Polyamine pathway contributes to the pathogenesis of Parkinson disease

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/pnas.1011751107">http://dx.doi.org/10.1073/pnas.1011751107</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
<td>Final published version</td>
</tr>
<tr>
<td>Accessed</td>
<td>Fri Jun 23 21:51:52 EDT 2017</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/84961">http://hdl.handle.net/1721.1/84961</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Article is made available in accordance with the publisher's policy and may be subject to US copyright law. Please refer to the publisher's site for terms of use.</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td></td>
</tr>
</tbody>
</table>
Polyamine pathway contributes to the pathogenesis of Parkinson disease

Nicole M. Lewandowskia,b, Shulin Ju,a Miguel Verbitskya,d, Barbara Rossa, Melissa L. Geddiee, Edward Rockensteinf,g, Anthony Adame1, Alim Muhammadad, Jean Paul Vonsattela,h, Dagmar Ringec, Susan Lindquistb, Eliezer Masliahb,c, Gregory A. Petsko1, Karen Marderc, Lorraine N. Clarkad,b, and Scott A. Smallia,j,1

*Taub Institute for Research on Alzheimer’s Disease and the Aging Brain, dCenter for Human Genetics, and Departments of bCellular, Molecular, and Biophysical Studies, aNeurology, ªPathology, and Psychiatry, Columbia University College of Physicians and Surgeons, New York, NY 10032; cDepartments of Biochemistry and Chemistry and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02454; Whitehead Institute for Biomedical Research and Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; and Departments of Neurosciences and ªPathology, University of California at San Diego, La Jolla, CA 92093

The full complement of molecular pathways contributing to the pathogenesis of Parkinson disease (PD) remains unknown. Here we address this issue by taking a broad approach, beginning by using functional MRI to identify brainstem regions differentially affected and resistant to the disease. Relying on these imaging findings, we then profiled gene expression levels from postmortem brainstem regions, identifying a disease-related decrease in the expression of the catabolic polyamine enzyme spermidine/spermine N1-acetyltransferase 1 (SAT1). Next, a range of studies were completed to support the pathogenicity of this finding. First, to test for a causal link between polyamines and α-synuclein toxicity, we investigated a yeast model expressing α-synuclein. Polyamines were found to enhance the toxicity of α-synuclein, and an unbiased genome-wide screen for modifiers of α-synuclein toxicity identified Tpo4, a member of a family of proteins responsible for polyamine transport. Second, to test for a causal link between SAT1 activity and PD histopathology, we investigated a mouse model expressing α-synuclein, DENSPM (N1, N11-diethylthiourespermine), a polyamine analog that increases SAT1 activity, was found to reduce PD histopathology, whereas Berenil (diminazene aceturate), a pharmacological agent that reduces SAT1 activity, worsened the histopathology. Third, to test for a genetic link, we sequenced the SAT1 gene and a rare but unique disease-associated variant was identified. Taken together, the findings from human patients, yeast, and a mouse model implicate the polyamine pathway in PD pathogenesis.

Functional MRI | SAT1 | Brainstem

Parkinson disease (PD) is the second most common neurodegenerative disease. Although many of the pathogenic molecules underlying the rare autosomal-dominant forms of PD have been identified (1), the full complement of pathogenic pathways involved in the common “sporadic” form of PD remains unknown (2). In principle, gene expression-profiling techniques such as microarray are well suited to identify molecular pathways contributing to the pathogenesis of complex diseases (3). In practice, however, microarray applied to diseases of the brain present a number of analytic challenges (4). Pinpointing regions within the same brain structure that are differentially targeted by and resistant to a disease can be used to address these challenges (4). Specifically, a 2 × 2 factorial ANOVA can be designed, including both within- and between-group factors, and this “double subtraction” is effective in improving signal-to-noise in a microarray experiment (5).

Although the basal ganglia have been clearly implicated in PD, we decided to focus on the brainstem. Postmortem studies mapping α-synuclein inclusions have suggested that brainstem nuclei, in particular the dorsal motor nucleus of the vagus (DMNV), might be affected early in the course of the disease (6). Notably, α-synuclein inclusions in the brainstem are associated with relatively less gliosis and cell death compared with the basal ganglia. Because gliosis and cell death affect expression profiles independent of pathogenic mechanisms, they act to confound the interpretation of gene expression studies.

Nevertheless, the lack of gliosis and cell death raises the question whether α-synuclein inclusions in the brainstem are functionally benign. Though it is true that early nonmotor PD symptoms might implicate the DMNV (7, 8), these clinical observations are only circumstantial. Accordingly, our first goal was to use a high-resolution variant of functional MRI (fMRI) that maps basal cerebral blood volume (CBV) with submillimeter resolution (9, 10) to show that the DMNV is dysfunctional in PD. Furthermore, brainstem fMRI identified the inferior olivary nucleus (ION) as a neighboring region relatively unaffected. Guided by these imaging findings, we generated gene expression profiles of the DMNV and ION harvested from postmortem brains with and without PD. A 2 × 2 factorial analysis, using ION expression levels as a within-brain control, identified a handful of molecules whose expression levels were differentially affected in the DMNV of PD cases.

Establishing whether a molecular pathway isolated by microarray is indeed pathogenic is difficult and requires additional studies (4). At the very least, studies in cell and animal models must show that manipulating the molecular pathway affects measures of the disease, or more ambitiously, genetic studies might identify disease-associated variants in genes involved in the pathway. Among the findings that emerged from our microarray analysis, we decided to dedicate further investigating into an observed decrease in the expression of spermidine/spermine N1-acetyltransferase 1 (SAT1). SAT1 is the rate-limiting catabolic enzyme in the polyamine metabolic pathway (11), and a decrease in SAT1 expression results in increased levels of higher-order polyamines, spermine and spermidine. Notably, previous studies have suggested that polyamines increase α-synuclein aggregation (12, 13) in vitro systems. Accordingly, we completed a wide range of additional studies using yeast models, mouse models, and human genetics to address separate but complementary questions about the pathogenicity of the polyamine pathway in PD.

