Neurexin 3 transmembrane and soluble isoform expression and splicing haplotype are associated with neuron inflammasome and Alzheimer’s disease

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Abstract

Background: Synaptic damage precedes neuron death in Alzheimer’s disease (AD). Neurexins, NRXN1, NRXN2, and NRXN3, are presynaptic adhesion molecules that specify neuron synapses and regulate neurotransmitter release. Neurexins and postsynaptic neuroligins interact with amyloid beta oligomer (AβO) deposits in damaged synapses. NRXN3 gene variants have been associated with autism, addiction, and schizophrenia, however, not fully investigated in Alzheimer’s disease. In the present study, we investigated an AD association of a 3′-splicing allele of rs8019381 that produces altered expression of transmembrane or soluble NRXN3 isoforms.

Methods: We carried out RT-PCR (reverse transcription polymerase chain reaction), PCR-RFLP (PCR and restriction fragment length polymorphism), Sanger sequencing, and in situ hybridization (ISH) assays for NRXN3 neuron expression and genotyping. Genetic associations were analyzed by $\chi^2$ tests, and ISH signals were analyzed by FISH v1.0 module of Indica Labs HALO software.

Results: We previously identified a functional haplotype in the 3′ region of neurexin 3 (NRXN3) gene that alters the expression ratios between NRXN3 transmembrane and soluble isoforms. In this study, we found that expression and ratio of transmembrane and soluble NRXN3 isoforms were reduced in AD postmortem brains and inversely correlated with inflammasome component NLRP3 in AD brain regions. The splicing haplotype related to the transmembrane and soluble NRXN3 expression was associated with AD samples with $P = 6.3 \times 10^{-5}$ (odds ratio = 2.48) and interacted with APOE genotypes.

Conclusions: We found that the SNP rs8019381 of NRXN3 that is located adjacent to splicing site #5 (SS#5) interacts with the APOE ε4 haplotype and alters NRXN3 transmembrane or soluble isoform expression in AD postmortem cortex. Dysregulation of presynaptic NRXN3 expression and splicing might increase neuron inflammation in AD brain.

Keywords: Alzheimer’s disease, Neurexins, Endocannabinoids, Apolipoprotein E, Alternative splicing

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Background

Non-familial and late-onset Alzheimer's disease (AD) is a common cause of dementia in the elderly. Emphases on classical AD neuropathological features, Aβ neuritic plaques (Aβ-NPs), neurofibrillary tangles (NFTs), and neuropil threads are increasingly acknowledged to be accompanied by disrupted synaptic contacts and impaired glutamatergic neurotransmission [1,2]. While the ε4 allele of apolipoprotein E (APOE) gene makes a large contribution to the genetic bases of interindividual differences in vulnerability to AD, the sizable genetic influences that remain after accounting for APOE are likely to arise from polygenic and/or rarer variants that each makes modest contributions to overall disease vulnerability.

Diffuse Aβ fibrillar plaques are often observed in post-mortem human brains with normal cognitive function [3–6]. Pathological Aβ plaque formation around synapses with AβO deposit correlates with memory loss and synapse dysfunction [7,8]. Neurexins were discovered as α-latrotoxin (venom of black widow spider) receptors [9] and function as presynaptic cell adhesion molecules [10] that help to regulate the release of neurotransmitters, specify, and stabilize classical synapses, including the glutamatergic synapses that provide a focus for research in AD [1,2]. Neurexin genes are among the largest genes (greater than one million base pairs) in the human genome, and the three mammalian neurexin genes NRXN1, NRXN2, and NRXN3 each display differential splicing events that provide thousands of neurexin isoforms on a background of longer α-neurexin and shorter β-neurexin that arise from the use of alternative promoters [11]. The larger α-neurexins contain three EGF-like (epidermal growth factor) domains each of which flanked by two LNS (lamin-neurexin-sex hormone-binding globulin) domains, a single transmembrane domain, and intracellular PDZ (PSD95-Dlg1-Zo1) domain that interact with intrasynaptic proteins [10,12]. Specifically, α-neurexins are coupled to presynaptic calcium channels to regulate neurotransmitter release [13] and interact with postsynaptic neureligins, leucine-rich repeat transmembrane proteins (LRRTMs), calsynatin (CLSTN), α-dystroglycan (DAG1), GABAA-receptors (GABARAs), latrophilins (ADGRLs), cerebellin (CBLN)-glutamate dehydrogenase (GLUD) complexes, synaptic cleft secreted neurexophilins (NXPHs), and intracellular PDZ-binding proteins [14,15]. Neurexins’ intracellular PDZ domains can bind to MINT1, MINT2, and CASK proteins [16,17] that themselves bind to and stabilize the transmembrane form of amyloid precursor protein (APP) [18]. MINT1 and MINT2 are adaptor proteins that complex with conserved motifs in APPs C-terminal region to stabilize APP transmembrane forms and reduce secretion of pathogenic Aβ cleavage products [19]. It is thus even possible that NRXNs-MINTs interaction complexes could alter APP protein processing. Soluble or secreted α-NRXN3 is produced by including extra exon 23 with four different intra-exonal spliced sites that encode four premature stop codons that abolishes the transmembrane and intracellular PDZ domains [20,21]. The smaller β-neurexin contains one LNS domain (no EGF domain), a transmembrane domain, and an intracellular PDZ domain. The β-neurexin acts as a brake for endocannabinoid 2-AG (2-arachidonylglycerol) synthesis that retrogradely regulates presynaptic cannabinoid receptor 1 (CB1R)-mediated depolarization-induced suppression of excitation on AMPA and NMDA receptors that are involved in excitatory postsynaptic currents (EPSCs) [22]. APP cleavage enzymes of α- and γ-secretases can process β-NRXN3 into an N-terminal extracellular domain (80 kDa) and a C-terminal intracellular domain (12 kDa). The enzymatic activities are altered by several single nucleotide polymorphisms (SNPs) of γ-secretase subunit presenilin 1 (PSEN1) that contribute to early-onset forms of familial AD [23]. Recent data identify roles of neurexin isoforms in several complex neuropsychiatric phenotypes that include autism [24–26], addiction [20,27,28], and schizophrenia [29,30].

