The genetic contributions of *SNCA* and *LRRK2* genes to Lewy Body pathology in Alzheimer's disease

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Received February 19, 2014; Revised March 21, 2014; Accepted April 24, 2014

The molecular genetic basis that leads to Lewy Body (LB) pathology in 15–20% of Alzheimer disease cases (LBV/AD) was largely unknown. Alpha-synuclein (*SNCA*) and Leucine-rich repeat kinase2 (*LRRK2*) have been implicated in the pathogenesis of Parkinson's disease (PD), the prototype of LB spectrum disorders. We tested the association of *SNCA* variants with LB pathology in AD. We then stratified the *SNCA* association analyses by *LRRK2* genotype. We also investigated the expression regulation of *SNCA* and *LRRK2* in relation to LB pathology. We evaluated the differences in *SNCA*-mRNA and *LRRK2*-mRNA levels as a function of LB pathology in the temporal cortex (TC) from autopsy-confirmed LBV/AD cases and AD controls. We further investigated the *cis*-effect of the LB pathology-associated genetic variants within the *SNCA* and *LRRK2* loci on the mRNA expression of these genes. *SNCA* SNPs rs3857059 and rs2583988 showed significant associations with increased risk for LB pathology. When the analyses were stratified by *LRRK2*-rs1491923 genotype, the associations became stronger for both SNPs and an association was also observed with rs2619363. Expression analysis demonstrated that *SNCA*- and *LRRK2*-mRNA levels were significantly higher in TC from LBV/AD brains compared with AD controls. Furthermore, *SNCA*-mRNA expression level in the TC was associated with rs3857059; homozygotes for the minor allele showed significant higher expression. *LRRK2*-transcript levels were increased in carriers of rs1491923 minor allele. Our findings demonstrated that *SNCA* contributes to LB pathology in AD patients, possibly via interaction with *LRRK2*, and suggested that expression regulation of these genes may be the molecular basis underlying the observed LB associations.

INTRODUCTION

Synucleinopathies are a group of neurodegenerative disorders that share a common pathological lesion composed of protein inclusions in the cytoplasm of selective populations of neurons and glia, known as Lewy bodies (LBs) and glial cytoplasmic inclusions (GCIs), respectively (1–4). Synucleinopathy disorders include Parkinson's disease (PD, MIM 168600), dementia with LBs (DLB, MIM 127750), Lewy bodies variant of Alzheimer disease (LBV/AD), pure autonomic failure (PAF, MIM 146500) and multiple system atrophy (MSA, MIM 146500).

Aggregates of the insoluble alpha-synuclein protein encoded by the *SNCA* gene are the major component of LBs (5). Among

synucleinopathies, PD has been studied most extensively, and *SNCA* has been identified as a highly significant genetic risk factor for idiopathic PD across multiple populations in genome-wide association studies (GWAs) and candidate gene association studies (6–18,19). Unequivocal association between *SNCA* SNPs and increased risk for MSA was also reported (20,21). However, the genetic etiology of LB pathology and the broader spectrum of synucleinopathies are largely unclear. In addition, there is accumulating evidence, which suggests that elevated levels of wild-type alpha-synuclein, resulted from triplication and duplication of the *SNCA* locus, lead to neuronal dysfunction and are sufficient to cause the Mendelian form of PD, with disease onset age inversely correlated with *SNCA*

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dose (22–28). Furthermore, elevated levels of *SNCA* mRNA have been reported in midbrain tissues (29) and in individual substantia nigra dopaminergic neurons from sporadic PD post-mortem brains compared with controls (30). In transgenic mice, overexpression of human alpha-synuclein leads to the formation of neuronal aggregates reminiscent of LBs (31). Collectively, these observations demonstrate the importance of *SNCA* overexpression in PD etiology; however, the broader impact to other synucleinopathies is largely unknown.

