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Cyclin-G-associated kinase modifies α-synuclein expression levels and toxicity in Parkinson's disease: results from the *GenePD* Study

Alexandra Dumitriu[1,†], Chris D. Pacheco[2,†], Jemma B. Wilk[1,†], Katherine E. Strathearn[3],
Jeanne C. Latourelle[1], Stefano Goldwurm[4], Gianni Pezzoli[4], Jean-Christophe Rochet[3],
Susan Lindquist[2,5,6] and Richard H. Myers[1,*]

[1]Department of Neurology, Boston University School of Medicine, 72 East Concord Street, E-304, Boston, MA 02118,
USA, [2]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA, [3]Department of Medicinal
Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, USA, [4]Parkinson Institute,
Istituti Clinici di Perfezionamento, Milano, Italy and [5]Howard Hughes Medical Institute and [6]Department of Biology,
Massachusetts Institute of Technology, Cambridge, MA 02142, USA

**Although family history is a well-established risk factor for Parkinson's disease (PD), fewer than 5% of PD cases
can be attributed to known genetic mutations. The etiology for the remainder of PD cases is unclear; however,
neuronal accumulation of the protein α-synuclein is common to nearly all patients, implicating pathways that
influence α-synuclein in PD pathogenesis. We report a genome-wide significant association ($P = 3.97 \times 10^{-8}$)
between a polymorphism, rs1564282, in the cyclin-G-associated kinase (*GAK*) gene and increased PD risk, with
a meta-analysis odds ratio of 1.48. This association result is based on the meta-analysis of three publicly
available PD case–control genome-wide association study and genotyping from a new, independent Italian
cohort. Microarray expression analysis of post-mortem frontal cortex from PD and control brains demonstrates
a significant association between rs1564282 and higher α-synuclein expression, a known cause of early onset
PD. Functional knockdown of *GAK* in cell culture causes a significant increase in toxicity when α-synuclein
is over-expressed. Furthermore, knockdown of *GAK* in rat primary neurons expressing the A53T mutation of
α-synuclein, a well-established model for PD, decreases cell viability. These observations provide evidence
that *GAK* is associated with PD risk and suggest that *GAK* and α-synuclein interact in a pathway involved in
PD pathogenesis. The GAK protein, a serine/threonine kinase, belongs to a family of proteins commonly
targeted for drug development. This, combined with *GAK*'s observed relationship to the levels of α-synuclein
expression and toxicity, suggests that the protein is an attractive therapeutic target for the treatment of PD.**

INTRODUCTION

Parkinson's disease (PD) is the second most common neurode-
generative disorder, trailing only Alzheimer's disease in
prevalence. PD presents clinically with resting tremor, muscu-
lar rigidity and bradykinesia (1). Pathologically, the disease is
characterized by the loss of dopaminergic neurons within the
substantia nigra pars compacta and the presence of Lewy
bodies (2). These hallmark intraneuronal inclusions are

primarily composed of the small, lipid binding protein
α-synuclein (3). Although a critical role for α-synuclein in
PD pathogenesis is universally accepted, <5% of all PD
cases can be attributed to genetic mutations in α-synuclein
and the other known PD genes.

While >95% of PD cases are considered idiopathic, it is
well established that between 16 and 27.5% have a positive
family history and first-degree relatives of PD patients
have a substantially increased risk for PD compared with

*To whom correspondence should be addressed. Tel: +1 6176385376; Fax: +1 6176385354; Email: rmyers@bu.edu
†These authors contributed equally to this work.

first-degree relatives of controls (4–6). These observations led us to undertake the *GenePD* Study, an evaluation of familial PD cases aimed at identifying genes that influence PD susceptibility. The *GenePD* Study, in collaboration with the PROGENI Study, performed a genome-wide association study (GWAS) (dbGaP Accession: phs000126.v1.p1) focusing on families with multiple affected relatives (7). This was the first GWAS to implicate non-coding single nucleotide polymorphisms (SNPs) within the genes encoding α-synuclein (*SNCA*) and the micro-tubule-associated protein tau (*MAPT*) in PD risk (7). Both findings were confirmed in the subsequent GWAS of idiopathic PD (8,9).

We hypothesized that our familial PD cohort (7) was positioned to identify genes that GWAS of primarily idiopathic PD failed to detect (8–12). The region producing the strongest *P*-value for familial PD risk included the cyclin-G-associated kinase (*GAK*) gene with a minor allele conferring a 1.7 odds ratio (OR) for PD, which, although suggestive, failed to reach the conservative level for genome-wide significance at $P < 5.0 \times 10^{-8}$ (13). Recently, Hamza *et al.* (14) performed a PD GWAS in 2000 cases and 1986 controls, and in a meta-analysis with our study (7), the *GAK* region reached genome-wide significance ($P = 3.2 \times 10^{-9}$). *GAK* was shown previously to be among 137 genes differentially expressed in the *substantia nigra pars compacta* of PD patients when compared with controls, with a 1.56-fold change (15). The combination of these independent findings prompted our further investigation of the involvement of *GAK* in PD pathogenesis.