Results

High-Resolution fMRI Identifies Brainstem Regions Targeted by and Resistant to PD. To determine whether the DMNV is dysfunctional in PD, CBV maps of the brainstem were generated with fMRI in five PD patients (mean age = 56.4 y) and five healthy age matched controls (mean age = 56.2 y). For each individual subject, ana...
Anatomical criteria were used to identify a single slice that contained the DMNV (Fig. 1A and SI Text). CBV maps of the brainstem slice that contained the DMNV were spatially coregistered, and an unbiased pixel-based analysis revealed a selective disease-associated CBV decrease in the general locale of the DMNV (Fig. 1B). To further confirm this finding, three ROIs were generated over the dorsal medulla, with the central ROI overlaying the DMNV (Fig. 1C). A repeated-measures ANOVA revealed a significant group by region interaction (F = 7.0; P = 0.011) driven by a selective disease-associated CBV decrease in the central ROI overlying the DMNV (Fig. 1 D). An ROI analysis of the ION further confirmed that this nucleus is relatively resistant to PD (Fig. 1D Right).

**Imaging-Guided Microarray Identifies a PD-Associated Decrease in SAT1 Expression.** Relying on these imaging findings, we harvested the DMNV and the ION from six postmortem brains with evidence of PD and five controls. The postmortem PD cases were evaluated for pathological changes (Lewy body-containing neurons and Lewy neurites evidenced with antibodies directed against α-synuclein aggregates) that matched the pattern proposed by Braak (6). Further neuropathological evaluation was performed to confirm the diagnosis of PD over related diseases (SI Text), and the DMNV in all cases was positive for α-synuclein aggregates. Microarray techniques were used to generate gene expression profiles for each of the 22 tissue samples.

A repeated-measures 2 x 2 factorial ANOVA model constructed for the imaging study was applied to the expression dataset, in which expression levels from two regions of the medulla (DMNV vs. ION) were included as the within-group factor, diagnosis (PD vs. Control) was the between group factor, and age and sex were included as covariates. Results revealed 10 transcripts whose expression levels showed a significant diagnosis by region interaction below a P value of 0.05. Five of these 10 transcripts were all down-regulated in PD vs. Control (Fig. 2A). Informed by previous studies (12, 13), we decided that among the transcripts that emerged from our microarray study, further investigation was warranted for spermidine/spermine N1-acetyltransferase 1 (SAT1), an enzyme that catalyzes the acetylation of polyamines spermidine and spermine. Because decreases in mRNA do not always indicate a decrease in protein levels (14), we harvested the DMNV and ION from a new set of postmortem PD cases and control brains in matched brain areas. SAT1 and Actin were examined by Western blot analysis. Replicating and extending the microarray finding (Fig. 2B Left), results revealed a PD-specific decrease in SAT1 protein, normalized to Actin, in DMNV (P = 0.045) but not in the ION (P = 0.19; Fig. 2B Center and Right). Because α-synuclein inclusions are found predominantly in neurons, we used immunohistochemistry to show that SAT1 is expressed in neurons of both the DMNV and the ION (Fig. 2C).

**Yeast Studies Establish a Link Between Polyamines and α-Synuclein.** A decrease in SAT1 increases levels of higher-order polyamines (spermidine and spermine). Previous in vitro studies have suggested that polyamines increase the aggregation of α-synuclein (12, 15, 16). To investigate the relationship between polyamines and α-synuclein in an in vivo system, we turned to an established yeast model of α-synuclein toxicity (17). In this model, yeast expressing one copy of α-synuclein grow normally (NovTox strain), cells expressing ~40% higher levels grow slowly (IntTox), and cells expressing twice as much protein die (HitTox), recapitulating the extreme dosage sensitivity this protein displays in association with PD in humans (18).

We examined the effects of exogenous spermine on yeast strains expressing either wild-type α-synuclein (Syn-WT) or the A53T α-synuclein mutant (Syn-A53T). This mutation enhances the toxicity of α-synuclein both in vitro (19) and in yeast (17), and is associated with the autosomal-dominant form of familial Parkinson’s disease in man (20). Spermine was found to be more toxic to yeast expressing α-synuclein (wild-type and A53T) than the strain carrying the empty vector (Fig. 3A), suggesting a relationship between polyamines and the cellular toxicity of α-synuclein.

IntTox, the strain with intermediate levels of α-synuclein expression, provides a sensitized background for detecting genes that enhance or suppress toxicity. When α-synuclein accumulates to toxic levels in this strain, one of the earliest detectable defects is a block in the trafficking of secretory vesicles from the endoplasmic reticulum to the Golgi. Proteins that promote forward-vesicle trafficking (e.g., the Rab1 GTPase, Ypt1) suppress α-synuclein toxicity, whereas those that inhibit it (e.g., the Rab1 GTPase activating protein, Gyp8) enhance it (16). In an unbiased genome-wide screen for modifiers of α-synuclein toxicity, we recovered Tpo4 as an enhancer of toxicity (21). Tpo4 is a member of a family of proteins responsible for polyamine transport in *Saccharomyces cerevisiae* (22). Quantitative tests of Tpo4, relative to Ypt1 and Gyp8, confirmed the effect of this polyamine transporter in enhancing the toxicity of α-synuclein when overexpressed (Fig. 3B).