Mutations in Leucine-rich repeat kinase 2 gene (*LRRK2*) are the most common genetic cause of PD (32,33), accounting for 4% of familial PD and 1% of sporadic PD across all populations (34). In addition, GWAs found that common genetic variants in *LRRK2* are associated with an increased risk for sporadic PD (13,14). Thus, there is a strong support for the genetic role of both *SNCA* and *LRRK2* in the pathogenesis of PD. However, the interplay of *SNCA* and *LRRK2* genes and its effect on predisposition of PD, and more generally, their roles in other synucleinopathies remain uncovered and are currently the focus of an intense investigation.

Herein, we aim to study the molecular basis that leads to LB pathology in Alzheimer disease. About 15–20% of demented patients with AD also have cortical and subcortical LBs (35,36). It has been suggested that AD subjects with LB comprise a distinct subset referred to as LBV/AD (37). Therefore, our control cohort consisted of pathologically confirmed AD with no evidence of LB in postmortem examinations. We investigated the roles of *SNCA* and *LRRK2* in LBV/AD by performing (1) a case–control association in an autopsy series of cases with LBV/AD compared with controls with AD only and (2) mRNA expression analyses of the two genes in subset groups of pathologically defined VLB/AD cases and AD controls.

RESULTS

Association of *SNCA* with LBV/AD

The *SNCA* locus (>110 kb) is divided into two major linkage disequilibrium (LD) blocks (HapMap) (13,14): (1) the 5' block contains the promoter and enhancer regions and extends to include the 5' sequence of intron 4; (2) the 3' block contains the 3' sequences of intron 4 and extends downstream of the gene. Figure 1 presents the LD structure of *SNCA* gene as determined by the analysis of our study cohort [all Caucasians, *N* = 511 (Table 1)]. In this study, we evaluated the role of *SNCA* in the risk to develop LB pathology in AD patients. Thus, for the purpose of this study, we defined cases as autopsy-confirmed LBs presentation co-occurring with AD pathology, and controls as confirmed AD only upon postmortem examination (i.e. free of LBs). The association between *SNCA* and the presence of LB in AD was tested with six tagging SNPs positioned across the *SNCA* locus (Fig. 1 and Table 2) and encompassing the two major, previously reported, LD blocks. The analyzed tagging SNPs were reported previously to be significantly associated with increased PD risk in GWAs and candidate gene-based studies (10,11,17–19). The genetic association tests were performed using a neuropathologically well-characterized Caucasian sample (*N*_{AD} = 404, *N*_{LBV/AD} = 107, Table 1). All models were adjusted for age, sex and Braak stage. Two SNPs showed significant associations for increased risk for LB

Figure 1. Linkage disequilibrium structure of the region of *SNCA* gene. Genotypes of six SNPs from the Caucasians sample set of this study (*N* = 511) were used to determine LD (*r*²) within the *SNCA* locus using HaploView software. *r*² values are shown within cells. Black cells, strong LD; shaded gray cells, intermediate; white cells, weak LD. The upper scheme represents the corresponding *SNCA* gene structure. Black line, non-coding regions (introns and intergenic sequences); narrow boxes, UTRs; wide boxes, translated exons. The 5' and 3' orientations of *SNCA* gene are also indicated.

Table 1. Demographic and neuropathology description

	AD	LBV/AD
Total no.	404	107
White %	100	100
Male %	34.9	43.9
Age at death (mean ± S.E.M)	78.2 ± 7.7	78.1 ± 6.5
B&B ≥ IV % ^a	88.1	81.3
CERAD score = severe % ^b	100	100
McKeith LB score ≥ moderate % ^c	NA	65.4

^aBraak and Braak stage (38); ^bConsortium to Establish a Registry for Alzheimer's Disease score (39,40); ^cLB pathology score based on McKeith (41). LB, Lewy body; AD, Alzheimer's disease; LBV/AD, Lewy body variant of Alzheimer's disease.