GAK is a ubiquitously expressed protein (16), containing highly conserved serine/threonine kinase (17,18), PTEN (19,20) and J-domains (17,21). Interestingly, the clathrin-binding C-terminal domain of GAK has been shown to bind pre-cathepsin D (CTSD) and to directly sort the zymogen into clathrin-coated vesicles destined for the lysosome (22). Recently, CTSD was implicated as the main lysosomal enzyme involved in α-synuclein degradation (23–25). Mutations in *CTSD* have been shown to induce the pathological accumulation of α-synuclein in mice, sheep and human infants afflicted with fatal CTSD-deficient forms of neuronal ceroid lipofuscinosis (OMIM #610127) (24,25). Together, these findings led us to investigate the interrelationships between *GAK*, *SNCA* and *CTSD* and their potential role in a previously undescribed pathway for PD pathogenesis. Here we report compelling evidence for interactions between *GAK, SNCA* and *CTSD* as well as biological evidence that reduced GAK function enhances α-synuclein-mediated toxicity.

RESULTS

To substantiate the association between the *GAK* SNP rs1564282 and increased PD risk, we performed a meta-analysis of three publicly available PD case–control GWAS, combined with our genotyping of a new, independent sample of 862 Italian PD cases and 517 controls. We used $P \leq 5 \times 10^{-8}$ as the threshold for genome-wide significance, a widely accepted threshold based on correction for 1 million independent variants in the genome (13). Our results confirm that the minor allele of the *GAK* SNP rs1564282 is associated

with a 1.48 increased odds of PD at a genome-wide level of significance ($P = 3.97 \times 10^{-8}$; Table 1). While all four studies independently show increased risk of PD associated with this SNP, the largest effect size is observed in the familial PD cohort. Interestingly, restricting analysis of the new Italian cases to those with reported family history ($n = 165$) shows a stronger effect size (OR = 1.9) and a stronger *P*-value ($P = 0.0026$) than the idiopathic cases alone (OR = 1.2, $P = 0.18$), emphasizing the advantages of focusing on familial cases in the search for genetic contributors to PD.

As reviewed above, *SNCA*, *GAK* and *CTSD* appear to interact either directly or indirectly. To evaluate whether there is a correlation between the expression of these genes, as reflected by messenger ribonucleic acid (mRNA) levels, in control or PD brains, we analyzed four probes (one each for exons 26 and 28 of *GAK*, one for the 3′-UTR of *SNCA,* and one for exon 6 of *CTSD*) present on a commercially available microarray (One-Color Agilent 60-mer Whole Human Genome Microarray). We screened the microarray with total RNA purified from frontal cortex of 33 PD cases and 29 controls (see Table 2 for characteristics of samples). Despite the expression levels of the two C-terminal *GAK* probes showing marked correlation with one another in both PD ($r^2 = 0.8$) and controls ($r^2 = 0.6$), the expression of *GAK* exon 26, but not exon 28, was observed to have a positive correlation with expression of *CTSD* exon 6 in both PD and control brains ($r^2 = 0.5$) (Table 3). Neither *GAK* nor *CTSD* expression was correlated with total *SNCA* expression in either PD or control brains.

We also used the microarray data from these four probes to analyze the relationship between several SNPs and expression levels of *GAK*, *CTSD* and *SNCA*, adjusting for PD/control status, RNA integrity number (RIN), post-mortem interval (PMI) and age at death (Table 4). In addition to the *GAK* SNP rs1564282, we evaluated three SNPs located within 100 000 bp of *SNCA,* which were also implicated in PD risk in our familial GWAS (7). In order to account for the 16 different association tests (four SNPs and four expression probes) that were conducted, we used an adjusted alpha-level of significance of 0.007; this value was determined by applying a modified Bonferroni correction (26) that accounts for the high degree of correlation between the expression levels of the used probes (mean $R^2 = 0.3$). We found that rs1564282 in *GAK* associated significantly with higher levels of total *SNCA* expression ($P = 0.003$). Additionally, an intronic *SNCA* SNP (rs356188) with a minor allele conferring a protective effect in the GWAS (7) was significantly associated with higher *CTSD* levels ($P = 0.007$). Some suggestive associations were also detected: rs1564282 *GAK* with lower levels of *GAK* exon 28 ($P = 0.03$), and rs356188 with higher levels of *GAK* exon 26 ($P = 0.02$). None of the SNPs within the *SNCA* gene region was significantly associated with *SNCA* expression levels (Table 4).

Previous reports have demonstrated that reduced expression of *CTSD* results in increased α-synuclein aggregation and toxicity (24,25), and that RNAi targeting of *GAK* in mammalian cell culture reduces CTSD protein levels in the lysosome (22). We decided to define the connection between GAK and α-synuclein by evaluating whether manipulating *GAK* expression affects α-synuclein-mediated toxicity and α-synuclein protein levels in a simple mammalian cell

Table 1. Meta-analysis of three publicly available GWAS and replication genotyping

SNP	Familial PD GWAS[7]			1st NIA PD-GWAS[10]			2nd NIA PD-GWAS[9]			Milan, Italy cohort			Meta-analysis	
	n	OR	*P*-value	*n*	OR	*P*-value	*n*	OR	*P*-value	*n*	OR	*P*-value	OR	*P*-value
rs1564282	1728	1.70	5.9×10^{-6}	505	1.55	0.047	1166	1.34	0.033	1379	1.32	0.0497	1.48	3.97×10^{-8a}

Each OR reflects the effect of the minor (T) allele, which has a frequency of ~11% in the overall sample.
[a]Achieves genome-wide significance.