NRXN3 mRNA is the second most reduced gene after vacuolar H⁺-ATPase subunit gene ATP6V1E1 in AD hippocampus identified by bioinformatic analysis of AD and aging Gene Expression Omnibus (GEO) databases [31,32]. We have identified 3’ region of NRXN3 haplotypes that are tagged by alleles of the SNP rs8019381, which is located near the end of NRXN3’s exon 23 at a key splicing site [20]. Alleles of rs8019381 tagged NRXN3 produce the splice variants that include or exclude exon 23 coding for a single transmembrane domain; thus, transmembrane or soluble NRXN3 isoforms are transcribed and translated, respectively [20]. NRXN3 is expressed in neurons in brain regions that are implicated in mnemonic processes and in dementia-associated AD pathologies. For example, NRXN3 is expressed in the cerebral cortex and in the hippocampus that contains AD-related senile plaques and neurofibrillary tangles [33,34]. Differences in the properties of synapses in these regions could alter brain connectivity, and the altered ratio of transmembrane and soluble NRXN3 isoforms could lead to pathological Aβ accumulation at synapses.

We have thus characterized the patterns of expression of total NRXN3, α-NRXN3 and β-NRXN3, and four NRXN3 transmembrane and soluble splice variants in mRNAs extracted from postmortem middle frontal gyrus from pathologically confirmed AD and control individuals. We have sought AD-related differences in frequencies of NRXN3 haplotypes and tested whether the NRXN3 associations are dependent on the APOE genotype. Finally, we have found evidence for NRXN3 association and interaction with APOE genotypes in previously
reported genome-wide association datasets and for NRXN3 inverse correlation with inflammasome component NLRP3 in neurons of the AD hippocampus and cortex. Taken together, these data support contributions for common human NRXN3 haplotypes and altered NRNX3 transmembrane and soluble isoform expression in AD brain.

Methods

Subjects: human samples

Middle frontal gyrus postmortem brain samples of 121 European-American AD (38 men and 83 women with mean age 80.3 ± 9.6) were obtained from the Division of Neuropathology, the Department of Pathology, the Johns Hopkins University School of Medicine (JHUSOMI), whose diagnoses were all confirmed by autopsy [35]. Additional European-American comparison groups comprising 349 subjects were examined to document the distribution of NRXN3 polymorphism in the general American Caucasian population of the USA. One hundred sixty samples (107 men and 53 women with mean age 36.1 ± 16.2) were obtained from Maryland Brain Bank (UMD) whose geographical location is close to that of AD subject collection. One hundred eighty-nine unrelated subjects (42 men and 147 women with mean age 51.2 ± 14.9) were selected from pedigrees from the Collaborative Study on the Genetics of Alcoholism (COGA) [36]. We explored mRNA levels of NRXN3 isoforms in middle frontal gyrus postmortem samples from 58 pathologically confirmed AD subjects and 48 control subjects. We also explored the association between this NRXN3 haplotype and AD with 121 pathologically confirmed AD subjects and 349 control subjects.

RNA isolation and cDNA synthesis

Total RNA was extracted from the larger sized middle frontal gyrus samples available from 58 of the autopsy-confirmed European-American AD patients and 48 normal individuals dying without neurological disease (40 females/18 males, mean age ± SD; 81.2 ± 10.0, PMI; 11.4 ± 6.4 from JHMI and 11 females/37 males, mean age ± SD; 47.6 ± 19.7, PMI; 11.9 ± 6.0 from JHMI and UMD) using Trizol (Thermo Fisher, Waltham, MA) protocol. Single-strand cDNA was synthesized from total RNA using SuperScript™ III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). Electrophoresis of all RNAs used for quantitative PCR revealed sharp 18S and 28S ribosomal RNA bands; four samples with evidence for RNA degradation were eliminated on this basis.

Quantitative RT-PCR

For quantitative real-time PCR assessments of NRXN3 mRNAs, isoform-specific primers and minor groove-binding (MGB) TaqMan probes were designed using Primer Express Software (Table 1), common NRXN3 TaqMan probe (Hs01028186_m1), and endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Vic-labeled) which were ordered from Thermo Fisher (Cat#4326317E, Thermo Fisher Scientific, Waltham, MA, USA). The relative fold change is calculated using the formula $2^{\left(-\frac{\Delta\Delta C_t}{\Delta C_t}\right)}$.