pathology (Table 3). SNP rs3857059 positioned at the 3' LD block of *SNCA* was significantly associated with LB pathology in AD brains (*P* = 0.01, OR = 1.92, 95% CI = 1.16–3.18). The minor allele G conferred increased risk to the development of LBs. At the 5' *SNCA* LD block, SNP rs2583988 showed a marginally significant association with LBV/AD (*P* = 0.05, OR = 0.7, 95% CI = 0.49–1.01). SNP rs2619363 that is in high LD with rs2583988 (*r*² = 0.93, Fig. 1) demonstrated suggestive evidence of association (OR = 0.74, 95% CI = 0.52–1.06) although

Table 2. The analyzed SNPs within SNCA region

rs number	^a Map position (bp)	Distance from previous	SNP type	Allele (minor/major)	^b HW (<i>P</i>)
rs2583988	Chr4:90979851		Upstream	T/C	1
rs2619363	Chr4:90978070	1781	Upstream	T/G	0.645
rs2736990	Chr4:90897564	80506	Intron4	G/A	0.4061
rs3857059	Chr4:90894261	3303	Intron4	G/A	0.9911
rs356165	Chr4:90865909	392	3'UTR	G/A	0.4312
rs356219	Chr4:90856624	9285	Downstream	G/A	0.8434

^aMap position based on dbSNP b126 build; ^b*P*-values for Hardy–Weinberg Equilibrium (*N* = 511).

Table 3. Association of SNCA SNPs with LB pathology in AD

rs number	MAF case, control	Risk associated allele	LBV/AD (<i>P</i>)	OR	95% CI
rs2583988	0.22, 0.29	C	0.05	0.70	0.49–1.01
rs2619363	0.23, 0.29	G	0.10	0.74	0.52–1.06
rs2736990	0.44, 0.47	A	0.56	0.91	0.67–1.24
rs3857059	0.107, 0.063	G	0.01	1.92	1.16–3.18
rs356165	0.36, 0.37	A	0.95	0.99	0.72–1.35
rs356219	0.37, 0.38	A	0.75	1.05	0.77–1.43

LBV/AD, Lewy body variant of Alzheimer’s disease; MAF, the minor allele frequency; Bold *P* value, indicates significant association; *N* = 511.

Table 4. Association of SNCA SNPs with LB pathology in AD stratified by LRRK2

rs number	LBV/AD (<i>P</i>)	OR	95% CI
rs2583988	0.009	0.46	0.26–0.83
rs2619363	0.01	0.51	0.29–0.89
rs3857059	0.006	2.61	1.31–5.20

Number of subjects in the subset group stratified by rs1491923 minor allele carriers, *N* = 252.
LBV/AD, Lewy body variant of Alzheimer’s disease.

did not reach statistical significance (*P* = 0.1). The minor alleles of these 5’ LD tagging SNPs demonstrated a protective effect from LB pathology. After correction for multiple testing, only SNP rs3857059 remained significant (*P* = 0.04).

The effect of LRRK2 on the association of SNCA with LBV/AD

In order to understand whether the observed associations between polymorphisms in the SNCA region and LB pathology are depended on the LRRK2 genotype, we tested for the interaction of the LB-associated SNCA SNPs with LRRK2 genotype. We conducted further analyses with these three SNCA SNPs (Table 4) stratified by LRRK2-rs1491923 genotype. SNP rs1491923 is located 0.17 Mb upstream of LRRK2 and is used in PD GWAs to tag LRRK2 and demonstrated the strongest significant association with PD risk (13,14). The minor allele C was reported as the ‘risk’ allele for PD. The results indicated significant associations among the carriers of the minor allele with the genotype LRRK2-C/+, i.e. combined groups of homozygotes of the minor allele and compound heterozygotes

Table 5. Brain samples for expression analyses: demographic description

	AD	LBV/AD
Total no.	36	67
White %	100	100
Male %	44.4	49.3
Age at death (mean ± S.E.M)	77.6 ± 8.2	78.0 ± 6.1
PMI hr. (mean ± S.E.M)	9.2 ± 6.9	11.4 ± 8.5

PMI, postmortem interval; AD, Alzheimer’s disease; LBV/AD, Lewy body variant of Alzheimer’s disease.