Table 2. Characteristics of RNA samples for microarray analysis

Sample type (n)	Age at death, years (range)	PMI, hours (range)	RIN (range)	Tissue pH[a] (range)
Control (29)	75.21 (58–97)	13.70 (1.50–39.67)	7.36 (4.8–8.5)	6.641 (6.26–7.32)
PD (33)	77.67 (64–94)	7.43 (1.16–30.75)	7.182 (5.2–8.4)	6.652 (6.27–7.13)

[a]The pH was measured following a previously established protocol (46).

culture system. We performed siRNA knockdown of *GAK* in HEK 293 cells while overexpressing α-synuclein. Reducing *GAK* expression consistently increased toxicity in α-synuclein-overexpressing cells, as indicated by increased adenylate kinase release (Fig. 1A). Notably, knockdown of *GAK* had no effect on toxicity in the absence of α-synuclein overexpression, as demonstrated by the green fluorescent protein (GFP) overexpression control (Fig. 1A). Western blot analysis confirmed that *GAK* siRNA treatment dramatically suppressed GAK protein levels in these cells (Fig. 2A). ELISA revealed that this reduction in the GAK protein was accompanied by a significant increase in α-synuclein protein levels in α-synuclein-overexpressing cells (Fig. 1B), although the less-sensitive western blot analysis demonstrated only subtle changes in α-synuclein protein levels (Fig. 2B). Control siRNA had no effect on α-synuclein levels and *GAK* siRNA did not induce α-synuclein expression when GFP was overexpressed instead of α-synuclein (Fig. 1B). These cell culture results suggest a synergistic effect whereby an increase in α-synuclein combined with a decrease in GAK results in increased cytotoxicity.

We also investigated whether the interaction of *SNCA* and *GAK* was present for the A53T mutation in α-synuclein. In this experiment, we examined how the reduction in endogenous *GAK* influences α-synuclein-mediated toxicity in cultured primary rat midbrain neurons expressing the A53T form of α-synuclein, an *in vitro* model of PD (27–31). Primary rat midbrain neuron cultures consist of ~4–5% tyrosine hydroxylase positive (TH+) cells, which have been described as being exquisitely sensitive to α-synuclein levels in PD (31). Primary rat midbrain cultures were treated with sub-lethal levels of an adenovirus containing the A53T-*SNCA* variant associated with early onset PD, with or without lentiviruses encoding one of two *GAK*-specific short-hairpin RNAs (shRNAs) (each targeting a different coding region of rat *GAK*). Ninety-six hours after transduction, we assessed relative dopaminergic cell viability by immunocytochemistry for TH, a marker of dopaminergic neurons and microtubule-associated protein 2 (MAP2), a general neuronal

marker. Transduction with the A53T-*SNCA* virus or with either *GAK* shRNA virus alone did not affect dopaminergic cell viability compared with untransduced cultures. However, simultaneous exposure to the A53T-α-synuclein adenovirus and either of the *GAK*-specific shRNA lentiviruses dramatically reduced the number of TH+ neurons relative to MAP2+ neurons, suggesting that α-synuclein neurotoxicity is triggered or enhanced by the combination of overexpression of mutant *SNCA* and reduction in GAK (Fig. 1C and D). In other (control) experiments, we found that a non-specific lentivirus (lenti-LacZ) failed to exacerbate dopaminergic cell death elicited by the A53T adenovirus (Strathearn and Rochet, data not shown). To confirm that transduction with the *GAK* shRNA lentiviruses reduces GAK protein levels, we examined GAK expression in MES23.5 dopaminergic cells by immunoblot analysis and found that each of the shRNAs reduced GAK protein levels by ~90–95% relative to control and 80–90% relative to cells expression A53T α-synuclein (Fig. 2C). These results are compatible with those obtained for the HEK 293 cells, indicating that GAK reduction plays an important role in cell toxicity not only when α-synuclein is overexpressed, but also for A53T mutant-α-synuclein.

DISCUSSION

Emerging evidence implicates endocytic pathways in the pathogenesis of the most common neurodegenerative disorders. Highlighting this concept are the results from a recent Alzheimer's disease GWAS implicating *PICALM* (32), a gene involved in clathrin-mediated endocytosis. In PD, the relationship between disrupted endocytic trafficking and α-synuclein accumulation has received increasing attention in a diverse number of models for the disorder (30,33,34). Notably, GAK (also known as auxilin-2) and PICALM have similar canonical interactions with clathrin (16,17,19,21,35) and both proteins are critical to clathrin-mediated endocytosis (36). Here we report that a SNP in the *GAK* gene (rs1564282), previously implicated as the top hit in a GWAS for familial PD (7), is significantly associated with PD risk at a genome-wide level of significance ($P = 3.97 \times 10^{-8}$). These results position *GAK* alongside *SNCA* and *MAPT* as a PD risk-associated gene, and also implicate clathrin-mediated endocytosis in the etiology of PD.

Identification of the *GAK* gene in a GWAS of strictly familial PD supports the importance of studying PD cohorts enriched for family history of disease. The Italian sample studied for replication had detailed data on family history, allowing us to observe the enhanced effect and improved *P*-value among those cases with family history of PD. Hamza *et al.* (14) recently confirmed our association with

Table 3. Correlation between mRNA expression levels

	SNCA (probe 29 939)	*CTSD* (probe 52 556)	*GAK* exon 26 (probe 155 700)	*GAK* exon 28 (probe 397 150)
SNCA		0.10 (0.58)	0.15 (0.42)	−0.13 (0.47)
CTSD	0.28 (0.14)		0.45 (0.009)	0.18 (0.31)
GAK exon 26 (probe 155 700)	0.18 (0.34)	0.50 (0.006)		0.79 (<0.0001)
GAK exon 28 (probe 397 150)	−0.27 (0.16)	0.02 (0.93)	0.58 (0.001)	

Correlations were computed separately for PD cases (above diagonal) and controls (below diagonal). *P*-values are indicated in parentheses.