![Image](https://example.com/image.png) Fig. 1. MRI identifies brainstem regions targeted by and resistant to PD. (A) Anatomical criteria used to identify an MRI slice of the medulla that contains the DMNV. As shown by the sagittal scout image (left), MRI slices were acquired from anterior to posterior (three consecutive MRI slices are shown together with their accompanying histological slices), perpendicular to the long axis of the brainstem (black line in scout image). Using strict anatomical criteria (Materials and Methods and SI Text), a single slice (red line in scout image and red-boxed middle images) was identified in each individual subject, containing the DMNV (red circle in the middle boxed histological slice). (B) CBV MRI maps of the brainstem slice that contains the DMNV were coregistered, and statistical maps comparing PD patients and controls were generated. For anatomical reference, the right half of the figure shows the statistical maps, which are color-coded such that cooler colors indicate less function, and the left half of the figure shows a histological slice where the DMNV is circled in red and the ION is circled in blue. (C) ROI analysis of the DMNV and the ION. The gray vertical line demarcates the midline of the medulla. The red boxes represent the three ROIs in the dorsal medulla, where the box furthest from the midline is the lateral measure, the box closest to the midline represents the medial measure, and the middle box represents the central measure, which contains the DMNV. The blue ovals represent the ROI of the ION. Again, as an anatomical reference, the right half of the figure is the MRI scan, and the left half of the figure is the histological slice. (D) Group data analysis shows selective PD-related dysfunction in the central dorsal medulla (Left). The y-axis is %CBV for each ROI (lateral, central, and medial), averaged for PD cases and controls, of both the left and right side of the medulla. No group differences were observed in the ION (Right).
Notably, in the IntTox strain, overexpression of Tpo4 caused α-synuclein to form intracellular foci more rapidly than it otherwise would (Fig. 3C). These α-synuclein foci were previously shown to occur at sites of stalled vesicles (18). Deletion of Tpo4 had no effect on α-synuclein toxicity, likely due to the redundancy of polyamine transporters in yeast.

**Mice Studies Establish a Link Between SAT1 Activity and α-Synuclein Histopathology.** To investigate the relationship between SAT1 activity and α-synuclein histopathology in the mammalian brain, we turned to genetically modified mice that express wild-type human α-synuclein and develop histological inclusions (23). Berenil (dimethyl benzene aceturate) is a pharmacological agent that reduces SAT1 activity (24), whereas DENSPM (N1, N11-diethylnorspermine) is a pharmacological agent that reduces SAT1 activity (24). We chronically delivered these agents via osmotic minipumps to the transgenic mouse model. Results showed that, compared with the transgenic mice treated with PBS, neuronal accumulation of α-synuclein in the basal ganglia (Fig. 4A) and focused within the substantia nigra (Fig. S1) was increased with Berenil (P = 0.009) and decreased with DENSPM (P = 0.043). Berenil worsened the effects on tyrosine hydroxylase (TH) fibers (P = 0.033), whereas DENSPM partially rescued the deficits (P = 0.022; Fig. 4B). Alterations in the dendritic complexity in the basal ganglia of the α-synuclein transgenic mice were worsened by Berenil (P = 0.013) and ameliorated by DENSPM, as detected by MAP2 (Fig. 4C). Therefore, overall α-synuclein pathology in these mice was worsened with Berenil and partially rescued with DENSPM. Although comparing drugs to PBS showed no significant effect in nontransgenic mice, general toxicity was associated with the route of administration. Infusion caused

![Graphs](image)

**Fig. 2.** Imaging-guided microarray identifies a PD-associated decrease in SAT1 expression. (A) Ten molecules whose expression levels via microarray analysis were differentially affected in PD cases vs. controls, comparing the DMNV to the ION. Notably, in all 10 transcripts, the expression level was down-regulated in PD vs. controls; P values were calculated by a repeated-measures 2 × 2 factorial ANOVA. (B) Mean expression levels are shown on the graph for SAT1 for mRNA where n = 22 (six DMNV and six ION for PD, and five for each region in controls). Expression within the DMNV was significantly down in PD compared with controls (P = 0.002) (Left). In a new set of brains, protein expression via Western blot, where n = 20 (five DMNV and five ION for each group, control and PD), showed a significant decrease of SAT1 expression (P = 0.045) in the DMNV of PD samples compared with controls, but not in the ION, as seen in the blot image of representative samples and quantified normalized to actin depicted in the right graph. (C) Immunohistochemistry for SAT1 protein in the DMNV (Left) and the ION (Right) showed positive expression within the neuronal cell bodies of both regions, at 40× (Left) and 400× (Right), respectively.

![Graphs](image)

**Fig. 3.** Yeast studies establish a link between polyamines and α-synuclein. (A) Yeast cells integrated with empty vector (Vec), wild-type α-synuclein (Syn-WT), or mutant α-synuclein (Syn-AS3T) were grown in YPGal medium supplemented with spermine at concentrations of 0 mM (Top), 0.2 mM (Middle), and 0.4 mM (Bottom). OD600 was monitored at 1 h intervals using the Bioscreen system. The growth curves shown are representative of three independent experiments. (B) In an unbiased genome-wide yeast screen, Tpo4 was shown to enhance toxicity in cells expressing α-synuclein at a ∼40% higher level (IntTox), in comparison with a known toxicity enhancer (Gyp8) and suppressor (Ypt1). (C) Tpo4 caused α-synuclein to form intracellular foci more rapidly than it otherwise would in the IntTox strain.
death before the termination of the experiment in some of the control and pharmacologically treated mice.

**Genetic Studies Identify a PD-Associated Variation in the SAT1 Gene.** To investigate a genetic link between SAT1 and PD, all exons and adjacent exon/intron boundaries of the SAT1 gene were sequenced in a total of 92 PD patients. We identified a variant, c.786_788delTGT (NM_002970), in the 3’UTR of the SAT1 gene in two PD patients (Fig. 5). One patient was heterozygous and one hemizygous for the c.786_788delTGT variant. To evaluate the frequency of the 3’UTR variant, we extended the analysis to genotyping of additional patients and controls enrolled in the genetic epidemiology of PD, GEPD (total: n = 797 subjects; patients: n = 389; and controls: n = 408). Overall, a total of four PD patients were identified that carried the c.786_788delTGT variant, including three heterozygotes and one hemizygote. The c.786_788delTGT variant was absent in all 816 control chromosomes (n = 408 controls). The mean age of the four PD patients carrying the c.786_788delTGT variant was 63.3 (range 51–72), the mean age at onset was 54 yr (range 38–68, with two having onset <50), and mean duration of PD was 9.25 yr (SD 7.5). There were three women and one man, and three Caucasian and one “Other” ethnicity. None reported a family history of PD in a first-degree relative. Three of the four had tremor at rest as a first symptom, and all were levodopa responsive. None of the four were demented at the time of evaluation. None of the cases carried mutations in Parkin, LRRK2, or DJ1. Lymphoblasts were available in only one PD case carrying the SAT1 c.786_788delTGT variant of the gene, and four noncarrier male patients. In this limited sample, a reliable effect of the variant on SAT1 mRNA expression, degradation, or stability could not be documented (for more experimental detail, see SI Text).