(Table 4). Associations of the dataset stratified by LRRK2 were significant for rs3857059 (*P* = 0.006, OR = 2.61, 95% CI = 1.31–5.20), rs2583988 (*P* = 0.009, OR = 0.46, 95% CI = 0.26–0.83) and rs2619363 (*P* = 0.01, OR = 0.51, 95% CI = 0.29–0.89). After correction for multiple testing, all three SNPs remained significant.

The effect of LB pathology on SNCA- and LRRK2-mRNAs expression

SNCA- and LRRK2-mRNA fold levels [SNCA/(ENO2, SYP); LRRK2/(ENO2, SYP)] were measured in 67 temporal cortex (TC) samples obtained from LBV/AD cases and in 36 TC tissues from AD subjects. All brain tissue donors were Caucasian. Table 5 summarizes the demographics of the tissue donors that represent a subset group of the entire study samples.

We first tested for associations with confounding factors that might affect RNA levels.

Expression levels for SNCA increased with Braak and Braak AD stage, but this effect was not statistically significant (*P* = 0.1), and we did not detect significant associations of SNCA-mRNA levels with age, sex or postmortem interval (PMI) (data not shown). LRRK2-mRNA expression levels were affected by gender; females demonstrated significantly higher levels of LRRK2 transcripts compared with males (*P* = 0.02). We did not observe associations of LRRK2-mRNA levels with age, Braak and Braak, or PMI (data not shown). All of the subsequent analyses were adjusted for age, sex, Braak and Braak and PMI.

Next, we assessed the effect of LB pathology state on the expression of SNCA and LRRK2 genes. SNCA-mRNA levels were significantly increased in TC of LBV/AD-cases compared with AD control (*P* = 0.0002; Fig. 2A). The mean SNCA-mRNA level was ~25% elevated in samples with LB pathology.

Figure 2. The effect of LB pathology on *SNCA*- and *LRRK2*-mRNAs expression levels in human brain tissues. The study cohort consisted of LBV/AD and AD TC tissues from Caucasian donors. Fold levels of human *SNCA*-mRNA (**A**) and *LRRK2*-mRNA (**B**) in TC were assayed by real-time RT-PCR using TaqMan technology and calculated relative to the geometric mean of *SYP*- and *ENO2* mRNAs reference control using the $2^{-\Delta\Delta C_t}$ method (i.e. results presented are relative to a specific brain RNA sample). The values presented here are mean levels $\pm$ SE adjusted for age, gender, PMI and Braak and Braak stage.

LRRK2-mRNA levels were significantly higher, ~30% in TC from LBV/AD brains compared with AD controls ($P = 0.02$) (Fig. 2B).

The *cis*-genetic effect on *SNCA*- and *LRRK2*-mRNA levels

For analysis of the *cis*-genetic effect on mRNAs expression, LBV/AD and AD cases were pooled and the models were adjusted for disease status as well. We analyzed the effect of the two SNPs that were significantly associated with LBV/AD risk on *SNCA* transcript levels. *SNCA* SNP rs3857059 was associated with *SNCA*-mRNA expression levels in the TC ($n = 103$, $P = 0.003$). Homozygotes for the minor allele 'G' demonstrated the highest mean *SNCA*-mRNA levels (Fig. 3). We did not detect any effect of SNP rs2583988 on *SNCA* transcript levels ($P = 0.83$).