Genetic testing

Genomic DNA was extracted from blood or tissue samples from the individuals noted in “human samples” above. DNA from most AD and control samples was extracted from the middle frontal gyrus brain tissues using Qiagen genomic DNA kits. Genomic DNA for other subjects was extracted from peripheral leukocytes as previously described [37]. APOE polymorphisms were genotyped using PCR-RFLP assays as described [38]. The rs8019381 SNP was genotyped by direct Sanger sequencing as described [20].

RNAscope in situ hybridization (ISH)

Human postmortem hippocampus (1 control sample of Braak 0 and 3 AD samples of Braak 6) and middle temporal gyrus (2 control samples of each Braak 0, 1, and 2 and 2 AD samples of each Braak 4, 5, and 6) were used for triplex fluorescent ISH. Human RNAscope ISH probes were ordered from Advanced Cell Diagnostics Inc. (ACD, Hayward, CA, USA) for NRXN3 in C2 channel (20 ZZ pairs targeted region 1095–2035 of NM_001105250.2; Cat No. 525431-C2), NLRP3 in C1 channel (30 ZZ pairs targeted region 1095–2035 of NM_001105250.2; Cat No. 525431-C2), NLRP3 in C1 channel (30 ZZ pairs targeted region 1095–2035 of NM_001105250.2; Cat No. 525431-C2).

| Table 1 | Real-time PCR primers and MGB Fam-TaqMan probes for NRXN3 isoforms |
|---------|--------------------------------------------------|
| NRXN3   | Forward primers | Reverse primers | MGB probes |
| Hs01028186_m1 NRXN3 TaqMan gene expression assay | TGAATCCCATCTGCTGAAAG | AAGGTTGCAACAGTAAGCAATAG | CCGAGTGACCGAGT |
| ex 22a24a TGACTTTGTTTCATCTCGTCAGATG | TGGTCAGTACCCCTCAGCA | CCGAGTGACCGAGT |
| ex 22a24b TGACTTTGTTTCATCTGCTGAAATG | CCGGAAACCGCTGATTT | CCGAGTGACCGAGT |
| ex 22a24c TGACTTTGTTTCATCTGCTGAAATG | CCCGGAACCGCTGATTT | CCGAGTGACCGAGT |
| ex 22a23a AGATGATCTGTTTTCATCTGCTGAA | CCGGAGTGATCTACCCCTGATTAGA | CCGAGTGACCGAGT |
| ex a1-2 GACATACACAGACGATCCCAAATCTTTC | TCAATGCGGCGGCAAGAA | AACTGGAAAGGTCTTTTC |
| ex β1-18 TTTTCTCTTCTCTTC | GCCACCACCTTCTCCAAA | AGACAGCGTCGCG |
targeted region 2627–4008 of NM_004895.4; Cat No. 478021), and NELIN/RBFOX3 in C3 channel (20 ZZ pairs target region 720–2217 of NM_001082575.2; Cat No. 415591-C3). The positive control probes (Cat No. 320868) were POLR2A (C1 channel), PPIB (C2 channel), and UBC (C3 channel). The negative control probe was bacterium (Bacillus subtilis) gene Dapb (Cat No. 320871). The cryostat sectioning of postmortem human brain samples, fixation, protease pretreatment, probe hybridization, pre-amplification, amplification, horseradish peroxidase reaction, and fluorescent labeling steps were described previously [39]. Zeiss LSM 880 confocal microscope was used to image fluorescent labeling. Amplification × 20 images (two to three images for each brain sample) were analyzed by FISH v1.0 module included in HALO software with RNAscope ISH setting (Indica Labs, Corrales, NM, USA). The H-score \[\Sigma_{\text{bin0-4}} \times \text{percentage of cells per bin}\] were used to calculate mRNA expression for each probe based on the minimum intensity threshold (a value between 0 and 400).

### Results

#### α-NRXN3 and β-NRXN3 mRNA expression in AD middle frontal gyrus

We compared expression of α-NRXN3 and β-NRXN3 in mRNAs extracted from the middle frontal cortices of human postmortem brain samples of AD and controls with different “splicing site 5 (SS#5)” NRXN3 haplotypes defined by alleles of the rs8019381 SNP. Since controls displayed few rs8019381 T alleles, we compared control samples with CC genotypes to AD samples with CC genotype and to AD samples with either one or two T alleles (CT/TT). There were no significant differences between expression of α-NRXN3 mRNA in CC controls vs AD patients with either CC or CT/TT genotypes (Fig. 1a; two-tailed Mann-Whitney \(P = 0.067\) and \(P = 0.127\), respectively). By contrast, when compared with control individuals with CC genotypes, β-NRXN3 mRNA expression levels decreased by 30% and 48% in AD patients with CC and CT/TT genotypes, respectively (Fig. 1b; \(P = 0.0004\) and \(P < 0.0001\), respectively). We identified a mild significant correlation between age and either α-NRXN3 or β-NRXN3 mRNA expression levels in the combined groups (Fig. 2a; \(P = 0.0473\), Spearman \(r = 0.193\) and \(P = 0.0061\), Spearman \(r = 0.2648\), respectively). However, we identified neither a trend nor a significant correlation between age and either α-NRXN3 or β-NRXN3 mRNA expression levels in the control group (Fig. 2b; \(P = 0.787\), Spearman \(r = 0.040\) and \(P = 0.386\), Spearman \(r = 0.128\), respectively). We also did not identify significant correlations between α-NRXN3 and β-NRXN3 mRNA expression levels in the AD group and age (Fig. 2c; \(P = 0.253\), Spearman \(r = 0.153\) and \(P = 0.186\), Spearman \(r = 0.176\), respectively). In these AD samples, the positive slope of this regression line does indicate trends toward less NRXN3 expression in older individuals with AD phenotype. Neither gender nor postmortem intervals were correlated with these expression levels in control or AD groups (data not shown). In the AD subjects, there were no differences in expression of either α-NRXN3 (\(P = 0.751\)) nor β-NRXN3 isoforms (\(P = 0.863\)) in individuals with haplotypes marked by CC vs CT/TT genotypes.

