 12% 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 1 hr at room temperature, and then incubated overnight at 4°C with primary mouse monoclonal antibody against α -syn (1:1000; BD Biosciences) or mouse monoclonal antibody against actin (1:10,000; Sigma). After five washes in TBST, blots were incubated with HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 2 hrs at room temperature and then washed in TBST six times for 10 min each. Blots were developed by the enhanced chemiluminescence method (Pierce, Rockford, IL).

Statistical analysis

For densitometric quantification of immunoblots, data from three independent blots was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. For all real-time quantitative PCR and luciferase assays, data was obtained from three independent experiments with two or three replicates in each experiment. In each experiment, the data was normalized to control condition. All sets of data were examined graphically and statistically and had reasonably normal distributions. Two and three way ANOVA was employed depending on the number of grouping factors in a given experiment. Experimental run was included as a grouping factor in the analyses to adjust for any variances between experimental runs for some studies. The lack of an interaction between experimental run and other variables (treatment or cell line) confirmed that while the values may be different from experimental run to run the relationship between levels of the factor were similar for all experiments. Post hoc analyses for most experiments were performed using Tukey's multiple comparison test. Comparisons of luciferase activity in control and α -syn stable cell line for each luciferase reporter construct were performed using t-tests. Comparison of 14-3-3 mRNA levels in wildtype and mutant huntingtin transgenic mice was performed using t-test. Comparisons of 14-3-3 mRNA levels in untreated or sodium butyrate-treated control cells and α -syn knockdown cells were performed using two-way ANOVA. Pooled data of all experimental runs were shown in graphed figures for simplicity.

Results

14-3-3 σ mRNA levels are decreased in α -syn-overexpressing stable cells

Previous gene array studies and quantitative real-time PCR showed that expression of certain 14-3-3 isoforms (σ , τ , and ζ) was altered in the substantia nigra and cortex of an α -syn transgenic mouse model (Yacoubian et al. 2008; Yacoubian et al. 2010). 14-3-3 σ was the most significantly downregulated isoform in this model. To examine whether 14-3-3 σ s are also altered in a cellular model, we turned to stable SH-SY5Y cell lines that overexpressed α -syn. Immunoblot showed α -syn overexpression in three different α -syn stable clones compared to control cells (Figure 1A). We then analyzed the mRNA levels of 14-3-3 σ , τ , and ζ in these cell lines using quantitative PCR. We found that 14-3-3 σ mRNA levels were decreased by 50%, 35% and 40% in three different α -syn stable clones compared to control cells, respectively (Figure 1B). 14-3-3 τ mRNA levels were also significantly reduced by 29% and 26% in two of the three tested α -syn stable clones compared to control (Figure 1C). The mRNA levels of 14-3-3 ζ were not significantly different between control and α -syn stable clones (Figure 1D).

To examine whether 14-3-3 σ expression could also be affected by other Parkinson's disease related or unrelated insults, we analyzed the mRNA levels of 14-3-3 σ in rotenone-treated SH-SY5Y cells and in BACHD mice expressing human mutant huntingtin (Gray et al.

2008). Quantitative PCR showed that rotenone treatment caused a reduction of 14-3-3 mRNA level by 46% (Figure 2A). However, overexpression of mutant huntingtin did not change 14-3-3 mRNA level when compared to wildtype mice (Figure 2B).

14-3-3 θ promoter activity is decreased in α -syn-overexpressing stable cells

As 14-3-3 is the most affected isoform in both α -syn transgenic mice and α -syn overexpressing cells, we focused on 14-3-3 in this study. To test whether overexpression of α -syn downregulates 14-3-3 at transcriptional level, we created luciferase reporter constructs under control of the 14-3-3 promoter. While the promoter for 14-3-3 has yet to be characterized, we created five putative promoter constructs that included varying lengths of the 5' region upstream of the translation start site (F2: 1.5 kB, F9: 2.22 kB, F12: 2.55 kB, F15: 2.9 kB and F17: 3.95 kB) based on the promoter region for 14-3-3 (Brunelli et al. 2007) (Figure 3A). To test the luciferase activity of these reporter constructs at baseline, we transfected the control pGL4 vector or the reporter constructs into control SH-SY5Y cells and measured luciferase activity. The results showed that the luciferase activity of control pGL4 vector was barely detectable (data not shown) and the shorter constructs (F2, F9, F12 and F15) drove luciferase expression at a comparable level, while the longest construct F17 showed 86% reduction in basal luciferase activity compared to F2 (Figure 3B, white bars). This suggests that negative regulatory elements may exist between 2.9 kB and 3.95 kB upstream of the translation start site of 14-3-3.

