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# Genetic Study of Neurexin and Neuroligin Genes in Alzheimer's Disease

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**Abstract**. The interaction between neurexins and neuroligins promotes the formation of functional synaptic structures. Recently, it has been reported that neurexins and neuroligins are proteolytically processed by presenilins at synapses. Based on this interaction and the role of presenilins in familial Alzheimer's disease (AD), we hypothesized that dysfunction of the neuroligin-neurexin pathway might be associated with AD. To explore this hypothesis, we carried out a meta-analysis of five genome-wide association studies (GWAS) comprising 1, 256 SNPs in the *NRXN1*, *NRXN2, NRXN3,* and *NLGN1* genes (3,009 cases and 3,006 control individuals). We identified a marker in the *NRXN3* gene (rs17757879) that showed a consistent protective effect in all GWAS, however, the statistical significance obtained did not resist multiple testing corrections (OR = 0.851, $p = 0.002$). Nonetheless, gender analysis revealed that this effect was restricted to males. A combined meta-analysis of the former five GWAS together with a replication Spanish sample consisting of 1,785 cases and 1,634 controls confirmed this observation (rs17757879, OR = 0.742, 95% CI = 0.632–0.872, $p = 0.00028$, final meta-analysis). We conclude that *NRXN3* might have a role in susceptibility to AD in males.

Keywords: Alzheimer's disease, genetics, genome-wide association study, meta-analysis, neurexins, neuroligins, *NRXN3*

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[1]Some data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.ucla.edu/). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.ucla.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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## INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative human pathology. It is considered a complex disorder that renders synaptic and memory defects and a progressive neuronal loss in the brain, causing a devastating cognitive phenotype.

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As in other complex diseases, genetic factors play a role in AD etiology. In fact, a common allele near the *APOE* transcript is almost universally associated with non-Mendelian AD [1]. Recently, other genetic markers have been consistently associated with AD in genome-wide association studies (GWAS), which are located near or within the following genes: *CLU*, *PICALM*, *CR1*, *BIN1*, *MS4A*, *CD2AP*, *ABCA7*, *EPHA1*, and *CD33* [2, 3]. (For more information, visit http://www.alzgene.org/ [4]).

On the other hand, the majority of familial cases of AD (FAD) are caused by mutations in *APP* [5], *PSEN1* [6], and *PSEN2* genes [7]. *PSEN1* and *PSEN2* genes encode presenilin 1 and 2 respectively. Presenilins (PS) are the catalytic component of γ-secretase, a protease complex that cleaves a number of transmembrane proteins, including the amyloid-β precursor protein (AβPP). Accumulation of the amyloid-β (Aβ) peptide is a hallmark of AD and most FAD-linked PS mutations affect Aβ generation [8]. In animal models, the loss of PS function results in synaptic plasticity defects and memory impairment through an unknown synaptic mechanism independent of Aβ accumulation [9]. In addition, it has also been reported that PS regulates neurotransmitter release during synaptic transmission [10]. Taken together, these observations indicate that defects in synaptic function may be associated with the memory impairment observed in AD.

Neurexins (NRXN) and neuroligins (NLGN) are synaptic cell-adhesion molecules important for synapse function [11–13]. At the synapse, NLGN are localized postsynaptically and interact with presynaptic NRXN [14]. Recently, a functional link between PS function and NRXN has been shown. NRXN are proteolytically processed by PS in neurons and, interestingly, FAD-linked PS mutations affect the normal processing of NRXN [15]. Novel findings have shown that NLGN are also processed by metaloproteases and PS at the synapse [16, 17]. NLGN and NRXN are encoded by five and three genes respectively: *NLGN1* at 3q26, *NLGN2* at 17p13, *NLGN3* at Xq13, *NLGN4X* at Xp22, and *NLGN4Y* at Yq11 for NLGN; and *NRXN1* at 2p16, *NRXN2* at 11q13, and *NRXN3* at 14q24-q31 for NRXN. Importantly, mutations in NRXN and NLGN genes have been linked to autism and other brain disorders [18–21].

Based on the molecular interaction between PS and NRXN at synapses, it has been suggested that NRXN can participate in the neuronal and memory defects associated with a loss of PS function [15]. Interestingly, the processing of neuroligin 1 can be stimulated by the proteolytic shedding of neurexins and it regulates synaptic function [16, 17]. For that reason we hypothesized that NRXN and NLGN coding genes could also have a role in the etiology of sporadic AD. To explore this possibility, we have carried out a candidate gene meta-analysis using genotypic data at autosomal NRXN and NLGN gene regions that were extracted from five different published GWAS performed in Caucasian population-based samples. One marker within the *NRXN3* gene showed a modest but uniform effect across the five GWAS studies. This result was validated in a new association study.