**Discussion**

To date, uncovering pathogenic molecular pathways associated with neurodegeneration has relied primarily on biochemical analysis of protein aggregates or on linkage analysis to isolate genetic mutations. These two approaches have been important in clarifying the molecular biology of neurodegeneration (2), and have been successful in identifying pathogenic molecules underlying rare, monogenic forms of disease. The introduction of gene expression profiling techniques has allowed the focus of molecular discovery to shift from aggregates and genes to brain cells themselves. Reflecting an interaction among multiple genetic and epigenetic factors, expression profiles of affected cells are, in principle, well suited for uncovering pathogenic pathways underlying complex disorders of the brain (4). When applied to neurodegenerative diseases, however, gene expression studies present a number of analytic challenges, including poor signal-to-noise and high false-positivity (4). Furthermore, observed expression differences are only correlative and cannot, by themselves, establish a pathogenic link to disease.

**fmRI-Guided Microarray.** In attempting to address the analytic challenges of microarray, we were guided by our previous studies that used microarray to identify retromer trafficking defects as pathogenic in late-onset Alzheimer’s disease (AD) (26, 27). First, we needed to identify neighboring regions within the same brain

---

**Fig. 4.** Mice studies establish a link between SAT1 activity and α-synuclein histopathology. Transgenic mice that express wild-type human α-synuclein and nontransgenic controls (nontg) were treated by intracranial infusion via osmotic pumps with PBS, DENSPM, or Beninil for 6 wk. Results for immunoreactivity within the basal ganglia are shown. (A) Mean α-synuclein cell-pixel intensity (cell mean) in the caudoputamen was increased with Beninil (*P = 0.009) and decreased with DENSPM (*P = 0.043) in comparison with transgenic α-synuclein mice treated with PBS (syntgPBS). (B) Tyrosine hydroxylase (TH) fibers were decreased with Beninil treatment (*P = 0.033), and increased with DENSPM (*P = 0.022), compared with syntgPBS, as shown by the cell mean (TH corrected optical density in the caudoputamen). (C) Beninil caused a decrease in MAP2 (*P = 0.013), compared with syntgPBS, whereas DENSPM rescued the transgenic α-synuclein effects. Percent cell mean represents the percentage of the neuropil covered by MAP2 immunoreactive dendrites in the caudoputamen.
structure, differentially affected and resistant to PD. Deciding to focus on the brainstem, a structure purposefully identified as affected early on in the disease course, we exploited the high-resolution capabilities of basal CBV mapping with fMRI. Previous studies have established the utility of CBV fMRI in mapping brain function (28) and have shown that basal CBV is tightly correlated with disease-associated metabolic dysfunction (29). Most importantly, compared with other hemodynamic variables used in fMRI—i.e., cerebral blood flow or deoxyhemoglobin—CBV generates functional maps with the highest spatial resolution (9).

Just as we have used CBV fMRI to identify hippocampal subregions differentially affected and resistant to AD, we reasoned that this high-submillimeter resolution might be used to map dysfunction in small regions of the brainstem. Indeed, our first fMRI study identified the DMNV as a brainstem region differentially affected by PD, and the ION as a neighboring region relatively resistant to the disease. Future large-scale imaging studies, in which brainstem CBV maps are generated in a larger number of PD patients across different stages of the disease, are required to determine whether observed brainstem dysfunction correlates with signs and symptoms of the disease, whether it antedates dysfunction in other brain regions, and whether it can be used for diagnostic purposes.

Guided by our fMRI results, we harvested the DMNV and ION in postmortem brains with and without PD, and by profiling expression levels in each individual tissue sample we were able to perform a “double subtraction” factorial ANOVA. As previously described, when applied to a microarray dataset, this statistical design improves the odds that the isolated molecules are linked to the disease (4, 5).

Validating the Microarray Finding. Although the list of molecules that emerged from the factorial ANOVA are likely linked to PD, this analysis cannot identify which molecular findings play a causal role in disease pathogenesis. As in all neurodegeneration, the disease process in PD exists many years before the patient’s death, and it is impossible to know whether a postmortem finding reflects an upstream defect that affects α-synuclein toxicity, or a downstream effect reflecting protracted neuronal dysfunction. Thus, a range of additional studies are required to interpret the mechanistic and causal role of any microarray finding identified in postmortem samples (4). For example, in our previous studies, imaging-guided microarray applied to AD identified deficiencies in the retromer trafficking pathway (27). However, only by systematically manipulating retromer-related molecules—in cell culture (27), animal models (26)—and by linking genetic variance to the disease in human patients (30), was this trafficking pathway pathogenically linked to AD (31).

Among the transcripts identified in the microarray study, we decided to focus our attention on the observed decrease in SAT1 expression. SAT1 is the rate-limiting enzyme in polyamine catabolism, and a reduction in SAT1 leads to an increase in higher-order polyamines—in particular, spermidine and spermine (24, 32). A previous study showed that PD patients have elevated levels of spermidine and spermine in red blood cells (13), which do not themselves produce polyamines. Another study showed that an α-synuclein, exogenously added spermidine and spermine accelerate the aggregation of α-synuclein (12). Furthermore, previous studies have shown that SAT1 is expressed in numerous brain regions implicated in PD, including the substantia nigra pars compacta (33).