We then tested the effect of α -syn overexpression on the luciferase reporter constructs driven by 14-3-3 promoter. The five reporter constructs described above were also transfected into α -syn stable cells and luciferase activities were measured and then compared to control cells. We found that luciferase activities driven by all of the tested promoter regions were significantly reduced by α -syn overexpression (34% for F2, 28% for F9, 30% for F12, 35% for F15 and 43% for F17) (Figure 3B, black bars). This result suggests that α -syn regulates the expression of 14-3-3 at the transcriptional level, and that the pattern of regulation is the same within the first 3.95 kB of the putative promoter region of 14-3-3.

14-3-3 θ promoter activity is decreased in cells expressing inducible α -syn

To further test the effect of α -syn overexpression on 14-3-3 transcription, we created a SH-SY5Y cell line in which α -syn expression is induced by doxycycline treatment and compared 14-3-3 reporter gene expression in uninduced and induced cells. Immunoblot showed α -syn overexpression after induction with 1 μ g/ml doxycycline for 48 hours (Figure 4A). To perform luciferase reporter assay, we induced α -syn overexpression with doxycycline for 1 or 4 days, and then transfected cells with the F9 reporter construct. Doxycycline was maintained in induced cells for 2 days after transfection, at which time luciferase activity was measured. We found that luciferase activity driven by 14-3-3 promoter was decreased in cells with α -syn induced for a total of 6 days (24%) but not for a total of 3 days when compared to uninduced cells (Figure 4B).

α -Syn knockdown does not alter 14-3-3 θ expression

To further examine the relationship between α -syn and 14-3-3, we analyzed 14-3-3 expression in α -syn-knockdown cells. Transduction of SH-SY5Y cells with recombinant α -syn shRNA lentivirus caused a dramatic reduction in the expression of α -syn (Figure 5A). Quantitative PCR showed that there were no significant differences in 14-3-3 mRNA levels between α -syn knockdown and control pLKO.1 cells (Figure 5B, black bars).

HDAC inhibition decreases 14-3-3 σ mRNA level and increases α -syn protein level

α -Syn has been shown to inhibit histone acetylation when overexpressed in cell culture or *Drosophila* (Kontopoulos et al. 2006). Since histone acetylation is critical in regulating gene expression, we hypothesized that α -syn may downregulate 14-3-3 σ transcription by inhibiting histone acetylation. If this is true, then we would expect that increasing histone acetylation should enhance 14-3-3 σ transcription, opposite to the effect of α -syn overexpression. We treated SH-SY5Y cells with varying doses of two different histone deacetylase (HDAC) inhibitors, sodium butyrate (SB) and trichostatin A (TSA), and measured 14-3-3 σ mRNA level by quantitative PCR. Surprisingly, we found that treatment of SH-SY5Y cells alone with either SB or TSA caused a reduction in 14-3-3 σ expression, comparable to the effect of α -syn overexpression alone (Figures 6A and 6B). Since SB and TSA are less specific and different HDAC inhibitors may have drastic difference in their effects (Dietz and Casaccia 2010; Jia et al. 2012), we then performed similar experiments with a more selective HDAC inhibitor, MS-275, which has been shown to preferentially inhibit HDAC1 (Hu et al. 2003). Treatment with MS-275 also caused reduction in 14-3-3 σ mRNA levels (Figure 6C), suggesting that inhibition of HDAC1 alone is sufficient to induce this reduction in 14-3-3 σ expression.