### Genetic analysis

Table 2 shows the genotype distribution and allele frequency of rs8019381 SNP for AD and control groups. The genotype distributions differed remarkably between the AD and control groups (\(\chi^2 = 15.587, \text{df} = 2, P = 0.000413\)). The minor allele frequency of the rs8019381 T allele was significantly greater for the AD group than for the control group (0.157:0.070, respectively). We also did not identify significant correlations between α-NRXN3 and β-NRXN3 mRNA expression levels in the AD group and age (Fig. 2c; \(P = 0.253\), Spearman \(r = 0.153\) and \(P = 0.186\), Spearman \(r = 0.176\), respectively). In these AD samples, the positive slope of this regression line does indicate trends toward less NRXN3 expression in older individuals with AD phenotype. Neither gender nor postmortem intervals were correlated with these expression levels in control or AD groups (data not shown). In the AD subjects, there were no differences in expression of either α-NRXN3 (\(P = 0.751\)) nor β-NRXN3 isoforms (\(P = 0.863\)) in individuals with haplotypes marked by CC vs CT/TT genotypes.

### Statistical analysis

Genetic associations were analyzed by \(\chi^2\) tests. Deviations from Hardy-Weinberg equilibrium (HWE) were examined by \(\chi^2\) test with \(P < 0.05\) as a deviation from HWE. Correction for multiple testing was not applied because of the a priori reason to focus on rs8019381 in this study. Power analyses used the program PS v2.1.31 [40]. Comparison of the ages in AD between the rs8019381 SNP genotype groups was analyzed using ANOVA. Logistic regression analysis was also applied using phenotype as the dependent variable, and the age, gender, APOE ε4 allele, and rs8019381 genotypes as the independent variables. Statistical analyses of mRNA expression RT-PCR and ISH data were performed using PRISM (GraphPad Software, CA, USA) software. Differences in the mRNA expression levels based on phenotype (control vs AD or genotype CC vs CT and TT) were examined using two-tailed Mann-Whitney tests. Two-way ANOVA and two-tailed/unpaired Student’s \(t\) test using H-scores of ISH intensities were tested for any significant differences between NRXN3 and NLRP3 expressions in different Braak stages of MTG and HIP samples. Linear regression of H-scores of each ISH probe was used to fit straight lines through control and AD data sets with different Braak staining stages and statistically calculated for any significant differences. \(P < 0.05\) was considered significant for comparisons of expression levels. Spearman’s rank correlation coefficient analyses were used to assess the contributions of age, sex, and postmortem interval to the mRNA expression levels of each splice variant.
rs8019381 SNP, the current samples yielded the power of 0.894 for detecting nominally significant results. There was no significant effect of age on the distribution of rs8019381 genotypes in the AD group (P = 0.562 by one-way ANOVA). The rs8019381 genotype distributions also displayed no significant deviation from Hardy-Weinberg equilibrium in either the AD or control groups (data not shown).

We next investigated the APOE genotypes in these samples and sought possible interactions with the effects of the NRXN3 haplotypes marked by the rs8019381 SNP (Table 2). As expected, the APOE genotype and allele frequency distributions of the AD samples differed significantly from those of the control group (χ² = 87.146, df = 5, P = 2.671 × 10⁻¹⁷ and χ² = 92.374, df = 2, P = 8.735 × 10⁻²¹, respectively; Table 2).

When we sought interactions between the rs8019381 SNP and APOE genotypes in the AD group, the rs8019381 SNP and APOE genotype distributions displayed significant differences between APOE ε4 non-carriers and APOE ε4 carriers (χ² = 8.043, df = 2, P = 0.0179). Among AD individuals, rs8019381 TT homozygotes were found only in those who did not carry APOE ε4 alleles. This difference provided significance for recessive analysis (comparing CC + CT vs TT) of the differences between AD APOE ε4 non-carriers and AD APOE ε4 carriers (χ² = 6.317, df = 1, P = 0.012), though not for analyses of allele frequencies (χ² = 0.429, df = 1, P = 0.513). These results contrasted with those in control samples, where we found that neither genotype distributions nor allele frequencies were significantly different between APOE ε4 non-carriers and APOE ε4 carriers (P = 0.428 and 0.541 for genotype and allele comparisons, respectively).