**Table 4.** Association of SNPs with mRNA expression levels[a]

| SNP	Position on chrom 4	Gene	MAF	GWAS OR[b]	*SNCA* (probe 29 939)		*CTSD* (probe 52 556)		*GAK* exon 26 (probe 155 700)		*GAK* exon 28 (probe 397 150)	
					Beta	*P*-value	Beta	*P*-value	Beta	*P*-value	Beta	*P*-value
rs1564282	842,313	*GAK*	0.14 (T)	1.7	0.35[c]	0.003	−0.06	0.49	−0.12	0.16	−0.15	0.03
rs356229	90,825,620	*3' SNCA*	0.44 (C)	1.35	−0.03	0.70	0.02	0.73	−0.01	0.87	−0.01	0.83
rs356188	90,910,560	*SNCA*	0.29 (C)	0.7	−0.08	0.27	0.12	0.007	0.11	0.02	0.07	0.1
rs3775478	91,061,863	*MMRN1*	0.12 (G)	1.7	0.05	0.67	0.04	0.55	−0.05	0.53	−0.04	0.56

MAF, minor allele frequency; Beta, beta coefficient.
[a]Adjusted for PD/control, RIN, PMI and age at death.
[b]OR previously reported for SNPs association to PD in our Familial PD GWAS (7) with *P*-values ranging from 5.9×10^{-6} to 8.4×10^{-5}.
[c]eSNP beta estimate in a subset of 32 brain samples with available ethnicity information (all Caucasians) was similar to the beta estimate value in the whole set of brains (Beta = 0.33).
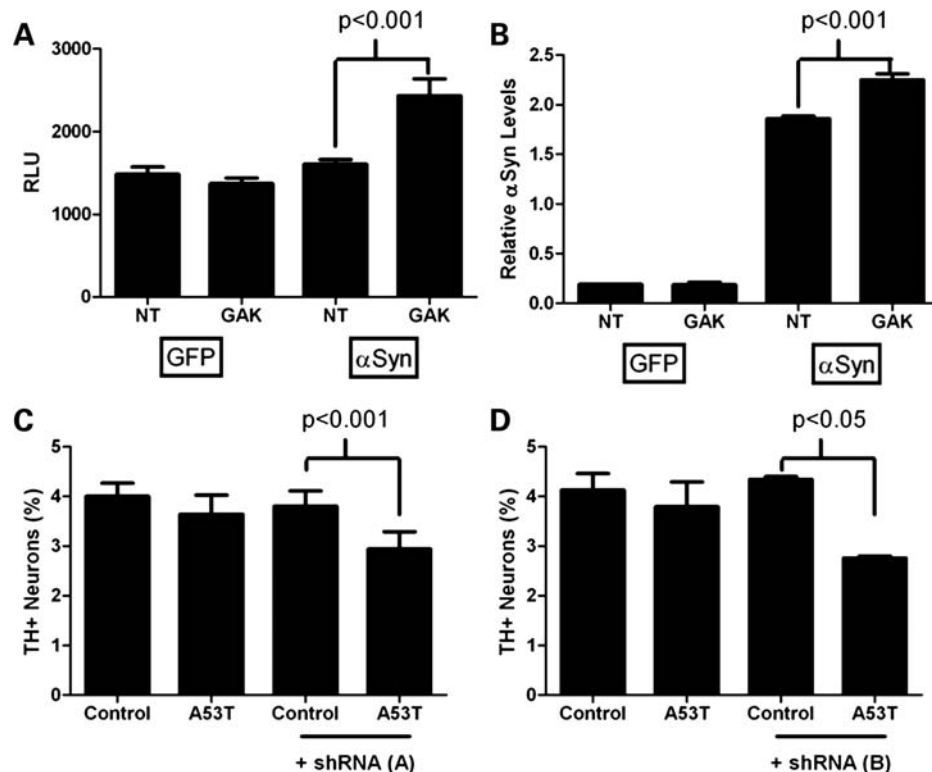
Figure 1. Potentiation of α-synuclein-mediated toxicity in cell culture systems with reduced GAK expression. (**A** and **B**) HEK 293 cells were transiently transfected with CMV-GFP, CMV-α-synuclein and either non-targeted (NT) or *GAK*-targeted pools of siRNA. (A) Toxicity was assessed by release of adenylate kinase into the media 72 h after transfection. Enzymatic release was detected by luciferase reaction and quantified by relative luminescent units (RLU) compared with controls (Toxilight). (B) α-Synuclein protein levels in siRNA-treated cell lysates were quantified by ELISA. (**C** and **D**) Primary midbrain cultures were transduced with A53T adenovirus (MOI = 3) with or without lentivirus encoding either (C) GAK shRNA 'A' (MOI = 3) or (D) GAK shRNA 'B' (MOI = 1). Control cells were incubated in the absence of virus. Dopaminergic cell viability was determined by staining with antibodies specific for MAP2 and TH and is expressed as the percentage of MAP2-positive neurons that were also TH-positive.
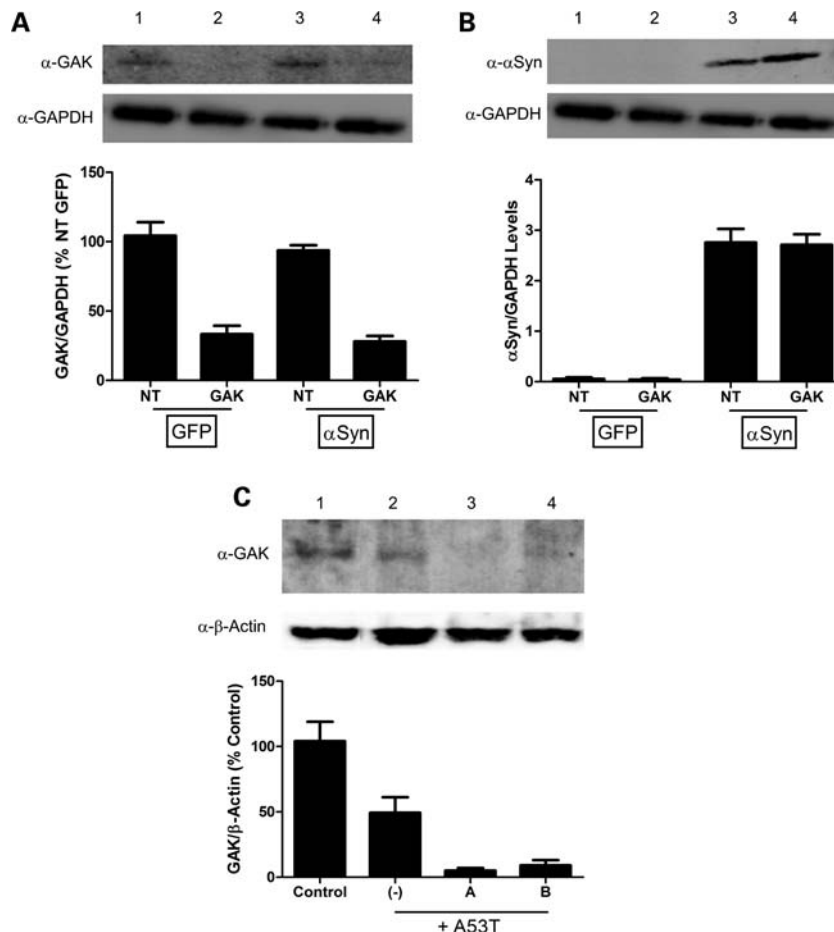
Figure 2. Western blot analyses confirming reduced GAK expression and enhanced α-synuclein accumulation. (**A**) GAK protein levels and (**B**) α-synuclein protein levels in HEK 293 cells transfected with either CMV-GFP (lanes 1 and 2) or CMV-α-synuclein (lanes 3 and 4) and non-targeted (lanes 1 and 3) or GAK-specific (lanes 2 and 4) pools of siRNA's. Western blot quantifications were performed via densitometric analysis using NIH ImageJ software. GAK band intensities were normalized first to the GAPDH signal and then to the values for non-targeted, GFP-transfected cells lane (set to 100%) (A), while α-synuclein band intensities were reported as relative expression levels of α-synuclein compared to GAPDH (B). Each experiment was performed in triplicate. (**C**) Representative western blot depicting shRNA-mediated GAK knockdown in the MES23.5 dopaminergic cell line ($n \geq 2$ for each of the different treatment conditions). Lysates were prepared from control cells (lane 1), and cells transduced with A53T-α-synuclein adenovirus alone (lane 2, MOI = 30) or with A53T-α-synuclein adenovirus (MOI = 30) plus lentivirus encoding GAK-specific shRNA 'A' (lane 3, MOI = 30) or 'B' (lane 4, MOI = 15). The graph below the blot shows relative GAK protein levels determined via densitometric analysis using NIH ImageJ software. GAK band intensities were normalized first to the β-actin signal and then to the normalized GAK band intensity in the control lane (set to 100%). The data suggest that (i) A53T expression induces GAK downregulation in the MES23.5 dopaminergic cell line, and (ii) shRNAs A and B induce a further decrease in GAK levels in A53T-expressing cells.