As HDAC inhibition has been previously demonstrated to increase α -syn protein levels (Leng and Chuang 2006), we tested whether SB or TSA alter α -syn protein levels in SH-SY5Y cells. Immunoblotting for α -syn showed 2.4 and 2.8-fold increase in α -syn protein level after treatment with 2 mM SB and 200 nM TSA, respectively (Figures 6D and 6E).

α -Syn knockdown does not reverse the effect of HDAC inhibition on 14-3-3 σ expression

To examine whether the increase in α -syn protein is solely responsible for the effect of HDAC inhibition on 14-3-3 σ expression, we tested whether α -syn knockdown by shRNA could reverse the effect of HDAC inhibition. We found that the 14-3-3 σ mRNA level in SB-treated SH-SY5Y cells transduced with α -syn shRNA lentiviruses was slightly reduced by 9% when compared to those transduced with pLKO.1 control viruses (Figure 5B, white bars). Thus, α -syn knockdown did not reverse the effect of HDAC inhibition on 14-3-3 σ level. This finding implicates parallel pathways induced by HDAC inhibition that can also reduce 14-3-3 σ mRNA levels besides α -syn overexpression.

Discussion

In this study, we examined whether α -syn can regulate 14-3-3 expression in a cellular model. We found that 14-3-3 σ and, to a lesser extent, 14-3-3 τ mRNA levels were reduced in SH-SY5Y cells overexpressing α -syn. This is in agreement with our gene array study showing that 14-3-3 σ is the most significantly downregulated isoform in α -syn transgenic mice (Yacoubian et al. 2010). This downregulation of 14-3-3 σ occurs at the transcriptional level as the activities of the 14-3-3 σ promoter were decreased both in α -syn stable cells and in α -syn inducible cells in a luciferase reporter assay. Together with the previous gene array studies, our data show that increased α -syn levels can lead to transcriptional dysregulation of 14-3-3 σ expression and point to an additional level of interaction between 14-3-3s and α -syn beyond direct protein-protein interactions.

14-3-3s have been identified as a major hub of dysregulated proteins in a transcriptional analysis of PD patients using the DEGAS (DysRegulated Gene set Analysis via Subnetworks) method (Ulitsky et al. 2010). Alterations in 14-3-3 expression have also been previously observed in multiple microarray studies involving PD models, yet the magnitude, direction, and isoform involved differ among the studies (Miller et al. 2004; Bassilana et al. 2005; Zhang et al. 2005; Yacoubian et al. 2008; Simunovic et al. 2009; Storvik et al. 2010;

Botta-Orfila et al. 2012). This is possibly due to the differences in PD models evaluated, differences in cell types examined, or differences in methods of microarray analysis. Indeed, there has been a lack of consistency among PD microarray studies in terms of changes in specific genes in general (Sutherland et al. 2009; Greene 2012). However, most of studies in which 14-3-3 alterations are observed do demonstrate a reduction in 14-3-3 proteins, including 14-3-3 σ , 14-3-3 η , 14-3-3 ζ , and 14-3-3 ϵ (Miller et al. 2004; Zhang et al. 2005; Yacoubian et al. 2008; Simunovic et al. 2009; Storvik et al. 2010). 14-3-3 σ was reduced in a microarray study involving laser capture microdissection of dopaminergic neurons from the nigra of PD brains (Simunovic et al. 2009).

In our study, we examined the specificity of the observed changes in 14-3-3 in α -syn overexpression model by testing whether similar changes could also be observed in a different PD model, the rotenone neurotoxin model, and in a different neurodegenerative disease model, the BACHD mutant huntingtin mouse model (Gray et al. 2008). Similar to our findings seen with α -syn overexpression, rotenone treatment also reduced 14-3-3 mRNA level. The mechanisms by which α -syn overexpression and rotenone affect 14-3-3 are still unclear. Interestingly, since rotenone has been shown to cause α -syn accumulation and aggregation (Sherer et al. 2002; Betarbet et al. 2006), it is interesting to speculate that rotenone and α -syn may be working through a common mechanism.

