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

Colton Linnertz1, Michael W. Lutz1,2, John F. Ervin1, Jawara Allen4, Natalie R. Miller4, Kathleen A. Welsh-Bohmer2, Allen D. Roses1,2,3 and Ornit Chiba-Falek1,2,4,*

1Department of Neurology, Duke University Medical Center, Durham, NC 27710, USA, 2Joseph and Kathleen Bryan Alzheimer’s Disease Research Center, Duke University Medical Center, Durham, NC 27710, USA, 3Zinfandel Pharmaceuticals, Chapel Hill, NC, USA and 4Institute for Genome Sciences and Policy, Duke University Medical Center, Durham, NC 27708, USA

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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 genomewide 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.
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). 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 their 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 VLDBV/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 genome-based studies (10,11,17–19). The genetic association tests were performed using a neuropsychologically well-characterized Caucasian sample (NAD = 404, NLBV/AD = 107, Table 1). All models were adjusted for age, sex and Braak stage. Two SNPs showed significant associations for increased risk for LB 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 (r2 = 0.93, Fig. 1) demonstrated suggestive evidence of association (OR = 0.74, 95% CI = 0.52–1.06) although...
We conducted further analyses with these three SNPs stratified by *LRRK2* in PD GWAs to tag rs3857059 remained significant (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 *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 dependent 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 was 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 carriers of the minor allele with the genotype *LRRK2*-C+/+. i.e., combined groups of homozygotes of the minor allele and compound heterozygotes did not reach statistical significance (*P* = 0.1). The minor alleles of these LD tagging SNPs demonstrated a protective effect from LB pathology. After correction for multiple testing, only SNP rs3857059 remained significant (*P* = 0.04).
LRRK2-mRNA levels were significantly higher, \( \approx 30\% \) 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 \((n = 103 \quad P = 0.003)\). 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 \((P = 0.15, \text{Fig. 4})\). 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 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 postmortem and received scores of absent, mild, moderate and severe. Neurpathology 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 (Ct) 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 \( 2^{-\Delta\Delta Ct} \) (57); \( \Delta Ct = [\text{Ct(SNCA)}] - \text{Ct (reference)} \). \( \Delta\Delta Ct = [\Delta Ct\text{(sample)}] - [\Delta Ct\text{(calibrator)}] \). The calibrator was a particular brain RNA sample used repeatedly in each plate for normalization within and across runs. The variation of \( \Delta Ct \) 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-rs1419123. 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 ± 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.

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Conflict of Interest statement. None declared.

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