Whether polyamines can modify the cellular toxicity of α-synuclein in vivo was unknown. To address this issue, we turned to a yeast model of PD and showed in an unbiased screen that Tpo4, responsible for polyamine transport in S. cerevisiae, and exogenous spermidine accelerated the toxicity of yeast expressing human α-synuclein. Importantly, because we are interested in sporadic PD, in which non-mutated forms of α-synuclein play a mechanistic role, acceleration of the phenotype was found in yeast expressing wild-type α-synuclein.

The next question was whether SAT1 activity can modify α-synuclein histopathology in the mammalian brain. To address this question, we turned to a transgenic mouse model of PD that expressed wild-type human α-synuclein, and treated them with pharmacological agents. Berenil and DENSPPM. Berenil (diminazene aceturate) has been shown to inhibit polyamine metabolism by reducing SAT1 activity via competitive inhibition of the enzyme (24). Berenil was favored above other SAT1 inhibitors due to its selectivity for effecting SAT1 alone, and no other enzymes involved in polyamine metabolism (34). DENSPPM (N1, N11-diethylnorspermine) is a polyamine analog and the most potent known inducer of SAT1. Interestingly, it demonstrated the ability to down-regulate polyamine biosynthetic enzyme activities and suppress transport of polyamines, as well as its potent up-regulation of SAT1, resulting in depletion of intracellular polyamine pools (35, 36). Using these pharmacological agents that mediate SAT1 activity, findings showed that Berenil worsened the PD phenotype, whereas DENSPPM partially improved the PD phenotype in these mice.

Finally, we decided to sequence SAT1 in human patients, even though a genetic variant in this gene was not necessarily assumed. In fact, as a complex disorder, we believe that polyamine abnormalities observed in PD are more likely to reflect a complex interaction between genes, epigenetics, and the environment rather than defects in the SAT1 gene itself. However, because any genetic finding in human patients would strengthen a pathogenic link, we reasoned that it was worth exploring this issue. Remarkably, a unique variant, c.786_788delTGT, in the SAT1 gene was indeed found. It is important to note that this variant is rare, occurring in less than 1% of the patients examined, but was exclusive to patients and absent in all 816 of the control chromosomes genotyped. Additionally, based on a small number of samples, we have not yet determined whether this genetic variant affects expression levels or otherwise affects the function of SAT1. Beyond the scope of the current body of work, future genetic studies genotyping a larger number of cases and controls are required to determine the exact frequency of c.786_788delTGT, and future molecular studies will determine whether and how this variant affects the enzymatic function of SAT1. Despite these unanswered questions, the surprising disease-associated mutation identified in SAT1 does, we believe, strengthen the link between the polyamine pathway and PD.

Conclusions. Although spanning a broad range, the studies presented in patients, yeast and mouse models, are unified in their attempt to confirm the pathogenicity of the microarray findings. In summary, these studies, as a whole, these complementary studies suggest that defects in the polyamine pathway play a role in PD pathogenesis, and the findings have immediate clinical implications. First, because polyamines can be measured in cerebral spinal fluid and blood, serological analysis might be able to detect polyamine abnormalities and can be used for early detection of PD and for monitoring therapeutic interventions. Second, and perhaps more importantly, a number of pharmaceutical agents have already been developed that enhance the function of SAT1 and polyamine metabolism (37). Based on our present findings, we have begun exploring whether any of these agents cross the blood–brain barrier, and if so, plan on initiating drug studies to de-
term whether reductions of brain polyamine levels ameliorate this common and undertreated disease.

Materials and Methods

Brain Imaging Studies. Subjects, data acquisition, and processing. See SI Text. Data analysis. Unbiased pixel-based analysis. All CBV maps of the target slice were spatially filtered and coregistered; on a pixel-by-pixel basis, a t-test was used comparing the grouped PD images to the grouped control images. The yielded z-score was then colorized onto the standard anatomical image for visual inspection (Fig. 18).

Hypothesis-driven ROI-based analysis. Performed as previously described (38); for details, see SI Text.

Postmortem Studies. Gene expression profiling. Total RNA was extracted from each of the 22 tissue samples with TRIzol reagent (Invitrogen) and purified with RNeasy mini columns (Qiagen). All subsequent steps followed Affymetrix’s Eukaryotic Target Preparation Protocol found in the GeneChip Expression Analysis Technical Manual (for details, see SI Text).

Western blot analysis. A new set of five PD and five control brain samples were obtained from the New York Brain Bank at Columbia University, following the criteria described in SI Text. Within each brain, the DMNV and ION were identified and sectioned using strict anatomical criteria. Tissue samples were prepared as previously described (27); for details, see SI Text.

Immunohistochemistry. Axial blocks of human medulla oblongata were frozen-sectioned using a Microm cryostat at 8-μm thickness as previously described (27); for details, see SI Text.

Yeast Studies. Yeast strains, media, and growth-rate assays. See SI Text. Yeast screen. Tp04 was identified in an unbiased genome-wide screen investigating modifiers of α-synuclein toxicity (21); see SI Text for details.

α-Synuclein growth with Tp04. α-Synuclein strains and control strains were grown overnight to saturation in glucose-containing media. Cultures were normalized for their OD and serially diluted fivefold before spotting onto yeast media plates containing either glucose or galactose.

α-Synuclein microarray. Cells were grown overnight in glucose-containing media. Culture densities were normalized, washed with water, and induced for 6 h in galactose. Cells were fixed [4% formaldehyde, 50 mM KPi (pH 6.5), 1 mM MgCl2] for 1 h on ice, washed twice with PBS, and imaged.

Transgenic Mice Studies. α-Synuclein transgenic mice and infusion. For this study, heterozygous transgenic (tg) mice (line 61) expressing wild-type human α-synuclein under the regulatory control of the mThy1 promoter were used (23). For details on these mice and the infusions, which were described previously (39), see SI Text.

Immunohistochemical analysis of α-synuclein accumulation and neurodegeneration. Immunohistochemistry was performed as described previously; for details, see SI Text.