Overexpression of mutant huntingtin in mice had no effect on 14-3-3 mRNA expression. The finding that similar changes were not observed in a model for a different neurodegenerative disorder may suggest that 14-3-3 could be differentially regulated depending on the disease context. However, the findings that HDAC inhibition also caused a decline in 14-3-3 mRNA suggests that other insults are also capable of inducing alterations in 14-3-3 expression, and decline in 14-3-3 is not necessarily specific to PD only.

While α -syn overexpression caused a reduction in 14-3-3 expression, knockdown of α -syn has no effect on 14-3-3 expression. This finding suggests that α -syn, when expressed at endogenous levels, may not regulate 14-3-3 transcription and that alteration in 14-3-3 level is likely a pathological consequence of α -syn overexpression. However, one limitation of our study is that we did not achieve complete knockdown of α -syn in our cell system. It is of interest to note that an increase in 14-3-3 expression at the protein level was observed in a triple knockout of α -synuclein mouse model (Gretchen-Harrison et al. 2010) – the converse of what we observed in an α -syn overexpression mouse model.

With α -syn overexpression in α -syn inducible cells, we did not observe 14-3-3 reduction until after α -syn being induced for longer period of time (six versus three days). This delay in α -syn effects could reflect the need for a certain threshold of α -syn levels or could reflect that it requires time for α -syn to exert its effects on the transcription of 14-3-3. In the α -syn mouse model in which we previously examined 14-3-3 expression levels, changes were observed in 3 month old mice, but it is not known how early these changes may develop (Yacoubian et al. 2008; Yacoubian et al. 2010).

How α -syn overexpression may regulate 14-3-3 transcription is not known. In this study, we examined whether α -syn overexpression could repress 14-3-3 transcription by inhibiting histone acetylation. Kontopoulos *et al.* have demonstrated that α -syn in the nucleus can inhibit histone acetylation in both cellular and *Drosophila* models and that α -syn toxicity is dependent on inhibition of histone acetylation in these models (Kontopoulos et al. 2006). When we tried to test whether increasing histone acetylation by inhibiting HDACs may have an effect on 14-3-3 level opposite to the effect of α -syn overexpression, we found that treatment with HDAC inhibitors SB, TSA, or more selective MS-275 caused a decrease in 14-3-3 expression. This unexpected finding indicates that excessive α -syn may negatively

regulate 14-3-3 transcription through a mechanism other than inhibition of the level of histone acetylation. However, the possible off-target effects of HDAC inhibitors and the effect of α -syn on the association of acetylated histones with specific genes such as 14-3-3 can not be completely ruled out.

Interestingly, we also observed an increase in α -syn protein expression in cells treated with HDAC inhibitors. This effect of HDAC inhibition on α -syn expression has been previously described (Leng and Chuang 2006). While we postulated that this increase in α -syn caused by HDAC inhibition may be responsible for the reduction in 14-3-3 expression, knockdown of α -syn did not reverse this effect of HDAC inhibition on 14-3-3 expression. It is possible that other proteins affected by HDAC inhibition along with α -syn could regulate 14-3-3 expression such that α -syn knockdown is not sufficient to reverse this effect.

Other potential mechanisms by which α -syn could regulate 14-3-3 transcription include effects on promoter methylation, transcription factors and microRNAs. The 14-3-3 isoform has been shown to be regulated by CpG island methylation (Ferguson et al. 2000; Iwata et al. 2000; Suzuki et al. 2000), and the 14-3-3 promoter region has CpG-rich regions which may serve as targets for DNA methylation. Our gene array work has previously demonstrated that transcription is the predominant biological function altered in the α -syn transgenic mice, and the expression of several transcription factors are altered in this mouse model (Yacoubian et al. 2008). Thus, α -syn could potentially downregulate 14-3-3 by either recruiting transcription repressors or by inhibiting expression of transcription activators necessary for 14-3-3 expression. Another level of regulation may be microRNA effects. Overexpressing A53T mutant α -syn in worms could modulate the expression of specific microRNAs (Asikainen et al. 2010). Meanwhile, certain species of microRNAs were shown to regulate the levels of 14-3-3 isoforms including 14-3-3 (Scheibner et al. 2012; Min et al. 2013). One could postulate that α -syn may regulate 14-3-3 expression through its effects on microRNAs.