## MATERIALS AND METHODS

### *Datasets*

In order to maximize the power of the study, we included the following GWAS datasets in the analysis: a) The Murcia study [22]; b) The Alzheimer's Disease Neuroimaging Initiative (ADNI) study [23]; c) The GenADA study [24]; d) The NIA study [25]; and e) The TGEN study [26]. For GWAS dataset details, see Supplementary data (available online: http://www.j-alz.com/issues/35/vol35-2.html#supplementarydata05).

Datasets from ADNI, GenADA, NIA, and TGEN studies were obtained from dbGAP (http://www.ncbi.nlm.nih.gov/gap), Coriell Biorepositories (http://www.coriell.org/), or ADNI (http://adni.loni.ucla.edu/). Prior to the genetic association analysis, each dataset (Murcia, ADNI, GenADA, NIA, and TGEN) was subjected to both an extensive quality control analysis and a principal component analysis. In addition, since different platforms were used in the five GWAS analyzed, we imputed genotypes using HapMap phase 2 CEU founders ($n = 60$) as the reference panel. These approaches have been previously described [22].

After quality control and preparatory steps, the Murcia study included 1,034,239 single-nucleotide polymorphisms (SNPs) in 319 cases and 769 controls; the ADNI dataset 1,794,894 SNPs in 164 cases and 194 controls; the GenADA dataset 1,436,577 SNPs in 782 cases and 773 controls; the NIA dataset 1,738,663 SNPs in 987 cases and 802 controls; and the TGEN dataset 1,237,568 SNPs in 757 cases and 468 controls. A total of 696,707 SNPs were common to all GWAS studies.

Overall, a total of 3,009 cases and 3,006 controls were included in the meta-analysis.

*SNP selection*

To select SNPs within genomic regions of NRXN and NLGN genes, we employed the UCSC Table Browser data retrieval tool [27], release genome assembly: Mar. 2006 (NCBI36/hg18), from the UCSC Genome Browser database (http://genome.ucsc.edu/) [28]. Selected SNPs were extracted from GWAS datasets using Plink v1.06 software [29].

*Linkage disequilibrium blocks*

Linkage disequilibrium (LD) blocks were determined along the genomic regions studied using Haploview software [30] and genotyping data from the largest dataset used (NIA dataset). Haplotypes in the selected LD blocks were determined by the Haploview selected tag-SNPs.

*Association analyses*

Unadjusted single-locus allelic (1 df) association analysis within each independent GWAS sample was carried out using Plink software. We combined data from these five GWAS datasets using the meta-analysis tool in Plink selecting only those markers common to all studies. Similarly, unadjusted haplotypic (1 df) association analyses, in which each haplotype was compared to all other haplotypes, were carried out on each GWAS dataset using Plink tools. Then we combined OR (95%CI) estimates obtained for each haplotype across studies using the meta-analysis command *metan* in Stata 12 (College Station, TX). Stata was also used to obtain Forest plots.

For all, single locus, haplotypic and stratified (age, APOE, and gender) meta-analyses, fixed effects models were employed when no evidence of heterogeneity was found. Otherwise random effects models were employed.

Multiple-testing correction was applied taking into account the number of different LD blocks detected. Thus, the $p$-value threshold was established by the following formula: $p = 0.05$/number of LD regions in the meta-analysis.

To test Hardy-Weinberg equilibrium and compare genotypic frequencies between groups, we used tests adapted from Sasieni [31]. These calculations were performed using the online resource at the Institute for Human Genetics, Munich, Germany (http://ihg.gsf.de). In these studies, the $p$-value threshold was established at 0.05.

*Subjects in the validation study*

For the replication study, blood samples were collected from consecutive AD patients in medical centers in Barcelona, Madrid and Murcia (Spain). The referral center's ethics committees and Neocodex have approved the research protocol, which was in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association. Written informed consent was obtained from all individuals included in this work.