Analysis of the *LRRK2* expression in TC ($n = 98$) showed a trend towards association with rs1491923, but these results were not statistically significant ($P = 0.15$, Fig. 4). The genetic association for LBV/AD risk was stratified by pooling carriers of the minor rs1491923 allele; thus, we next compare *LRRK2*-mRNA expression of that combined group to the homozygotes of the major allele group. The observed difference was marginally

Figure 3. The effect of rs3857059 genotypes on *SNCA*-mRNA expression levels in human brain tissues. The study cohort consisted of TC tissues from Caucasian donors. The subjects were genotyped for rs3857059. Fold levels of human *SNCA*-mRNA in TC from LBV/AD and AD donors were assayed by real-time RT-PCR using TaqMan technology and calculated relative to the geometric mean of *SYP*- and *ENO2* mRNAs reference control using the $2^{-\Delta\Delta C_t}$ method (i.e. results presented are relative to a specific brain RNA sample). General Linear Model (GLM) analysis showed that SNP rs3857059 was significantly associated with *SNCA*-mRNA expression levels ($P = 0.003$). The values presented here are mean levels $\pm$ SE adjusted for age, gender, PMI and Braak and Braak stage.

Figure 4. The effect of rs1491923 genotypes on *LRRK2*-mRNA expression levels in human brain tissues. The study cohort consisted of TC tissues from Caucasian donors. The subjects were genotyped for rs1491923. Fold levels of human *LRRK2*-mRNA in TC from LBV/AD and AD donors were assayed by real-time RT-PCR using TaqMan technology and calculated relative to the geometric mean of *SYP*- and *ENO2* mRNAs reference control using the $2^{-\Delta\Delta C_t}$ method (i.e. results presented are relative to a specific brain RNA sample). General Linear Model analysis showed that carriers of rs1491923 minor allele (CC and TC) had significantly higher expression levels of *LRRK2*-mRNA compared with homozygotes of the major allele (TT) ($P = 0.05$). The values presented here are mean levels $\pm$ SE adjusted for age, gender, PMI and Braak and Braak stage.

significant ($P = 0.05$); carriers of the minor allele (CC and TC) had higher average expression levels of *LRRK2*-mRNA than homozygotes of the major allele (TT) (Fig. 4).

DISCUSSION

This is the first study to assess the role of the *SNCA* gene in pathologically proven LBV/AD samples. Our control group consisted of autopsy-confirmed AD cases that showed no evidence for LB; thus, we directly evaluated the risk to develop LB pathology.

We detected significant associations for LB pathology with two SNPs rs3857059 and rs2583988 positioned in *SNCA* 3' and 5' LD blocks, respectively. Interestingly, SNP rs3857059 was also associated with *SNCA*-mRNA levels in our study sample, suggesting that this SNP may regulate, or tag another SNP that regulates, *SNCA* steady-state transcript level. This data provided support to the concept that *SNCA* overexpression may be the molecular mechanism leading to the formation of LB.

Among synucleinopathies, PD has been studied most extensively, and *SNCA* role in PD has been established in many studies. *SNCA* has been identified as a highly significant genetic risk factor for idiopathic PD across multiple populations in GWAs and candidate gene association studies (6–19). There is accumulating evidence, which suggests that elevated levels of wild-type *SNCA* lead to neuronal dysfunction and are sufficient to cause the Mendelian form of PD, with disease onset age inversely correlated with *SNCA* dose (22–28). Furthermore, elevated levels of *SNCA* mRNA have been reported in midbrain tissues (29) and in individual substantia nigra dopaminergic neurons from sporadic PD postmortem brains compared with controls (30). Collectively, these observations demonstrate the importance of *SNCA* overexpression in PD etiology. We demonstrated significant association of *SNCA* variants with increased risk to develop LBV/AD. We also showed that *SNCA* transcript levels were significantly elevated in LBV/AD brains compared with AD brains. In addition, the LBV/AD-associated *SNCA* SNP was associated with increased *SNCA* expression. Collectively, our data provided support for the contribution of *SNCA* gene to another LB disorder, the LBV/AD. Furthermore, our results suggested that overexpression of *SNCA* is the underlying molecular mechanism of the observed genetic association.