The relevance of this study to PD is that 14-3-3 downregulation by increased α -syn levels could be a potential mechanism for promoting α -syn-induced toxicity in PD. In support of this hypothesis, we have previously demonstrated that overexpression of 14-3-3 reduces α -syn aggregation in H4 cells and reduces α -syn-induced toxicity in a worm model (Yacoubian et al. 2010). Enhancing 14-3-3 expression could be a potential strategy to reduce neurotoxicity in PD. More studies on the regulation of 14-3-3 expression will be helpful in finding genetic or pharmacological means to modulate the level of 14-3-3s.

Acknowledgments

We thank Mary Ballestas and the UAB Neuroscience Core Center (P30 NS47466) for preparation of the pLKO.1 control and α -syn shRNA lentiviruses and the doxycycline-inducible α -syn pSLIK lentivirus. Consultative services on biostatistics were supported by the National Center for Advancing Translational Research of the NIH under award number UL1TR00165. Research reported in the publication was supported by the NINDS of the NIH under award number NS060948 and by the Parkinson's Association of Alabama.

References

- Asikainen S, Rudgalvyte M, Heikkinen L, et al. Global microRNA expression profiling of *Caenorhabditis elegans* Parkinson's disease models. *J Mol Neurosci.* 2010; 41:210–218. [PubMed: 20091141]
- Athanassiadou A, Voutsinas G, Psiouri L, et al. Genetic analysis of families with Parkinson disease that carry the Ala53Thr mutation in the gene encoding alpha-synuclein. *Am J Hum Genet.* 1999; 65:555–558. [PubMed: 10417297]

- Bassilana F, Mace N, Li Q, et al. Unraveling substantia nigra sequential gene expression in a progressive MPTP-lesioned macaque model of Parkinson's disease. *Neurobiol Dis.* 2005; 20:93–103. [PubMed: 16137570]
- Berg D, Riess O, Bornemann A. Specification of 14-3-3 proteins in Lewy bodies. *Ann Neurol.* 2003a; 54:135. [PubMed: 12838532]
- Berg D, Holzmann C, Riess O. 14-3-3 proteins in the nervous system. *Nat Rev Neurosci.* 2003b; 4:752–762. [PubMed: 12951567]
- Betarbet R, Canet-Aviles RM, Sherer TB, et al. Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. *Neurobiol Dis.* 2006; 22:404–420. [PubMed: 16439141]
- Botta-Orfila T, Tolosa E, Gelpi E, et al. Microarray expression analysis in idiopathic and LRRK2-associated Parkinson's disease. *Neurobiol Dis.* 2012; 45:462–468. [PubMed: 21946334]
- Brunelli L, Cieslik KA, Alcorn JL, Vatta M, Baldini A. Peroxisome proliferator-activated receptor-delta upregulates 14-3-3 epsilon in human endothelial cells via CCAAT/enhancer binding protein-beta. *Circ Res.* 2007; 100:e59–71. [PubMed: 17303761]
- Dawson TM, Dawson VL. Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J Clin Invest.* 2003; 111:145–151. [PubMed: 12531866]
- Dietz KC, Casaccia P. HDAC inhibitors and neurodegeneration: at the edge between protection and damage. *Pharmacol Res.* 2010; 62:11–17. [PubMed: 20123018]
- Ferguson AT, Evron E, Umbricht CB, et al. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci U S A.* 2000; 97:6049–6054. [PubMed: 10811911]
- Gray M, Shirasaki DI, Cepeda C, et al. Full-length human mutant huntingtin with a stable polyglutamine repeat can elicit progressive and selective neuropathogenesis in BACHD mice. *J Neurosci.* 2008; 28:6182–6195. [PubMed: 18550760]
- Greene JG. Current status and future directions of gene expression profiling in Parkinson's disease. *Neurobiol Dis.* 2012; 45:76–82. [PubMed: 21056669]
- Greten-Harrison B, Polydoro M, Morimoto-Tomita M, et al. alphetagamgamma-Synuclein triple knockout mice reveal age-dependent neuronal dysfunction. *Proc Natl Acad Sci U S A.* 2010; 107:19573–19578. [PubMed: 20974939]
- Hu E, Dul E, Sung CM, et al. Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J Pharmacol Exp Ther.* 2003; 307:720–728. [PubMed: 12975486]
- Iwata N, Yamamoto H, Sasaki S, et al. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene.* 2000; 19:5298–5302. [PubMed: 11077447]
- Jia H, Pallos J, Jacques V, et al. Histone deacetylase (HDAC) inhibitors targeting HDAC3 and HDAC1 ameliorate polyglutamine-elicited phenotypes in model systems of Huntington's disease. *Neurobiol Dis.* 2012; 46:351–361. [PubMed: 22590724]
- Kawamoto Y, Akiyuchi I, Nakamura S, Honjyo Y, Shibasaki H, Budka H. 14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains. *J Neuropathol Exp Neurol.* 2002; 61:245–253. [PubMed: 11895039]
- Kontopoulos E, Parvin JD, Feany MB. Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Hum Mol Genet.* 2006; 15:3012–3023. [PubMed: 16959795]
- Kruger R, Kuhn W, Muller T, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet.* 1998; 18:106–108. [PubMed: 9462735]
- Leng Y, Chuang DM. Endogenous alpha-synuclein is induced by valproic acid through histone deacetylase inhibition and participates in neuroprotection against glutamate-induced excitotoxicity. *J Neurosci.* 2006; 26:7502–7512. [PubMed: 16837598]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Miller RM, Callahan LM, Casaceli C, et al. Dysregulation of gene expression in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse substantia nigra. *J Neurosci.* 2004; 24:7445–7454. [PubMed: 15329391]