This analysis comprised 1,785 unrelated sporadic AD patients [526 males, mean (SD) age = 82.0 (7.81), and 1,259 females, mean (SD) age = 82.0 (7.81)] and 1,634 unrelated population controls with unknown cognitive status [542 males, mean (SD) age = 50 (10.9), and 1,092 females, mean (SD) age = 53.2 (12.1)] that were previously used to evaluate other SNPs associated with AD [32, 33]. Additionally, 17 cases and 131 controls from the Murcia study were included as a genotyping quality control group. Control subjects were recruited from the general population. All AD patients fulfilled DSM-IV criteria for dementia and were diagnosed according to the NINCDS-ADRDA criteria for possible and probable AD [34]. All patients received a thorough clinical and neurological examination and a comprehensive neuropsychological evaluation including tests for general cognition, memory, language, perceptual and constructional abilities, and executive functions. Complete blood analysis and neuroimaging studies were performed in all subjects to exclude other potential causes of dementia following the guidelines for the diagnosis of AD from the Study group on Behavioral Neurology and Dementia of the Spanish Neurological Society. Spanish AD patients were consecutively recruited at the three participating centers: Fundació ACE-Institut Català de Neurociències Aplicades, Barcelona; Hospital Universitario La Paz-Cantoblanco, Madrid; Hospital Virgen de la Arrixaca, Murcia and Fundación Alzheimur, Murcia. All individuals enrolled in this study were white Mediterranean with registered Spanish ancestors (two generations) as recorded by clinical researchers.

*DNA extraction and rs17757879 genotyping in the validation sample*

We obtained 5 ml of peripheral blood from all patients to isolate germline DNA from leukocytes. DNA extraction was performed automatically according to standard procedures using the Magnapure DNA

isolation system (Roche Diagnostics, Mannheim, Germany). Aliquots of DNA at a concentration of 10 ng/μl were prepared for PCR amplification.

The genotypes of the rs17757879 marker were obtained using real-time PCR in a LightCycler 480 System (Roche Diagnostics). Primers and probes employed for this genotyping protocol are summarized in Supplementary Table 1. Briefly, 10 ng of genomic DNA, 0.1 μM of forward amplification primer, 0.5 μM of reverse amplification, 0.2 μM each detection probe, and 4 μl of LC480 Genotyping Master (5X, Roche Diagnostics, Germany) were used in a 20 μl-final reaction volume. PCR conditions were as follows: an initial denaturation step of 95°C for 7 min, followed by 45 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and a final extension step of 72°C for 2 min. After amplification, specific conditions to obtain melting curves were 95°C for 30 s, 45°C for 20 s, and 75°C for 0 s (with a temperature transfer speed of 4.4°C/s except for the last step in which the temperature transfer was 0.4°C/s). A continuous fluorometric register was performed during last step. In order to confirm the genotypes selected PCR amplicons were bi-directionally sequenced using standard capillary electrophoresis techniques.

## RESULTS

### *SNP meta-analysis*

Using UCSC Table Browser data retrieval tool, we identified 6,004 SNPs within the intragenetic regions of *NGLN1*, *NLGN2*, *NLGN3*, *NLGN4X*, *NRXN1*, *NRXN2*, and *NRXN3*. Genotypes were available in all datasets for a subset of 1,256 SNPs located in *NLGN1*, *NRXN1*, *NRXN2*, and *NRXN3* genes (Table 1). The genotype data corresponding to these SNPs were extracted from each GWAS dataset using Plink tools. Then, we carried out an association analysis of these SNPs in all five case-control datasets, and a meta-analysis combining these five studies. Sixty-nine SNPs, located within *NLGN1* and *NRXN3* genes, showed a meta-analysis association $p < 0.05$. The best *p*-value (0.002) was observed for rs17757879 marker within the *NRXN3* gene. This SNP showed a modest (15% decreased risk) but remarkably consistent effect across the five studies (Table 2 and Supplementary Table 2).

Considering that the genomic regions studied included 66 LD blocks, the *p* value cut off for statistical significance was set in 7.5 × 10E-4. Consequently, our results did not remain significant after the multiple testing corrections.

### *Haplotypic analysis*

To test whether those SNPs with a low *p*-value were tagging a genomic region associated to AD we carried out a haplotypic association analysis. First, we selected the most significant SNPs ($p < 0.01$) ($n = 30$) (Supplementary Table 2). These SNPs clustered into discrete regions of *NLGN1* and *NRXN3* genes, specifically in LD blocks 2 and 9 of *NLGN1* gene and in LD blocks 8 and 16 of *NRXN3* gene (Supplementary Figure. 1). Using Haploview software and the genotype data from the NIA study, we selected the tag SNPs of those LD blocks and performed a haplotypic analysis on each independent GWAS study, as well as a meta-analysis of all of them. None of them reached the association *p* value cut off established at 7.5 × 10E-4 (Supplementary Table 3) or had a consistent effect across the five studies.