SNCA and *LRRK2* are the major genes involved in PD. Mutations in *SNCA* are the most potent cause of autosomal dominant PD (42,43) whereas mutations in *LRRK2* gene are the most common cause (32,33). In addition, common genetic variants in *SNCA* and *LRRK2* were associated with an increased risk for sporadic PD and reported to have the strongest significant signals of association (13,14). Recent reports show association between *SNCA* SNPs and increased risk for MSA (20,21), providing support for a common genetic etiology between MSA and PD. It was also suggested that variants in all three members of the synuclein gene family influence the risk to develop DLB (44). Other investigations suggested the involvement of glucocerebrosidase gene (*GBA*) in both PD and DLB (45,46). However, overall little is known about the genetic susceptibility of other synucleinopathies and whether they share common molecular genetic mechanisms. Here, we extended the molecular genetics knowledge to an additional synucleinopathy disorder, which has not been sufficiently studied LBV/AD. Our data provided support for *SNCA* contribution to LBV/AD and hence established the broader genetic role of *SNCA* gene in another synucleinopathy disorder. Moreover, we showed that *SNCA* associations with LB pathology in AD are stronger when stratified by *LRRK2* genotype, suggesting that *LRRK2* may also play a role in susceptibility to LBV/AD. Overall these results suggested a common genetic etiology between LBV/AD and PD that warrant further investigation in other synucleinopathies.

There are some reports that suggested a functional link between *SNCA* and *LRRK2* proteins. The fact that *SNCA* protein is heavily phosphorylated in LBs and that *LRRK2* is

a protein kinase suggests that a link, not necessarily direct, exists between the two. *SNCA* deposited in LBs is highly phosphorylated at serine 129 (47,48), and phosphorylated proteins seem to be more prone to aggregation *in vitro* (47), suggesting that abnormally high levels of phosphorylated proteins may trigger the neurodegenerative process. It was reported that recombinant *SNCA* is directly phosphorylated by cell lysates overexpressing *LRRK2* (49), suggesting that *LRRK2* is the kinase that mediates phosphorylation of *SNCA*. Another study by this group (50) successfully co-immunoprecipitated *LRRK2* and *SNCA* from pathological tissue of diffuse LB (DLB) cases or from HEK293 cells upon exposure to oxidative stress. Their data implied that the two proteins localize upon stress to the same cellular compartment where they participate in a common biological process and *LRRK2* kinase activity might, directly or indirectly, influence *SNCA* phosphorylation state. However, there is no evidence that *LRRK2* causes increased *SNCA* phosphorylation in cell or animal systems. Thus, further investigations using pathological brain tissue from *LRRK2* cases to show increased levels of phosphorylated *SNCA* are needed to support this hypothesis. We demonstrated significantly higher levels of *LRRK2* in brains with LB pathology (LBV/AD) compared with brains with no evidence for LB presence (AD). Our observation indirectly supports this hypothesis; we suggest that increased *LRRK2* expression resulted in greater phosphorylation activity and may lead to increased levels of phosphorylated *SNCA* and, therefore, enhanced aggregation and LB formation. Moreover, we showed that *LRRK2*-rs1491923C/+ genotype is associated with higher *LRRK2* expression and stratification by that genotypes also strengthen the association of *SNCA* with LB pathology. Collectively, these evidences support our hypothesis and suggest that an increase in *LRRK2* expression accelerated the effect of *SNCA* overexpression on risk of LB pathology. Different mechanisms for the functional link of *SNCA* and *LRRK2*, other than phosphorylation, were also proposed (51).

Our study examined associations of SNPs with gene expression in *cis* (Figs 3 and 4). Our limitation was a relatively small cohort ($n = 103$; and homozygotes for the minor alleles were rare $n = 3, 11$). Therefore, P -values should be interpreted with caution. After Bonferroni adjustment for two hypotheses (two variants), the association of *SNCA* expression with variant rs3857059 remained significant ($P = 0.006$). The association of *LRRK2* expression was tested with a single SNP but using two models (additive and dominant) and after corrections became non-significant. Nevertheless, the results of this study suggest an association between expression of *SNCA* and *LRRK2* and specific variants in *cis* that warrants further investigation using larger cohorts and in follow-up functional studies.