$GAK (P = 3.1 \times 10^{-4})$ in a GWAS for 2000 PD cases and 1986 controls from the NeuroGenetics Research Consortium (NGRC). Thus, while the evidence for *GAK* SNPs associated with PD was first seen in a cohort of familial PD (7) and reported here in the subset of Italian families with family history of PD, the association of *GAK* in PD risk appears to be present in random PD samples as well. Nevertheless, our results support the power of the familial PD sub-population in identifying genetic contributors to PD. Our replication and functional studies further suggest that new pathways identified in this way will likely prove relevant to the pathogenesis of idiopathic PD.

We also found that rs1564282 was associated with higher levels of *SNCA* expression, as reflected by the mRNA level, in the total sample of brains, adjusting for case/control status. Using a stratified analysis, we observed this eSNP

relationship to be stronger in PD (beta = 0.473, *P* = 0.013), than in control samples (beta = 0.163, *P* = 0.267), although the effect of the SNP on expression is consistent in the two groups. Altered mRNA levels could be an influence on stability or degradation of the transcripts, and may not reflect altered mRNA expression. However, higher *SNCA* levels are consistent with the previously reported increased PD risk observed in duplication and triplication of the *SNCA* gene, a rare, but recognized, cause of early-onset PD (37–39). We examined the expression levels of two C-terminal *GAK* exons, exons 26 and 28, in post-mortem brains. Exon 26 is known to encode part of the clathrin-binding domain of GAK, which is critically involved in the sorting of the CTSD pro-enzyme, while exon 28 encodes the J-domain of the protein (22). We found exon 26 to be correlated with *CTSD* exon 6 expression, which was not observed for *GAK*

exon 28. This suggests the presence of alternative splicing of *GAK* brain transcripts, whereby transcripts that contain exon 26, but not exon 28, have distinct relationships to *CTSD* levels.