Overall, significant associations between the rs8019381 genotypes and AD thus remained in both APOE ε4 non-carriers (P = 0.000403) and in APOE ε4 carriers (P = 0.00331). The allele frequencies for the rs8019381 SNP also differed significantly in AD vs control comparisons in both the APOE ε4 non-carriers (P = 0.00252) and APOE ε4 carriers (P = 0.00827). We also confirmed a significant effect of rs8019381 polymorphism on the AD phenotype considering for age, gender, and APOE ε4 allele (P = 0.00157, Table 3).

As anticipated for loci on distinct chromosomes, APOE (19q13) and NRXN3 (14q24) markers displayed evidence for independent segregation in these samples. Neither the genotype distribution nor allele frequency of rs8019381 SNP was associated with the APOE allele frequency among the AD or control groups (P AD = 0.061, P CTL = 0.850 and P AD = 0.600, P CTL = 0.283 for genotypic and allelic comparisons, respectively).

**Genetic variation and NRXN3 SS5 splice variants in AD**

Since the NRXN3 haplotype tagged by rs8019381 (Fig. 3a) has been associated with altered patterns of expression of NRXN3 splice variants that encode...
Fig. 2  

a Correlation between age and either α-NRXN3 or β-NRXN3 mRNA expression levels in the control and AD combined groups ($P = 0.0473$, Spearman $r = 0.193$ and $P = 0.0061$, Spearman $r = 0.2648$, respectively).  
b Correlation between α-NRXN3 and β-NRXN3 mRNA expression levels in the control group and age ($P = 0.787$, Spearman $r = 0.040$ and $P = 0.386$, Spearman $r = 0.128$, respectively), and  
c in the AD group and age ($P = 0.253$, Spearman $r = 0.153$ and $P = 0.186$, Spearman $r = 0.176$, respectively).  
ΔCt values of each isoform expression were obtained after normalization to Ct values of GAPDH.
transmembrane vs soluble isoforms, we evaluated the distributions of these isoforms in control brains, which were virtually all from individuals with CC haplotypes, and in frontal cortex samples of AD brains from CC, CT, and TT individuals. We have previously noted that the predominant NRXN3 transmembrane isoforms that arise from alternative splicing at SS#5 are exon 22a-24b, exon 22a-24a, while the predominant soluble isoform comes from exon 22a-23a. We thus assessed the levels of these four isoforms (Fig. 3b).

The most prominent result of these assays, as with studies of total NRXN3, α-NRXN3, and β-NRXN3 mRNA levels, was the reduced expression that was found for most of the isoforms in the AD postmortem middle frontal gyrus (MFG), middle temporal gyrus (MTG), and hippocampus (HIP) (Fig. 3c). In comparison with one-half reduction of total NRXN3 expression in the AD cortex, the exon 22a-24b variant that encodes the major transmembrane isoform was expressed at levels that were decreased, by 85% in AD subjects with either CC or CC/TT genotypes \((P < 0.0001)\) by two-tailed Mann-Whitney tests for both comparisons (Fig. 4b). Levels of the exon 22a-24c variant, the second major transmembrane isoform were also decreased by 56% and 66% in AD patients with CC and with CC/TT genotypes when compared with control CC individuals (Fig. 4c; \(P < 0.0001\) by two-tailed Mann-Whitney test for both comparisons). Overall differences in mRNA expression levels for these two transmembrane isoforms between CC and CT/TT genotypes were not detected among the AD patients \((P = 0.611 \text{ and } 0.476)\) by two-tailed Mann-Whitney tests, respectively.

The major soluble NRXN3 isoform, encoded by the exon 22a-23a variant mRNA was also decreased by 63% and 71% in AD patients with CC and CC/TT genotypes when compared with those in control individuals with CC genotypes (Fig. 4d; \(P < 0.0001\) by two-tailed Mann-Whitney test for both comparisons). Differences in mRNA expression levels between CC and CT/TT genotypes were not detected among the AD brains \((P = 0.455)\) by two-tailed Mann-Whitney test.

Closer examination revealed evidence for interactions between the clinical phenotype, the AD susceptible rs8019381 T allele, and expression of the exon 22a-24a mRNA that encodes a major transmembrane isoform (Fig. 4a). Expression of exon 22a-24a mRNA was decreased by 46% in AD patients with CT/TT genotypes when compared with those in control individuals with CC genotypes \((P = 0.0002)\) by two-tailed Mann-Whitney tests). Within the AD group, exon 22a-24a mRNA expression levels were also decreased by 46% in AD patients with CT/TT genotypes when compared with those with CC genotypes \((P = 0.043)\) by two-tailed Mann-Whitney tests). By contrast, exon 22a-24a mRNA expression levels did not differ significantly between control and AD samples with CC genotypes \((P = 0.180)\) by two-tailed Mann-Whitney test). These differences correlated with differences in the ratios between transmembrane and soluble isoform expression in CC vs CT/TT AD individuals, and the ratio differences reached the margin of statistical significance (Fig. 5). In AD patients, the ratios of transmembrane vs soluble