- Min S, Liang X, Zhang M, et al. Multiple tumor-associated microRNAs modulate the survival and longevity of dendritic cells by targeting YWHAZ and Bcl2 signaling pathways. *J Immunol.* 2013; 190:2437–2446. [PubMed: 23355742]
- Ostrerova N, Petrucelli L, Farrer M, et al. alpha-Synuclein shares physical and functional homology with 14-3-3 proteins. *J Neurosci.* 1999; 19:5782–5791. [PubMed: 10407019]
- Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science.* 1997; 276:2045–2047. [PubMed: 9197268]
- Sato S, Chiba T, Sakata E, et al. 14-3-3eta is a novel regulator of parkin ubiquitin ligase. *Embo J.* 2006; 25:211–221. [PubMed: 16096643]
- Scheibner KA, Teaboldt B, Hauer MC, et al. MiR-27a functions as a tumor suppressor in acute leukemia by regulating 14-3-3theta. *PLoS One.* 2012; 7:e50895. [PubMed: 23236401]
- Sherer TB, Betarbet R, Stout AK, et al. An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. *J Neurosci.* 2002; 22:7006–7015. [PubMed: 12177198]
- Shin KJ, Wall EA, Zavzavadjian JR, et al. A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. *Proc Natl Acad Sci U S A.* 2006; 103:13759–13764. [PubMed: 16945906]
- Simunovic F, Yi M, Wang Y, et al. Gene expression profiling of substantia nigra dopamine neurons: further insights into Parkinson's disease pathology. *Brain.* 2009; 132:1795–1809. [PubMed: 19052140]
- Singleton AB, Farrer M, Johnson J, et al. alpha-Synuclein locus triplication causes Parkinson's disease. *Science.* 2003; 302:841. [PubMed: 14593171]
- Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature.* 1997; 388:839–840. [PubMed: 9278044]
- Storvik M, Arguel MJ, Schmieder S, et al. Genes regulated in MPTP-treated macaques and human Parkinson's disease suggest a common signature in prefrontal cortex. *Neurobiol Dis.* 2010; 38:386–394. [PubMed: 20206263]
- Sutherland GT, Matigian NA, Chalk AM, et al. A cross-study transcriptional analysis of Parkinson's disease. *PLoS One.* 2009; 4:e4955. [PubMed: 19305504]
- Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, Imai K. Inactivation of the 14-3-3 sigma gene is associated with 5 CpG island hypermethylation in human cancers. *Cancer Res.* 2000; 60:4353–4357. [PubMed: 10969776]
- Ulitsky I, Krishnamurthy A, Karp RM, Shamir R. DEGAS: de novo discovery of dysregulated pathways in human diseases. *PLoS One.* 2010; 5:e13367. [PubMed: 20976054]
- van Hemert MJ, Steensma HY, van Heusden GP. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays.* 2001; 23:936–946. [PubMed: 11598960]
- Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med.* 2002; 8:600–606. [PubMed: 12042811]
- Yacoubian TA, Cantuti-Castelvetri I, Bouzou B, et al. Transcriptional dysregulation in a transgenic model of Parkinson disease. *Neurobiol Dis.* 2008; 29:515–528. [PubMed: 18191405]
- Yacoubian TA, Slone SR, Harrington AJ, et al. Differential neuroprotective effects of 14-3-3 proteins in models of Parkinson's disease. *Cell Death Dis.* 2010; 1:e2. [PubMed: 21152247]
- Zhang Y, James M, Middleton FA, Davis RL. Transcriptional analysis of multiple brain regions in Parkinson's disease supports the involvement of specific protein processing, energy metabolism, and signaling pathways, and suggests novel disease mechanisms. *Am J Med Genet B Neuropsychiatr Genet.* 2005; 137B:5–16. [PubMed: 15965975]