We evaluated the relationship between expression of GAK and α-synuclein toxicity in two biological models: (i) a HEK 293 cell line transiently overexpressing α-synuclein, and (ii) primary rat neuronal cells transduced with the virus expressing *SNCA* with the A53T mutation. In both models, knockdown of *GAK* expression resulted in dramatic increases in cell death. Specifically, in the HEK 293 cells, siRNA knockdown of *GAK* increased toxicity and overall α-synuclein protein levels. In the primary neuronal cultures, expression of shRNA for *GAK* reduced the survival of TH$^+$ dopaminergic neurons relative to that of total (MAP2+) neurons. From these data we infer that down-regulation of GAK enhances the preferential toxicity of A53T α-synuclein to dopaminergic neurons [29–31,40]. Interestingly, the *substantia nigra* expression study performed by Grunblatt *et al.* [15] showed a 1.56-fold increase in *GAK* expression in PD compared with control samples. One possible explanation is the presence of a compensatory effect in the PD-affected brain region, which would lead to increased *GAK* expression. The minor allele of the rs1564282 *GAK* SNP is nominally associated with decreased expression of *GAK* exon 28 in frontal cortex, and decreased expression of *GAK* might be a possible mechanism by which this SNP is linked to increased risk for PD.

The described studies implicate *GAK*, *SNCA* and *CTSD* in PD pathogenesis, suggesting a role for these genes within a clathrin-mediated endocytic pathway involved in promoting neuronal toxicity. Additionally, the combination of *GAK* knockdown and α-synuclein over-expression may provide new *in vitro* models for PD, a disease that has been historically difficult to model. Finally, while the specific mechanisms by which GAK depletion enhances α-synuclein toxicity are not known, further studies targeted at enhancing GAK's function may provide novel approaches for PD treatment.

MATERIALS AND METHODS

Brain samples and microarray expression data

Brain tissue from the frontal cortex Brodmann 9 area was obtained from three different brain banks: the Harvard Brain Tissue Resource Center McLean Hospital, Belmont, Massachusetts; the Human Brain and Spinal Fluid Resource Center VA West Los Angeles Healthcare Center, California; and the Sun Health Research Institute Sun City, Arizona. Thirty-four PD and 29 control samples were identified to be the best available group of samples for a microarray study from an available pool of 118 PD and 87 control brains. We selected samples for inclusion in the micro-array by the following four criteria: (i) lack of Alzheimer disease pathology (specified by available neuropathological reports), (ii) pH value >6.25, (iii) similar age for PD cases and controls, and (iv) male gender. Total RNA for the 34 PD and 29 control samples was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was purified using the RNeasy MinElute Cleanup columns (Qiagen Sciences Inc, Germantown, MD, USA) and its quality was assessed with an Agilent Bioanalyzer Nano Chip (Agilent, Foster City, CA, USA). Two micrograms of each RNA sample were labeled and hybridized to the One-Color Agilent 60-mer Whole Human Genome Microarray at the Microarray Facility of the Whitehead Institute for Biomedical Research (Cambridge, MA, USA). The dye-normalized and post-surrogate processed signal for the green channel, gProcessedSignal, obtained from Agilent's Feature Extraction Software was used for downstream analyses. The QC reports for each of the 63 samples were individually inspected, and no systematic errors were determined for any sample. All the microarray processing analyses were performed in R (http://www.R-project.org).

To ensure that only the high-quality probes were retained for analysis, standard exclusion criteria were used as follows: (i) control probes were removed; (ii) each probe was flagged for a specific sample if (values explained in the Agilent Feature Extraction Reference Guide): gIsWellAboveBG equal to 0, gIsFeatNonUnifOL equal to 1, gIsBGNonUnifOL equal to 1, gIsFeatPopnOL equal to 1, gIsBGPopnOL equal to 1, or gIsSaturated equal to 1. If more than 50% of the PD samples or more than 50% of the control samples had a specific probe flagged, the respective probe was removed; (iii) each probe was flagged for a specific sample if the expression value for the probe was outside the range for the spike-in controls. A probe was removed if it was flagged in at least 50% PD cases and 50% controls; in addition, (iv) each probe was flagged for a specific sample if its expression was below the median expression value of the sample. If a probe was flagged in more than 80% cases and more than 80% controls, the probe was removed. To make sure we were not removing an excessive number of probes, we compared the list of genes corresponding to the retained probes with the list of ubiquitously expressed genes published by Ramskold *et al.* [41]. 86.98% of the genes determined as ubiquitously expressed by RNA-Seq in the Ramskold *et al.* study were present in our set; we considered this value to be acceptable. Out of the total 45 015 probes present on the microarray chips, 22 393 (corresponding to 16 567 genes) were analyzed.