### *Genetic analysis of rs17757879*

In order to study whether the consistent effect observed for SNP rs17757879 among studies could be due to an association of this SNP with a subgroup of

Table 1
Number of SNPs described within candidate genes, number of SNPs in all GWAS included in the meta-analysis study and LD blocks defined by them

| Gene | Chromosome | Number of SNPs (Base pair position range)* | Number of SNPs in the five GWAS dataset (Base pair position range) | LD blocks |
|---|---|---|---|---|
| *NLGN1* | 3 | 1303 (174602072–175485357) | 313 (174615674–175485357) | 19 |
| *NLGN2* | 17 | 9 (7251196–7263185) | 0 | |
| *NLGN3* | 23 | 17 (70284052–70309279) | 0 | |
| *NLG4X* | 23 | 372 (5816120–6158318) | 0 | |
| *NRXN1* | 2 | 1856 (50000674–51114564) | 511 (50000674–51100386) | 21 |
| *NRXN2* | 11 | 94 (64130079–64248645) | 13 (64130080–64220661) | 1 |
| *NRXN3* | 14 | 2353 (77938599–79403317) | 419 (77952969–79403317) | 25 |

*According to UCSC genome browser (NCBI36/hg18) and dbSNP build 130.

Table 2
rs17757879 meta-analysis results in all samples and in samples stratified by gender

| | A1 | A2 | *n* | *p* | *p* (R) | OR | OR (R) | Q | I | ADNI | Murcia | GenADA | TGEN | NIA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| All | T | C | 5 | 0.002009 | 0.002009 | 0.8514 | 0.8514 | 0.9367 | 0.00 | 0.8628 | 0.8446 | 0.7960 | 0.8584 | 0.8980 |
| Males | **T** | **C** | **4** | **0.001028** | **0.001028** | **0.7339** | **0.7339** | **0.6941** | **0.00** | **0.5455** | **0.6782** | **0.7823** | **N.I.** | **0.7702** |
| Females | T | C | 4 | 0.3841 | 0.6095 | 0.9362 | 0.9501 | 0.2027 | 34.93 | 1.627 | 0.9099 | 0.8063 | N.I. | 1.004 |

A1, reference allele; A2, alternative allele; *p*, fixed-effects *p*-value; *p* (R), random-effects *p*-value; OR, fixed-effects odds ratio; OR (R), random-effects odds ratio; Q, *p*-value for heterogeneity of OR; I, effect size for heterogeneity of OR; N.I., not included. The last five columns show the OR for each study. Bold values represent the most interesting results.

Table 3
rs17757879 genotype distribution in the Spanish validation study

| Parameters | Males | | | Females | | |
|---|---|---|---|---|---|---|
| | Controls $n = 542$ | Patients $n = 526$ | Statistical | Controls $n = 1092$ | Patients $n = 1259$ | Statistical |
| rs17757879 (*CC/CT/TT*) | 389/144/9 | 406/113/7 | OR = 0.752<br>CI = 0.570–0.991<br>$p = 0.042$* | 802/267/23 | 928/302/29 | OR = 0.986<br>CI = 0.525–1.587<br>$p = 0.884$* |

OR, odds ratio; CI, confidence interval. *Allele positivity test from Sasieni [31].

patients, we first performed a meta-analysis in males and females separately. Information on the gender of participants was available for all but the TGEN study. Interestingly, we observed that the effect was only detected in males but not in females (Table 2).

In order to test if the observed associations were dependent on *APOE*, we calculated the *APOE* ε4 stratum specific estimates for these markers. We observed no evidence of effect modification by APOE (data not shown).

To check whether the consistent effect of rs17757879 observed in males across the four studies could be replicated in a new and independent genetic association study, we genotyped 526 Spanish male AD cases and 542 Spanish male controls. Additionally, we also included 131 controls and 17 patients (total 148 samples) that were previously included in the Murcia study as a genotyping quality control group (these samples were not included in the validation study). All genotypes obtained in these 148 samples matched with those obtained in the Murcia study. The genotypic distribution of the rs17757879 marker was in accordance with the Hardy-Weinberg equilibrium law (all $p > 0.39$) in both the patient and control groups (data not shown). Analysis of the genotype data showed a lower frequency of CT and TT genotypes in the patient group (OR = 0.752, $p = 0.042$) (Table 3). These results are in agreement with our previous observation in the meta-analysis.

Again, in order to confirm that the observed associations were dependent on APOE, we calculated the *APOE* ε4 stratum specific estimates for these markers. We observed no evidence of effect modification by APOE in our validation sample (data not shown). The same study was performed in different age-strata (65 years old or younger) and, similarly, no evidence of effect modification by age was observed (data not shown).