### Table 2 Distribution of the NRXN3 rs8019381 C/T SNP and APOE allele frequencies among the rs8019381 genotypes

| Group          | Genotype* | Allele frequency | \(P\) value |
|----------------|-----------|------------------|-------------|
|                | CC        | CT               | TT          | C           | T           |
| Control (\(n = 336\)) | 291 (0.866) | 43 (0.128) | 2 (0.006) | 0.930 | 0.070 |
| AD (\(n = 121\))     | 86 (0.711)  | 32 (0.264) | 3 (0.025) | 0.843 | 0.157 |

rs8019381 genotypes among APOE e4 non-carriers and APOE e4 carriers

| Group          | APOE e4 carriers | Allele frequency | \(P\) value |
|----------------|------------------|------------------|-------------|
| Control        | 216 (0.857)      | 35 (0.139) | 1 (0.004) | 0.927 | 0.073 |
| AD             | 75 (0.893)       | 8 (0.095)  | 1 (0.012) | 0.940 | 0.060 |

rs8019381: Significant differences were found between the AD and the controls in either the genotype distribution \((\chi^2 = 15.587, df = 2, P = 0.000413)\) or the allele frequencies \((\chi^2 = 15.997, df = 1, P = 0.0000634)\).

### Table 3 Logistic regression analysis of rs8019381 C/T SNP on the AD phenotype considering for age, gender, and APOE e4 allele

| Variable        | Parameter | Standard error | Wald \(\chi^2\) | \(P\) value |
|-----------------|-----------|----------------|----------------|-------------|
| Intercept       | -13.917   | 1.528          | -9.107         | < 0.0001    |
| Age             | 0.179     | 0.020          | 8.750          | < 0.0001    |
| Gender          | -0.051    | 0.416          | -0.123         | 0.902       |
| APOE e4 allele* | 1.691     | 0.03268658     | 4.194          | 0.000033    |
| rs8019381 genotype** | 3.078 | 0.068 | 3.181 | 0.00157 |

*For APOE e4 allele analyses, e4 non-carriers were coded as 0 and APOE e4 carriers were coded as 1

**For rs8019381 genotype analyses, each SNP was coded as 0 for major allele homozygotes, 0.5 for heterozygotes, and 1 for minor allele homozygotes
isoforms were 25% greater in CT/TT than in CC subjects ($P = 0.053$ by two-tailed Mann-Whitney test and $P = 0.044$ by unpaired $t$ test) despite that the overall ratio of the transmembrane and soluble isoforms decreased in AD brains. Interestingly, these ratios did not differ between AD patients with CT/TT genotypes vs those in control CC individuals ($P = 0.331$ by two-tailed Mann-Whitney tests).

**Inverse correlation of NRXN3 with inflammasome component NLRP3 in AD brains**

We carried out an ultra-sensitive RNAscope ISH assay to study the altered NRXN3 expression at cellular levels in control and AD postmortem brain samples that were co-hybridized and co-stained with inflammasome component NLRP3 [41] and neuron marker NEUN/RBFOX3. All NRXN3 and the majority of NLRP3 signals were

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**Fig. 3** a) The approximate location of rs8019381 and splicing patterns at the SS#5 of the NRXN3 gene. The number of potential variants for the splicing site is 30. Exon 24 is the last exon of the gene that codes for the transmembrane region, cytoplasmic domain, and contains 3′UTR. Because any exon 23 isoforms include in-frame stop codons (depicted as asterisks), if it is inserted, soluble α-NRXN3 and β-NRXN3 can be produced from this gene. b) The three major transmembrane isoforms and one major soluble isoform are investigated in the prefrontal cortex in individuals with AD ($n = 58$) and controls ($n = 48$). Each probe for the RT-PCR was designed across both exons. c) Expression levels of total NRXN3 mRNA in the middle frontal gyrus (MTG), middle temporal gyrus (MTG), and hippocampus (HIP).
co-localized with NEUN/RBFOX3-positive neurons (Fig. 6). We found that the reduced NRXN3 mRNA was inversely correlated with the increased NLRP3 mRNA in NEUN/RBFOX3-positive neurons of the AD middle temporal gyrus (Fig. 6a–f) and hippocampus (Fig. 6g, h) samples. Two-tailed and unpaired Student’s t test using H-scores that represent NRXN3 neuron expression was significantly higher than that of NLRP3 at Braak 2 stage of MTG and significantly lower at Braak 6 stage in MTG and HIP (Fig. 7a). Two-way ANOVA analysis found significant differences of H-scores in HIP ($F_{1,14} = 6.07$; $P = 0.0273$) but not in MTG ($F_{1,28} = 0.45$; $P = 0.4515$) with different Braak stages; however, the interaction of NRXN3 and NLRP3 neuron expression in MTG and HIP at different Braak stages were very significant ($P < 0.0001$). Linear regression analysis using H-score for each Braak number (0, 1, 2, 4, 5, 6) found that the differences of slopes of NRXN3 and NLRP3 were significant ($F_{1,8} = 11.49$; $P = 0.0095$) in MTG samples. NLRP3 regression slope was significantly non-zero ($F_{1,4} = 18.32$; $P = 0.0128$), and NRXN3 regression slope was not significantly non-zero ($F_{1,4} = 0.98$; $P = 0.3773$). The correlation was also simulated in control (Braak = 0) and AD (Braak = 6) in HIP samples. The linear regression lines for NRXN3 and