Figure 1. 14-3-3 mRNA levels were reduced in stable SH-SY5Y cells overexpressing α -syn (A) Immunoblot of protein lysates from control and three different SH-SY5Y stable cell lines that overexpress α -syn (Syn-1, Syn-2 and Syn-5). (B, C, D) RNA was extracted from control and α -syn stable cell lines and reverse transcribed into cDNA. Quantitative PCR was performed using primers specific for 14-3-3 β , ϵ , or γ . Compared to control cells, 14-3-3 mRNA levels were significantly reduced in all three α -syn stable lines. 14-3-3 β mRNA levels were reduced in two out of the three α -syn stable lines, whereas 14-3-3 ϵ mRNA levels were not altered. Results were expressed as percentage of control. Experiments were performed three times with two or three replicates per experiment. ** $p < 0.01$, *** $p < 0.001$ compared to control (two-way ANOVA followed by Tukey's multiple comparison test). Error bars reflect S.E.M.

Figure 2. 14-3-3 mRNA levels were reduced by rotenone treatment but not mutant huntingtin overexpression

(A) SH-SY5Y cells were treated for 24 hrs with 0.2 μ M rotenone. RNA was extracted and quantitative PCR was performed using primers specific for 14-3-3. Treatment with rotenone caused significant reduction in 14-3-3 mRNA level. Results were expressed as percentage of the untreated group. Experiments were performed three times with three replicates per experiment. *** $p < 0.001$ compared to untreated (t-test). (B) RNA was extracted from brains of wildtype mice (WT) or BAC transgenic mice expressing human mutant huntingtin (BACHD). 14-3-3 mRNA levels were analyzed by quantitative PCR and normalized to HPRT. There was no significant difference between the WT (n=5) and BACHD mice (n=7). Results were expressed as percentage of wildtype. n.s. = not significant (t-test). Error bars reflect S.E.M.

Figure 3. 14-3-3 promoter activity was repressed in stable SH-SY5Y cells overexpressing α -syn (A) Schematic of luciferase reporter constructs (F2, F9, F12, F15 and F17) containing varying lengths of putative 14-3-3 promoter region. The nucleotide numbers are relative to the translation start site of 14-3-3. (B) Expression of luciferase reporter constructs in control and α -syn stable SH-SY5Y cells. Control and α -syn stable cell line Syn-1 were co-transfected with Renilla luciferase control vector and the control pGL4 vector or one of the five 14-3-3 luciferase reporter constructs. Luciferase activities were measured by dual luciferase reporter assay and expressed as percentage of the shortest construct F2 in control cells. In control condition, the shorter constructs F2, F9, F12 and F15 drove luciferase expression at a comparable level, whereas the longest construct F17 showed significantly lower basal luciferase activity (white bars, construct effect $p < 0.001$ by three-way ANOVA). Luciferase activities of all of the tested reporter constructs were significantly reduced in α -syn stable cells compared to their respective controls (black bars, *** $p < 0.001$, t-test). Experiments were performed three times with two replicates per experiment. Three-way ANOVA was first done to analyze the overall effect of each grouping factor (cell line, construct and experimental run). T-tests were then used to compare luciferase activities in control cells and α -syn stable cells for each reporter construct. Error bars reflect S.E.M.