Genetic Studies. Details on patient population can be found in SI Text. Sequencing and genotyping. PCR and amplification of all exons and exonic/ intron boundaries of the SAT1 gene were performed. The PCR and sequencing primers used for amplification of SAT1 are available upon request. Cycle sequencing in forward and reverse directions was performed on purified PCR products and run on an ABI 3700 genetic analyzer (Applied Biosystems). Sequence chromatograms were viewed and genotypes determined using Sequencer (GeneCodes). Statistical analysis was reviewed.

Protein, mRNA levels, and stability of splice variants. See SI Text.

ACKNOWLEDGMENTS. We thank those who willingly participated in the studies presented herein. This work was supported in part by the McKnight Endowment for Neuroscience (to S.A.S., D.R., and G.A.P.), National Institutes of Health Grants 1R01 GM06440, 1G02Z02074 (to E.M.), N02RR003837 (to S.L.), UL1 RR024156 (to K.M.), and N03RR0630, and the Parkinson’s Disease Foundation (to K.M. and L.N.C.). M.L.G. is supported by a National Institutes of Health postdoctoral fellowship.
Supporting Information

Lewandowski et al. 10.1073/pnas.1011751107

SI Text

Brain Imaging Studies. Subjects. Subjects were recruited from a cohort of patients seen and rated by Lucien Coté at Columbia University Medical Center, Department of Neurology. At the time of the scan, all patients were in early stages of PD, rated at stage 1 or 2 according to the clinical criteria outlined in Hoehn and Yahr’s Staging of Parkinson’s Disease, Unified Parkinson Disease Rating Scale (UPDRS). Age-matched subjects were acquired for the control group. Two of five PD and three of five controls were women. All controls underwent a neurological evaluation before the scans to ensure their “control” status.

Data acquisition and processing. Subjects were imaged with a 1.5-T Philips Intera scanner following a similar protocol as described (1, 2). For each subject, two sets of oblique axial 3D T1-weighted images (TR = 20 ms; TE = 6 ms; flip angle = 25 degrees; in-plane resolution = 0.86 × 0.86 mm; slice thickness = 2 mm) were acquired perpendicular to the long axis of the brainstem (Fig. 1A). The first series of images were acquired before, and the second acquired 4 min after, IV administration of a standard dose of gadolinium-pentate (Omniscan, 0.1 mmol/kg).

Images were then transferred to a workstation containing an analysis software package (MEDxSensor Systems). An investigator blind to subject grouping performed all imaging processing. The relatively short acquisition time minimized head motion; however, the AIR program was used to coregister the images. A Gnu plot was generated to assess the quality of the coregistration, and an individual study was rejected if a shift greater than 1 pixel dimension was detected.

Among the series of anatomical (i.e., precontrast T1-weighted) images, fixed criteria based on the external morphology of the slices were used to identify the single slice in each individual that contained the DMNV. The external morphology of the medulla is dominated by upper-quadrant protrusions, reflecting the inferior cerebellar peduncles, and lower-quadrant protrusions, reflecting the inferior olivary nuclei. Moving from anterior to posterior, the target slice was always the one in which the anatomy of the medulla transitioned from a triangular to a box-like morphology, reflecting an inward shift in the inferior cerebellar peduncles (Fig. 1A). Moving from posterior to anterior, the target slice was always the one in which protrusions of the inferior olivary nuclei became visible.

Steady-state CBV maps derived from the contrast-induced changes in T1-weighted signal were generated as previously described (1, 2). Briefly, subtraction maps were created by subtracting the precontrast from the postcontrast image. Then, percent CBV maps were generated by dividing the subtraction maps by a subtraction value measured from a region that is 100% blood, and multiplying by 100. In contrast to previous studies that used a value measured from the sagittal sinus (1, 2), we used a value measured from the sigmoid sinus because of its close proximity to the brain tissue of interest.

Data Analysis. Hypothesis-driven ROI-based analysis. In the absence of clear anatomical landmarks, ROIs cannot be simply drawn over the DMNV, or neighboring dorsal nuclei. An alternative approach was utilized. A tracing tool in the imaging software program was used to manually trace the midline of the medulla, originating from the anterior median fissure extending to the median sulcus of the forth ventricle (Fig. 1C). From this midline, rectangular strips were drawn, nine pixels in length and three pixels in width, that began from the lateral aspect of the dorsal medulla and extended to the midline (Fig. 1C). Then, each rectangular strip was divided into three equal segments—lateral, central, and medial—with the central segment overlying the DMNV (Fig. 1C). These ROIs were then coregistered onto the CBV maps, yielding three CBV values per subject by combining measures on the right and left sides into mean values (Fig. 1C).

A repeated-measures ANOVA was constructed to test the hypothesis that the DMNV ROI was differentially affected in PD cases vs. controls. CBV values from the three dorsal medulla ROIs were included, as the within-subject factors and diagnosis (PD vs. control) was included as the between-subject factor.

Postmortem Studies. Gene expression profiling, PD pathology. Parkinson disease (n = 12, 6 DMNV and 6 ION) and control brain samples (n = 10, 5 DMNV and 5 ION) were obtained from the New York Brain Bank (NYBB), snap-frozen in liquid nitrogen and stored at −80°C. All PD cases were confirmed to have α-synuclein-positive cytoplasmic inclusions. The pathological changes displayed in the postmortem PD cases matched the pattern proposed by Braak (3). Lewy body-containing neurons and Lewy neurites evidenced with antibodies directed against α-synuclein aggregates were used, and outsider cases were excluded. The clinicopathological correlation of the cases of interest further confirmed the diagnosis of idiopathic PD. The thorough neuropathological evaluation performed ruled out the presence of changes that could have caused parkinsonism (e.g., neuronal and glial tangles, as seen in progressive supranuclear palsy; synucleinopathic changes, as observed in multiple system atrophy; multiple infarcts centered within the striatum; or ubiquitinated, nuclear inclusions, as seen in fragile X-associated tremor/ataxia syndrome, which mimics PD clinically). Lewy body-containing neurons or Lewy neurites (most of the time, both) are almost always found within the reticular formation of the medulla oblongata, and this in addition to the involvement of the dorsal motor nucleus of the vagus. Control brains were evaluated with the same protocol as the one used for the evaluation of the brains from patients. Control brains were identified without diagnostic abnormality. Within each brain, the DMNV and ION were identified and sectioned using strict anatomical criteria following NYBB procedures.