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**Fig. 4 a–c** Expression levels of mRNAs encoding NRXN3 transmembrane isoforms (exon 22a-24a, exon 22a-24b, and exon 22a-24c variants, respectively) and d NRXN3 soluble isoform (exon 22a-23a variant) in the middle frontal gyrus of control individuals ($n = 44$ individuals with rs8019381 CC genotype) and AD patients ($n = 35$ individuals with rs8019381 CC genotype and $n = 23$ individuals with CT or TT genotypes). Relative levels of each mRNA expression were obtained after normalization to GAPDH. Then, percentile change is obtained respective to an average of β-NRXN3 mRNA in control individuals with CC genotype. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$, calculated using two-tailed Mann-Whitney tests.
PTM (posttranslational modifications), such as APP genes and their altered expression/splicing/translation/translation/nRXN3 component —— inversely correlated with that of inflammasome component. NRXN3 APOE gene haplotype interacts with nRXN3 and NLRP3 expression trajectories might serve as early diagnosis and therapeutic targets at early Braak 2—3 stages. NRXN3 SNP rs8019381 was found to contribute to AD susceptibility. There is no information available for rs8019381 in previously reported genome-wide association studies for AD [3, 55, 56] because of the relatively small haplotype block (14 kb) in the study. While no genomic markers that display strong linkage disequilibrium with rs8019381 are identified in the Translational Genomic Research Institute (TGen) datasets [56], we identified rs2067730 that lies about 6 kb 5′ to rs8019381 in genome-wide association studies of clinically diagnosed AD vs control subjects of European ancestries who were recruited from Canadian memory clinics [55]. Interestingly, like NRXN3 rs801938, rs2067730 displays association with AD in this sample and appears to interact with the APOE genotype (P = 0.027). The magnitude of rs8019381 association suggests an odds ratio of 2.48, with a broad 95% confidence interval that encompasses 1.6—3.9. While this effect is much less than the large, oligogenic influence of APOE haplotypes on AD vulnerability, it is larger than many of the effects of other proposed polygenic variants listed in systematic meta-analysis presented on the AlzGene database [57] or in two genome-wide association data-sets that compare AD vs control samples [3, 55, 56]. The effects of the NRXN3 haplotype may be even larger in individuals with specific APOE haplotypes. Both the current dataset and data reported by Li et al [55] provide evidence for significant interactions among APOE haplotypes and 3′ NRXN3 haplotypes in AD.

The reduced expression of total NRXN3, α-NRXN3, and β-NRXN3 in samples of the cerebral cortex and hippocampus from pathologically confirmed AD and control brains formed the initial basis for implicating NRXN3 in AD. These findings were accompanied by a significant reduction of ratios between transmembrane and soluble isoforms in AD individuals with rs8019381 CT or TT genotypes. These observations support the hypothesis that reduction of NRXN3 transmembrane isoform alters synapse homeostasis, reduces neurotransmitter release, and promotes Aβ oligomerization and APOE dysfunction in synaptic degeneration [7, 58]. Alternatively, the altered ratio might differentially interact with alternatively spliced isoforms of APP, causing increased Aβ production [59].

**Discussion**

We found that NRXN3 gene haplotype interacts with the APOE ε4 haplotype, and the expression and ratio of its transmembrane and soluble isoforms were reduced in AD postmortem MFG. NRXN3 mRNA level was inversely correlated with that of inflammasome component NLRP3 in MTG AD neurons. The linear regression of NRXN3 and NLRP3 signals that intersected at Braak 2.1 for HIP and Braak 2.6 for MTG might indicate differential progression of Aβ fibrils in different brain regions. Previous studies by array tomography and electron microscopy find that AβO forms halo at synapses that attracts Aβ fibrils around damaged neurites [42–44]. The most prominent AD-associated susceptible genes and their altered expression/splicing/translation/PTM (posttranslational modifications), such as APP and PSEN1 [23, 45], APOE and APOER2 [46], PTK2B [47], PPP3CA and PPP3R1 [48, 49], and PIN1 [50], are involved in synaptic homeostasis. Dysregulation of presynaptic NRXN3 might be an early event that triggers synaptic calcium dyshomeostasis and let AβO invasion at synapses. Subsequent dystrophic neurites and dysfunctional synapses stimulate NLRP3/caspase-1 and calcineurin/caspase-3 pathways that activate interleukin-1β and interleukin-18 [51, 52] and cause mitochondria impairment and apoptosis [53], respectively. Aβ fibrils are at their peak when AD symptom just appears [54], and NRXN3 and NLRP3 expression trajectories might serve as early diagnosis and therapeutic targets at early Braak 2—3 stages.