The expression data for the retained probes of the 63 arrays were quantile normalized, and the obtained values were ln (natural logarithm) transformed. Two different probes for *GAK* (A_23_P155700, targeting expression of the gene's exon 26, sequence: TCCTCCAGGTCTGACAAGAAAGGGCCAAAGACCATTGCAGAGATGAGGAAGCAGGACCTG and A_24_P397150, targeting expression of the gene's exon 28, sequence: CCGTACGAGCAGCACGCCAAGATGATCTTCATGGAGCTGAATGACGCCTGGTCGGAGTTT) were available on the microarray chip. One probe was available for *CTSD* (A_23_P52556, targeting expression of the gene's exon 6, sequence: GTATTACAAGGGTTCTCTGTCCTACCTGAATGTCACCCGCAAGGCCTACTGGCAGGTCCA). The *SNCA* gene was targeted by a single probe (A_23_P29939, targeting the 3′-UTR of the gene, sequence: TGACAGATGTTCCATCCTGTACAAGTGCTCAGTTCCAATGTGCCCAGTCATGACATTTCT), repeated 10 times on the microarray. All these probes passed our strict filtering criteria. The expression data for *SNCA* used in this manuscript is the average value for the 10 repeated probes.

Sixty-two out of the 63 microarray samples had an RIN value above 4.8, and only these 62 samples were analyzed in the current study (42) (Table 2).

Genotyping

The genotyping in the Milan, Italy cohort and the available brain samples was performed using the TaqMan technology implemented on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in the Neurogenetics Laboratory at Boston University School of Medicine. In brains, the *GAK* SNP rs1564282 and three SNPs around the *SNCA* gene on chromosome 4 (Table 4) were genotyped.

Statistical analysis of SNP data

The rs1564282 SNP genotyping was obtained from three publicly available GWAS data sets available from dbGaP: a sample of familial PD cases and NINDS controls (all self-reported white, non-Hispanic and screened for population outliers using the multidimensional scaling method; $n = 1728$, 865 cases, 863 controls) (7), an NIA sponsored case/control GWA (all self-reported white, non-Hispanic; $n = 505$, 256 cases, 249 controls) (10) and the second release of publicly available data from the NIA (all self-reported white, non-Hispanic; $n = 1166$, 644 cases, 522 controls) (9). Subjects in the three GWASs were cleaned with identical QC thresholds, requiring 98% or higher call rate and screening for sex inconsistency. The rs1564282 SNP met all quality control criteria including call rate above 98%, consistency with Hardy–Weinberg equilibrium (HWE) in controls, and did not appear differentially missing by genotype (the MISHAP test), by case/control status or by sex. Local genotyping in the Milan, Italy cohort [all white, non-Hispanic samples; case ($n = 862$), controls ($n = 517$)] was also consistent with HWE among controls and had a 96% call rate. SNP analysis was implemented in PLINK (43) separately for each of the four cohorts using logistic regression with adjustment for age (onset for cases/enrollment for controls) and sex. Meta-analysis was implemented with METAL using the inverse variance method.

The relationship of SNPs to expression levels (eSNP studies) was performed using linear regression in SAS v9.1. The normalized mRNA levels were modeled as the dependent variable and the association of SNPs was adjusted for PD/control status, RIN, PMI and age at death. The RIN and pH were the most highly correlated variables in our data (Spearman correlation coefficient = 0.403, *P*-value = 0.001) and we decided to include in the linear regression model only one of these two variables, to avoid the problem of over-adjustment. We chose the RIN variable, given its larger range of values compared with pH (Table 2). The results obtained with pH in the model instead of RIN are very similar (unreported). SNPs were modeled to estimate the effect of the minor allele with an additive genetic model except when fewer than five rare allele homozygotes were present in the data (corresponding to MAF ≤ 0.14), when a dominant model was implemented (rs1564282, rs3775478).

Knockdown of *GAK* in HEK 293 cells, cytotoxicity and ELISA assays

HEK 293 cells (ATCC) were maintained at 37°C, 5% CO$_2$ in DMEM supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine, 10 µg/ml penicillin and 10 µg/ml streptomycin (GIBCO). *GAK* knockdown experiments were conducted in 96-well plates by siRNA treatment with either ON-TARGETplus SMART pool L-005005-00-0005, human *GAK* or ON-TARGETplus Non-targeting Pool D-001810-10-05 (Dharmacon/Thermo), as well as plasmids DNA encoding cytomegalovirus (CMV)-α-synuclein or CMV-GFP by Lipofectamine 2000. The protocol provided by the manufacturer was followed for co-transfection of siRNA and plasmid DNA in 96-well format. Each experiment had six separate wells per condition and the experiments were repeated three times. Toxicity was accessed by adenylate kinase release (ToxiLight, Amaxa) 72 h post-transfection and subsequent luminescent readings were acquired on a Perkin Elmer Envision 2103 Multilabel reader. The α-human α-synuclein ELISA kit was obtained from Invitrogen and the protocol was described by the manufacturer.

Antibodies

The following antibodies were used in this study: mouse anti-β-actin (Clone AC-74, Sigma-Aldrich, St Louis, MO, USA); chicken anti-MAP2 (EnCor Biotechnology); rabbit anti-TH (Chemicon); mouse anti-GAK (clone 1C2, Medical and Biological Laboratories); rabbit anti-GAPDH (Abcam); mouse anti-α-synuclein (BD Biosciences); anti-rabbit IgG-Alexa Fluor 488 and anti-chicken IgG-Alexa Fluor 594 (Invitrogen) and anti-mouse IgG-conjugated with alkaline phosphatase (Promega, Madison, WI, USA).