Figure 4. 14-3-3 promoter activity was repressed in doxycycline-inducible SH-SY5Y cells overexpressing α -syn

(A) SH-SY5Y cells were transduced with a doxycycline-inducible α -syn lentivirus and selected with geneticin for stably-transduced cells. Immunoblotting showed increased α -syn expression after induction with 1 μ g/ml doxycycline for 48 hrs. (B) Luciferase activities of reporter constructs were significantly reduced in α -syn inducible cells. 14-3-3 luciferase reporter construct F9 and Renilla luciferase control vector were co-transfected into uninduced cells or cells that had been induced for 1 or 4 days, and then luciferase activity was measured 2 days after transfection. Therefore, cells were induced for a total of 3 or 6 days, respectively. Luciferase activities were expressed as percentage of uninduced cells. Experiments were performed three times with two or three replicates per experiment. *** p <0.001 compared to uninduced (two-way ANOVA followed by Tukey's multiple comparison test). Error bars reflect S.E.M.

Figure 5. Knockdown of α -syn had no effect on 14-3-3 mRNA level and did not reverse the effect of HDAC inhibition on 14-3-3 level

(A) SH-SY5Y cells were transduced with control pLKO.1 or α -syn shRNA lentiviruses and selected with puromycin for stably-transduced cells. Immunoblotting showed decrease in α -syn expression after lentiviral transduction. (B) SH-SY5Y cells stably transduced with the pLKO.1 control or with α -syn shRNA lentiviruses were untreated or treated with 2 mM SB for 24hrs. RNA was extracted and quantitative PCR was performed using primers specific for 14-3-3. In untreated cells, 14-3-3 mRNA level was not changed by the knockdown of α -syn when compared to pLKO.1 control (black bars). Treatment with SB caused significant reduction in 14-3-3 expression in both pLKO.1 and α -syn knockdown cells (black vs. white bars). 14-3-3 mRNA level in SB-treated cells transduced with α -syn shRNA

lentiviruses was slightly lower than those transduced with pLKO.1 control (white bars). Results were expressed as percentage of untreated pLKO.1. Experiments were performed three times with three replicates per experiment. Three-way ANOVA was first done to analyze the overall effect of each grouping factor (cell line, treatment and experimental run). Two-way ANOVA was then used to compare pairs of experimental groups. * $p < 0.05$, *** $p < 0.001$, n.s. = not significant. Error bars reflect S.E.M.

Figure 6. Treatment of HDAC inhibitors caused a decrease in 14-3-3 mRNA level and an increase in α-syn protein level
 (A, B, C) SH-SY5Y cells were treated for 24 hrs with varying doses of HDAC inhibitors sodium butyrate (SB), trichostatin A (TSA), and MS-275. RNA was extracted and quantitative PCR was performed using primers specific for 14-3-3. All three HDAC inhibitors caused decreases in 14-3-3 mRNA levels. Results were expressed as percentage of the untreated group. Experiments were performed three times with three replicates per experiment. *** $p < 0.001$ compared to untreated (two-way ANOVA followed by Tukey's multiple comparison test). (D, E) SH-SY5Y cells were treated with 2 mM SB or 200 nM TSA for 24 hrs. Proteins were collected and immunoblotted with antibody against α-syn or actin. Blots were quantified by densitometry. The levels of α-syn were normalized to the levels of actin. Treatment with SB and TSA increased α-syn expression by 2.4 and 2.8-fold, respectively. Densitometric quantification included three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to untreated (one-way ANOVA followed by Dunnett's multiple comparison test).