**Fig. 5** Ratios of expression levels in total transmembrane isoforms to soluble isoform. In AD groups, ratios of transmembrane to soluble isoform show a significant difference between individuals with CC genotype and those with CT or TT genotypes. The ratios were increased by 25% in AD patients with CT or TT genotypes compared with AD with CC genotype (*P = 0.053 by two-tailed Mann-Whitney test and P = 0.044 by unpaired t test).
Fig. 6 a–h RNAscope in situ hybridization of control and AD brain samples with different Braak numbers. Green represents NLRP3, yellow NRXN3, and magenta NEUN. The red arrow indicates colocalization of three probes in the same cell. H-score correlations of NRXN3 and NLRP3 intensities with Braak numbers. (a) MTG-Braak 0; (b) MTG-Braak 1; (c) MTG-Braak 2; (d) MTG-Braak 4; (e) MTG-Braak 5; (f) MTG-Braak 6; (g) HIP-Braak 0; (h) HIP-Braak 6.
The associated NRXN3 SNP rs8019381 is located at the junction of exon 23’s splicing donor site (23 base pairs downstream from the 3′ of exon 23), within a region that might alter splicing efficiency. The NRXN3 haplotype studied here is likely to be different from other ethnic samples. The rs8019381 “T” allele frequency (0.07) of the control samples reported here (based on genotypes from 672 chromosomes) is similar to values obtained in unselected Europeans and European-Americans available (0.094 based on genotypes from 224 chromosomes) from dbSNP. African Yoruban rs8019381 frequency (0.198) is (based on genotypes of 180 chromosomes) higher than European population, and East Asian rs8019381 frequency is much lower than European and African populations, with none of the “T” allele detected in Chinese (based on genotypes of 90 chromosomes) and 0.006 detected in Japanese (based on genotypes of 172 chromosomes) HapMap samples. Additional studies will be necessary to identify more informative NRXN3 rs8019381 for use in individuals with non-European heritage. Much of the evidence presented here provides an increased focus on the role of synaptic pathology in AD. Synapse losses can be documented with the first clear-cut evidence for dementia that are accompanied by synaptic toxicities conferred by APP [60] and APOE mutations [61]. The evidence in the current report suggests that Aβ and APOE synaptic pathologies are likely to interact with allele-specific alterations in gene expression of NRXN3 transmembrane and soluble isoforms.

An astronomical number of synapses derived from about 86 billion human brain neurons [62] are dynamic

![Fig. 7](image-url)
throughout the human life span, and their damage precedes neuron death due to Aβ oligomer (AβO) toxicity in AD [18, 63]. Substantial microscopic Aβ plaques are observed in old adult brains with intact cognition function [6]; however, nano-synaptic-space distribution of AβO is less known and neurexin complexes are known partners of Aβ [8]. Complex neurexin alternative splicing codes define synaptic specificity, strength, plasticity [15, 31], and vulnerability toward AβO [64]. The trans-synaptic anterograde and retrograde signaling of the neurexin-neuroligin-endocannabinoid system [13, 31, 65, 66] provides an attractive pathway for AD therapeutic development. Modulation of presynaptic and postsynaptic endocannabinoid tone through CB1R [67] and CB2R [68, 69], respectively, by their specific ligands might reduce neuron inflammasome and shift neurexin-neuroligin alternative splicing repertoire toward heathy synapses and reverse cognitive decline during aging [70, 71]. Neurexin peptides are significantly elevated in cerebrospinal fluid (CSF) of individuals with mild cognitive impairment (MCI), especially in patients with MCI progressing to AD dementia [72, 73]. Conceptually, targeted tryptic peptide panels of specific neurexin isoforms will improve CSF early diagnosis for pre-symptomatic AD. Screening of effective neurexin and cannabinoid receptor ligands [66, 74] and behavioral modulation of mental activities and nutrition intakes [75] might help to improve synaptic health and prevent cognitive decline 10 years or more before AD symptom appearance.

Conclusion

NRXN3 rs8019381 SNP located at SS#5 splicing site was found to contribute to AD susceptibility and interact with the APOE ε4 haplotype. The altered expressions of NRXN3 transmembrane and soluble isoforms were further reduced in susceptible rs8019381 heterozygous and homozygous alleles (CT or TT) in the AD postmortem cortex. The reduced NRXN3 expression was inversely correlated with the increase of inflammasome component NLRP3 expression in NELIN/RBFOX3-positive neurons in the AD brain.

Abbreviations

AD: Alzheimer’s disease; Aβ: Amyloid beta; AβO: Amyloid beta oligomer; CSF: Cerebral spinal fluid; HIP: Hippocampus; HWE: Hardy-Weinberg equilibrium; LNS: Lamin-neurexin-sex hormone-binding globulin domains; MCI: Mild cognitive impairment; MFG: Middle frontal gyrus; MTG: Middle temporal gyrus; NFT: Neurofibrillary tangles; PCR-RFLP: PCR and restriction fragment length polymorphism; PDZ: PSD95-Dlg1-ZO1 domains; PMI: Postmortem interval; PTM: Posttranslational modification; RT-PCR: Reverse transcription polymerase chain reaction; SNP: Single nucleotide polymorphism; SS#5: Splicing site 5

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Availability of data and materials

Data available on request from the author.

Authors’ contributions

QL, AH, and JME were responsible for the conception and design of the study. AH and QRL carried out the experiments and were responsible for the analysis, interpretation of the data, and drafting of the manuscript. DIL performed in the cryostat sectioning of the postmortem brain samples and RNAscope in situ hybridization. OP and JCT provided the postmortem AD and control brain samples with Braak staining stages and gave invaluable advice for the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments on the postmortem brain tissues were approved by John Hopkins Medicine Institutional Review Board (https://www.hopkinsmedicine.org/institutional_review_board/index.html).

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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