Western blotting

Protein expression levels in MES23.5 cultures were determined via western blot analysis. The cells were dislodged from the plate by pipetting, collected by centrifugation, washed with phosphate-buffered saline (PBS) (136 mM NaCl, 0.268 mM KCl, 10 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, pH 7.4), and lysed in RIPA buffer [25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (v/v) SDS, 1 sodium deoxycholate and protease inhibitor cocktail (Sigma)]. After centrifugation at 13 000*g*, the detergent-soluble (supernatant) fraction was recovered. The protein concentration in the soluble fraction was measured using the bicinchoninic acid Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), and equal amounts of protein were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 4–20% (w/v) polyacrylamide gel. The proteins were transferred to a polyvinylidenedifluoride membrane, which was then probed with a primary antibody specific for GAK (1:1000). The membrane was treated with a secondary anti-mouse alkaline phosphatase-conjugated antibody (1:6000). Chemifluorescence images were obtained and analyzed using a Typhoon imaging system (GE Health Sciences, Piscataway, NJ, USA). Densitometry analysis of the western blots was performed using NIH ImageJ software, where targeted proteins

(GAK or α-synuclein) were compared with house keeping genes, GAPDH or β-actin.

Preparation of lentiviral constructs

To ensure that changes in dopaminergic cell viability were due specifically to *GAK* knockdown and did not result from off-target effects, we conducted the shRNA experiments using three different shRNA constructs targeting different regions of the *GAK* gene. The use of multiple non-overlapping shRNAs ('multiplicity control') is an acceptable approach to ensure the validity (specificity) of an RNAi-mediated pheno-type (44). All three shRNA constructs produced similar effects on dopaminergic cell viability (the data obtained using two of these constructs are included here).

The pLenti shRNA constructs targeting rat *GAK* were purchased from OpenBioSystems. Clone identification numbers for the two constructs presented in this study are TRCN0000027669 and TRCN0000027605. These constructs are referred to here as 'shRNA A' and 'shRNA B', respectively. The ViraPower Lentivirus Expression System (Invitrogen) was used to generate lentivirus encoding rat shRNA GAK (A or B). Lentiviral constructs were packaged into viruses as described (31,45). Lentiviral particles were titered using the Traditional p24 ELISA kit purchased from Cell Biolabs.

Preparation of adenoviral constructs

The ViraPower Adenovirus Expression System (Invitrogen) was used to generate adenovirus encoding human A53T-aSyn (downstream of the CMV promoter) as described (29,31). Adenoviral constructs were packaged into the virus via lipid-mediated transient transfection of the 293A packaging cell line. Adenoviral particles were titered using the QuickTiter Adenovirus Titer ELISA Kit (Cell Biolabs).

Preparation of primary mesencephalic cultures

Primary midbrain cultures were prepared via dissection of day 17 embryos obtained from pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) as described previously (31). All of the procedures involving animal handling were approved by the Purdue Animal Care and Use Committee. The cells were plated on poly-L-lysine-treated 48-well plates at a density of 163 500 cells per well. Five days after plating, the cells were treated with cytosine arabinofuranoside (20 μM, 48 h) to inhibit the growth of glial cells. At this stage (i.e. 7 days *in vitro*), the glial cells accounted for ~50% of the total cell population, and the neurons appeared differentiated with extended processes.

Lentiviral and adenoviral transductions

Primary cultures (7 days *in vitro*) were transduced with lenti-virus and/or adenovirus in the presence of polybrene (6 μg/ml). The transductions were carried out for 72 h at a multi-plicity of infection (MOI) of 3 in the case of A53T adenovirus and GAK shRNA lentivirus 'A', or an MOI of 1 in the case of GAK shRNA lentivirus 'B'. The cells were then treated with

fresh media for an additional 24 h and analyzed by immuno-cytochemistry. Control samples consisted of untransduced primary rat midbrain cultures (Fig. 1C and D), as well as primary rat midbrain cultures treated with sub-lethal levels of the A53T adenovirus and with a non-specific lentivirus (lenti-LacZ) (data not shown). In addition, the use of multiple *GAK* shRNA lentiviruses (multiplicity control) ensured that the phenotype produced by each shRNA was due to *GAK* knockdown rather than non-targeting effects (44). MES23.5 cells were transduced with A53T adenovirus (MOI = 30) and GAK shRNA lentivirus (MOI = 15 or 30) for 24 h. After incubating the cells in fresh media for 48 h, lysates were prepared and analyzed via western blotting.

Immunocytochemistry

Primary cells were fixed, permeabilized and blocked as described (31,45). After washing with PBS, the cells were treated overnight at 4°C with the primary antibodies anti-MAP2 (1:1000) and anti-TH (1:500). After washing with PBS, the cells were treated with goat anti-rabbit Alexa Fluor 488 and goat anti-chicken Alexa Fluor 594 for 1 h at room temperature. Prolong gold antifade reagent with DAPI (Invitrogen) was then applied to each before adding a cover slip.

Measurement of primary neuron viability

MAP2- and TH-immunoreactive primary neurons were counted in 10 randomly chosen observation fields (~500–1000 neurons total) for each experimental condition using a Nikon TE2000-U inverted fluorescence microscope (Nikon Instruments, Melville, NY, USA) with a 20X objective. The data were expressed as the percentage of MAP2$^+$ neurons that were also TH$^+$ (this ratiometric approach was used to correct for variations in cell density). Each experiment was repeated three to four times using embryonic neurons isolated from different pregnant rats. Statistical analyses consisted of one-way analysis of variance with the Newman–Keuls *post hoc* test and were carried out using the program GraphPad Prism, Version 4.0 (http://www.graphpad.com/prism/Prism.htm).

Conflict of Interest statement. None declared.

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