Microarray experimental detail. All subsequent steps performed are outlined in Affymetrix's Expression Analysis Technical Manual, which may be found at the manufacturer’s website. Note all incubations were performed in a thermocycler (Mastercycler; Eppendorf) to allow for proper temperature distribution during workup. A preparation of Poly-A RNA controls for two-cycle cDNA synthesis was made using an eukaryotic poly-A RNA control kit (Affymetrix). These poly-A spike-in controls were added to the total RNA that was isolated and used to prepare double-stranded cDNA. The T7-Oligo(dT) primer for cDNA synthesis was added and the first-cycle first-strand master mix was added to the samples (Two-Cycle cDNA Synthesis Kit; Affymetrix). This same kit was used to prepare the first-cycle second-strand master mix. First-cycle IVT amplification of cRNA was performed on all samples using MEGAscript T7 kit (Applied Biosciences). First-cycle cRNA was cleaned up using a sample clean up module (supplied through Affymetrix). The cleaned cRNA was added to random primers and to the second-cycle first-strand master mix (using the two-cycle cDNA synthesis kit) to produce cDNA. After incubation, RNase H was added to each sample. T7-Oligo(dT) primer was added, followed by the second-cycle second-strand master mix. Following incubation, T4 DNA polymerase was added. A sample cleanup module was used to clean up the double-stranded cDNA (Affymetrix). Using the GeneChip IVT Labeling Kit (Affymetrix), biotin-labeled cRNA was synthesized. The
cRNA was cleaned and fragmented using the sample cleanup module (Affymetrix). Fragmenting was customized to a 100 array format. In the Gene Chip Analysis Facility of Columbia University, HG-U133A 2.0 microarrays (GeneChip; Affymetrix) were hybridized with fragmented cRNA for 16 h in a 45 °C incubator with constant rotation at 60 x g. Microarrays were washed and stained on a fluidics station, and scanned using a laser confocal microscope. HG-U133A 2.0 microarrays were analyzed with Affymetrix Microarray Suite v5.0 and GeneSpring v5.0 3 (Silicon Genetics) software. Transcripts whose detection levels had P < 0.05 were excluded, and raw data from the 18,400 included transcripts were found on the included CD-ROM (GeneChip Human Genome U133A 2.0 Array Library File). Microarray data were processed and analyzed as described (4). The microarray data has been submitted in a MIAME-compliant format to Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov GEO), series accession no. GSE19587.

Western blot analysis. Tissue samples were soaked in 5 vol of solution [20 mM Tris HCl (pH 7.9), 150 mM NaCl, 5% Nonidet P-40, 1 mM EDTA] supplemented with protease inhibitor mixture (Roche). Samples were homogenized on ice with 12 strokes at 900 rpm using a motor-operated Tephlon-paste homogenizer and held on ice for 30 min. Homogenate was centrifuged at 12,500 rpm for 20 min at 4 °C, and the supernatant was saved. Western blotting was performed on 25 μg of protein sample. Blots were incubated sequentially in Tris-buffered saline with 1% Tween 20 (TBS-T) and blocked with 0.1% BSA for 1 h; the mouse polyclonal anti-SAT1 (Novus Biologicals) was incubated overnight with constant rotation at 4 °C, washed with TBS-T, and the appropriate enzyme HRP-labeled secondary antibody was incubated for 1 h and then washed with TBS-T and ECL reagent (GE Healthcare Amersham). This procedure was repeated using Actin to normalize the samples.

Immunohistochemistry. Tissue was quick-frozen onto the slides, postfixed with 4% paraformaldehyde in PBS, washed with PBS, then treated with 3% H2O2, washed and preincubated for 1 h in block solution consisting of 2% horse serum (Vector Laboratories), 1% BSA (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Slides were then incubated 18 h at 4 °C in diluted (1:75 in block solution) polyclonal antisera to SAT1 (Proteintech Group Inc.). After washing with PBS, immunoreactivity was detected by an avidin-biotin-linked peroxidase method, using successive incubations and washes with anti-rabbit biotinylated IgG, Vectastain Elite ABC reagent (Vector Laboratories), and diaminobenzidine (Sigma-Aldrich) chromagen reagent. Sections were washed with PBS, dehydrated, and mounted using Permount (Fisher Scientific).

Yeast Studies. Yeast strains, media, and growth rate assays. All yeast studies used the W303 strain (MATa ade2-1 can1-100 his3-11,15 leu2-3,112, trp1-1, ura3-1). The α-synuclein strains used for growth rate assays were as follows: a control strain containing an empty vector supplemented with different concentrations of spermine at 30 °C. Growth curves were generated by growing cells in YPGal medium supplemented with different concentrations of spermine at 30 °C. OD600 was monitored at 1-h intervals using the Bicore C (Growth Curves).

Yeast screen. Briefly, 5,000 yeast ORFs were cloned individually into a plasmid with a galactoside-inducible promoter, pBY011 (CEN, URA3, AmpR). Cells expressing IntTox α-synuclein were transformed with the clones and grown 2 d on synthetic drop-out media. The cells were then spotted onto SD-Ura or SGal-Ura plates. Modifiers of α-synuclein toxicity were identified after 3 d of growth on galactose plates. Tpo4 was retested three times for confirmation.