LBV/AD shows both overlaps and differences with AD, and it has been suggested that AD subjects with LB comprise a distinct subset referred to as LBV/AD (52). At present, however, the nosological position of LBV/AD within the broad spectrum of AD and PD remains disputable. The more general question, whether PD, PDD and DLB are different disorders or represent a single entity with distinct clinical subtypes, recently has been discussed controversially (4). The current study demonstrated genetic and molecular genetic similarities between LBV/AD and PD and provides a model for future investigations that are needed to address this enigma.

MATERIALS AND METHODS

Study samples

The study cohort consisted of individuals with two autopsy-confirmed neuropathological diagnoses: (1) LB variant of AD cases, i.e. LB co-occurred with AD pathology (LBV/AD, $N = 107$) and (2) LB-free AD (AD, $N = 404$) as controls for the genetic association analyses. Demographics and Neuropathology for these samples are shown in Table 1. Neuropathology phenotypes were determined in postmortem examination following widely used and well-established methods. Two rating systems were used as a quantitative phenotype to confirm AD: (i) the severity degree of the intraneuronal neurofibrillary-tau pathology was scored using Braak and Braak (38) stage I–VI; (ii) neuritic plaques were scored according to Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Criteria (39,40). The extent of plaque pathology was recorded post-mortem and received scores of absent, mild, moderate and severe. Neuropathology of LB was identified following the method and clinical practice recommendations of McKeith and colleagues (41,53). The overall severity degree of the LB pathology (based on the examination of several affected brain regions) received scores of mild, moderate, severe and very severe.

DNA and genotyping

Genomic DNA extracted from brain tissues were obtained through the Kathleen Price Bryan Brain Bank (KPBBB) at Duke University. Genotype determination of each single-nucleotide polymorphism (SNP) was performed by allelic discrimination using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). Each genomic DNA sample (20 ng) was amplified using TaqMan Universal PCR master mix reagent (Applied Biosystems) combined with the specific TaqMan SNP genotyping assay mix corresponding to the genotyped SNP. The assays were carried out using the ABI 7900HT and the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles: 15 s at 95°C, and 1 min at 60°C. Genotype determination was performed automatically using the SDS version 2.2 Enterprise Edition Software Suite (Applied Biosystems). All genotypes were tested for Hardy–Weinberg Equilibrium (Table 2).

Brain samples for expression analyses

Temporal (TC) cortexes, from neuropathologies of LB variant of AD (LBV/AD: $n = 67$) and LB-free AD (AD: $n = 36$), were obtained through the KPBBB at Duke University. All PMIs were <24 h. Demographics for these samples are included in Table 3. All brain samples were collected from neuropathologically characterized cadavers who had confirmed diagnosis of AD at postmortem examination. The presence or absence of LB was also determined in neuropathological postmortem examination as described earlier.

RNA extraction and cDNA synthesis

Total RNA was extracted from brain samples (100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by

purification with an RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA concentration was determined spectrophotometrically at 260 nm, whereas the quality of the purification was determined by 260 nm/280 nm ratio that showed values between 1.9 and 2.1, indicating high RNA quality. Additionally, quality of sample and lack of significant degradation products was confirmed on an Agilent Bioanalyzer. The RNA Integrity Number measurements were >7 validating the RNA quality control. Next, cDNA was synthesized using MultiScribe RT enzyme (Applied Biosystems) under the following conditions: 10 min at 25°C and 120 min at 37°C.