Transgenic Mice Studies. α-Synuclein transgenic mice and infusion. The tg mice (line 61) were selected because they display abnormal accumulation of detergent-insoluble α-synuclein and develop h-α-synuclein immunoreactive inclusion-like structures in the basal ganglia, cortex, and limbic system (5). Furthermore, these animals also display neurodegenerative and motor deficits that mimic certain aspects of PD and dementia with Lewy bodies (6). To evaluate the effects of the polyamine pathway, h-α-synuclein tg mice (10–11 mo) received intraventricular infusions with a cannula implanted into the skull and connected to osmotic minipumps of vehicle, Beneril, or DENSMP. A total of 15 h-α-synuclein tg mice (n = 5 per group) were used for these experiments. Mice were treated for 6 wk; the compounds were dissolved in 0.9% NaCl at a concentration of 62 μM for DENSMP and 30 μM for Beneril at a pH of 7.2. Then, 200 μL of these solutions were filled into the osmotic minipump (Alzet) ensuring constant delivery (0.25 μL/h). The minipump was implanted s.c. on the back under light anesthesia. An additional group of control non-tg (n = 5) mice were implanted with minipumps filled with vehicle only. All procedures were completed under the specifications set forth by the Institutional Animal Care and Use Committee.

Immunohistochemical analysis of α-synuclein accumulation and neurodegeneration. Brains were removed and divided sagittally. One hemibrain was postfixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4 °C for 48 h and sectioned at 40 μm with a Vibratome 2000 (Leica) and placed in cryosolution; the other hemibrain was snap-frozen and stored at −70 °C for further analysis. Sections were first washed 3 x 5 min each in PBS. They were then pretreated with 1% Triton-X, 3% hydrogen peroxide in PBS for 15 min, washed again 3 x 5 min in PBS, and blocked in 10% serum matching the animal the secondary antibody was raised in for 1 h, and then washed with PBS. Sections were then placed in primary antibody: mouse anti-TH, rabbit anti-(h) α-synuclein, mouse anti-MAP2 (Chemicon; 1:500) overnight in 4 °C. Sections were washed 3 x 5 min in PBS and then placed in biotinylated secondary antibody (1:100; Vector Laboratories) for 2 h. After washing again three times in PBS, sections were placed in 20% diaminobenzidine (DAB) (Vector Laboratories) for 20 s. The reaction with DAB was halted by immersing the sections in double-distilled water. Sections were then mounted, dried, and coverslipped with Entellan (Fisher Scientific). Sections for fluorescent immunohistochemistry were treated with mouse monoclonal antimicrotubule-associated protein 2 (MAP2, 1:50; Roche Molecular Biochemicals) primary antibody. This antibody was then detected with the FITC-conjugated anti-mouse secondary antibody (Vector Laboratories). Coverslips were air-dried overnight, mounted on slides with antifading media (Vectashield, Vector Laboratories), and imaged with the LSCM (MRC1024, Bio-Rad). The immunolabeled blind-coded sections were imaged by LSCM (MRC1024; Bio-Rad) and analyzed with the ImageJ 1.43 program (http://rsbweb.nih.gov/ij/download.html), as previously described (6).

Genetic Studies. Patient population. The SAT1 gene was sequenced in 92 PD patients who participated in the Genetic Epidemiology of PD (GEPD) study (7–9). An additional 389 cases and 408 controls from GEPD were genotyped for the SAT1 c.786_788delTGT (NM_002970) variant. Cases were ascertained from the Center for Parkinson’s Disease and Other Movement Disorders at Columbia University based on age at onset of motor signs ≤50 (EOPD) or >50 (LOPD), with oversampling of the EOPD group (7). The majority of the controls were recruited by random-digit dialing, with frequency matching by age, gender, and ethnicity. All participants were seen in person and underwent an identical evaluation
Protein, mRNA levels, and stability of splice variants. Lymphocytes. RNA was extracted from cultures of lymphoblastoid cell lines established by transforming patients’ lymphocytes with Epstein–Barr virus using standard techniques (8). Cells were homogenized and RNA extracted using TRIzol reagent (Invitrogen). RNA was further purified using the RNaseasy Mini Kit (Qiagen) with on-column DNase digestion. cDNA was synthesized from 1 μg total RNA using SuperScript III First Strand Synthesis Supermix (Invitrogen). The following TaqMan Gene Expression assays (Applied Biosystems) were used for real-time PCR relative quantification of mRNA levels: Hs00161511, Hs00971737, Human ACTB Endogenous Control, and Human GAPDH Endogenous Control. The reactions were performed in triplicate on an AB7500 Real Time System using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. The data were normalized to the geometric mean of β-Actin and GAPDH, and the 2^−ΔΔCt method was used to obtain relative expression values. Comparisons were made between lymphoblastoid cell lines for each SAT1 mRNA variant separately.

HECK 293 cells. HEK 293 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, 100 μg streptomycin, and 0.1 mM nonessential amino acids (GIBCO, Invitrogen) in an atmosphere of 5% CO_2 at 37 °C. Cells were seeded in six-well plates at ≈5 × 10^4 cells/well, and transfections were performed the next day using 2.5 μg of construct (SAT1-s wt/del, SAT1-l-wt/del; GE-NEART) using Lipofectamine 2000 (Invitrogen). Each plasmid variant was cotransfected with a GFP plasmid (GeneScript) to control for transfection efficiency. A plate with each variant transfection was lysed for protein analysis via Western blot (following methods described previously) using anti-mouse GFP antibody (GeneScript) and anti-rabbit SAT1 (Novus Biologicals), normalized to tubulin. After 48 h, the remaining transfected cell plates were treated with actinomycin D (G-BioSciences) and RNA was extracted at different time points (0, 2, 4, 6, 12, 24, and 48 h) for real-time-PCR analysis as described for lymphoblasts. Data for each construct was normalized to t = 0.


Fig. S1. Mice studies validate that SAT1 activity modifies PD pathology. Transgenic mice that express wild-type human α-synuclein and nontransgenic controls (nontg) were treated by intracranial infusion via osmotic pumps with PBS, DENS PM, or Berenil for 6 wk. Results for immunoreactivity within the substantia nigra are shown. Mean α-synuclein cell pixel intensity (cell mean) was increased with Berenil (*P = 0.001), and a trending decrease seen with DENS PM in comparison with transgenic α-synuclein mice treated with PBS (syntgPBS).