Real-time PCR

Real-time PCR was used to quantify the levels of human *SNCA* mRNA (29,54–56). Briefly, duplicates of each sample were assayed by relative quantitative real-time PCR using the ABI 7900HT to determine the level of *SNCA* message in brain tissues relative to mRNAs encoding the human neuronal proteins Enolase 2 (*ENO2*) and Synaptophysin (*SYP*). ABI MGB probe and primer set assays were used to amplify *SNCA* cDNA (ID Hs00240906_m1, 62 bp) and the two RNA reference controls, *ENO2* (ID Hs00157360_m1, 77 bp) and *SYP* (ID Hs00300531_m1, 63 bp) (Applied Biosystems). Each cDNA (10 ng) was amplified in duplicate in at least two independent runs (overall ≥ 4 repeats), using TaqMan Universal PCR master mix reagent (Applied Biosystems) and the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles: 15 s at 95°C, and 1 min at 60°C. As a negative control for the specificity of the amplification, we used RNA control samples that were not converted to cDNA (no-RT) and no-cDNA/RNA samples (no-template) in each plate. No amplification product was detected in control reactions. Data were analyzed with a threshold set in the linear range of amplification. The cycle number at which any particular sample crossed that threshold (C_t) was then used to determine fold difference, whereas the geometric mean of the two control genes served as a reference for normalization. Fold difference was calculated as $2^{-\Delta\Delta C_t}$ (57); $\Delta C_t = [C_t(SNCA) - C_t(\text{reference})]$. $\Delta\Delta C_t = [\Delta C_t(\text{sample})] - [\Delta C_t(\text{calibrator})]$. The calibrator was a particular brain RNA sample used repeatedly in each plate for normalization within and across runs. The variation of the ΔC_t values among the calibrator replicates was <10%.

For assay validation, we generated standard curves for *SNCA* and each reference assay, *ENO2* and *SYP* using different amounts of human brain total RNA (0.1–100 ng). In addition, the slope of the relative efficiency plot for *SNCA* with each internal control (*ENO2* or *SYP*) was determined to validate the assays. The slope in the relative efficiency plot for *SNCA* and the reference genes was <0.1, showing a standard value required for the validation of the relative quantitative method. The described method and analysis were also used to quantify the levels of human *LRRK2*-mRNA (ID Hs00968209_m1, 144 bp).

Statistical analysis

All analyses were carried out using SAS statistical software, Version 9.3 (SAS Institutes, Cary, NC, USA). Statistical analyses were performed to assess association between each genetic variant and LB pathology. *SNCA* genotypes were coded with 0,

1 or 2 copies of the minor allele. Multivariable logistic regression analysis of the association between *SNCA* genotypes and LB phenotype was carried out using PROC LOGISTIC controlling for study age and sex. To test for interactions of each *SNCA* polymorphism with *LRRK2* genotype, we conducted further analyses stratified by *LRRK2*-rs1491923. Linkage disequilibrium within the *SNCA* genomic region was determined using Haploview software (Version 4.2) (58).

Expression levels of *SNCA*-mRNA and *LRRK2*-mRNA of each sample were measured in replicate, and the results were averaged. The mean expression of a group of samples was reported as mean $\pm$ SE. We assessed the associations of the expression traits (*SNCA*- or *LRRK2*-mRNA) with: (1) LB pathology and (2) genotypes of the corresponding gene, using the Generalized Linear Models procedure (PROC GLM). A log transformation (log2) was performed on all mRNA levels to assure normal distribution (59). An additive genetic model was used, and genotypes were coded with 0, 1 or 2 copies of the minor allele. A genetic dominant model in which homozygous and heterozygous genotypes of the minor allele were pooled was also used for *LRRK2* analysis. All models were adjusted for sex, age, PMI and Braak and Braak stage. Correction for multiple testing employed the Bonferroni method.

ACKNOWLEDGEMENTS

We thank the Kathleen Price Bryan Brain Bank (KPBBB) at Duke University funded by NIA AG028377 for providing us with the brain tissues.

Conflict of Interest statement. None declared.

FUNDING

This work was supported in part by the Ellison Medical Foundation New Scholar award (AG-NS-0441-08 to O.C.) and the National Institutes of Health/National Institute of Neurological Disorders and Stroke (NIH/NINDS) (R01 NS085011 to O.C.).

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