To confirm that the association was not detected in women, we also performed the same study in an available sample of 1,259 Spanish female AD cases and 1,092 Spanish female controls. The genotypic distribution of the rs17757879 marker was in the Hardy-Weinberg equilibrium (all $p > 0.90$) in both the case and control groups (data not shown). No genetic association was detected between the rs17757879 marker and AD in females (Table 3).

To maximize the statistical power and to obtain a valid summary estimate, we decided to perform a meta-analysis using all available data taking into account the genetic model observed in the validation study (CC versus CT and TT). Finally, we reached a final association $p$ value of $2.8 \times 10\text{E-}4$ for rs17757879 in males (OR = 0.742, CI = 0.632–0.872) but not in females (Fig. 1).

## DISCUSSION

Recent GWAS studies point to synaptic cell membrane processes, among others, as a new etiological pathway involved in the development of AD [35]. NRXN and NLGN are trans-synaptic cell adhesion membrane proteins involved in normal synaptic function and disease [13]. However, neither NRXN nor NLGN genes have been previously studied as candidate genes for sporadic AD. Regarding previous GWAS performed in AD, two markers within *NRXN3*

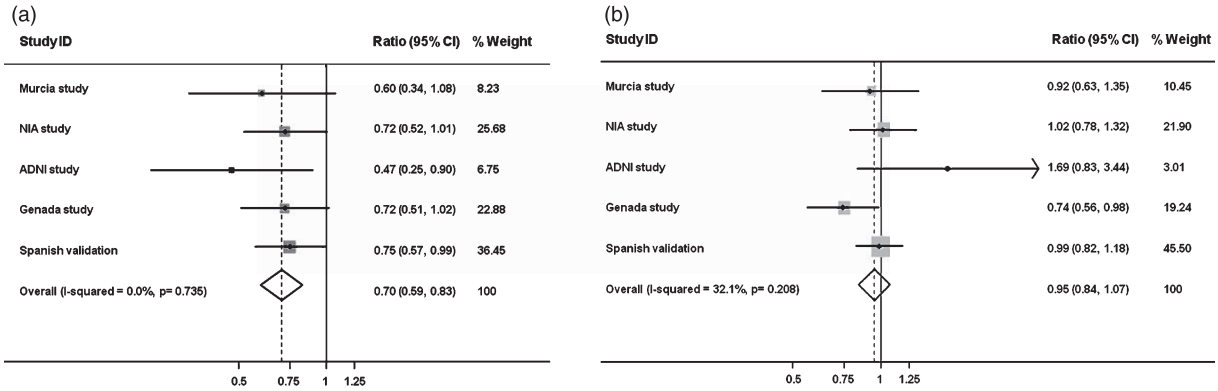


Fig. 1. Forest plot of meta-analysis including four GWAS datasets and the independent replication sample in males (a) and females (b).

were indeed listed as potentially associated in the first GWAS from the GERAD group at Stage I [36]. In their larger follow-up work [37], these SNPs no longer appear among the associated markers.

The GWAS strategy allows identifying novel genetic factors related to common diseases. However, this approach requires large samples to detect modest effects [38]. Meta-analysis of multiple GWAS followed by replication of the results in independent samples appears as an alternative strategy for overcoming this problem [39]. In spite of this, it is certainly possible that genetic variants with low effect or those showing a modest effect but just in a subgroup of patients fail to be unveiled by GWAS.

Taking into account the recent studies about the molecular interaction between PS with NRXN and NLGN [15–17, 40] and the physical interaction of NRXN with NLGN [41, 42], we decided to carry out a meta-analysis study using the five GWAS datasets as we included in a previous work by our group [22]. In order to maximize the power of the study, we selected those genetic markers in the target regions that were available at all five datasets. Thus the *NLGN2*, *NLGN3*, *NLGN4X*, and *NLGN4Y* were excluded from the analysis since they were not covered in the five GWAS.

In our first non-stratified meta-analysis we did not observed association of NRXN and NLGN with sporadic AD, in accordance with the results obtained by all GWAS performed previously. Since we have strictly limited our study to the *loci* regions of the selected genes, excluding 5′ and 3′regions, we cannot rule out the existence of genetic markers associated with sporadic AD located within the uncovered genomic regions or the NLGN genes that were not studied. In spite of this, we found an interesting and consistent effect—yet not statistically significant—of the rs17757879 marker within *NRXN3* gene across the five GWAS analyzed, suggesting that this finding was not random. Interestingly, this effect was only observed in males but not in females. The results of an independent replication sample were in line with these findings, showing that the T allele carriers of the rs1775779 marker showed a decreased risk of AD. Taking into account this genetic model, we performed a final meta-analysis, including the replication sample (OR = 0.742, CI = 0.632–0.872, $p = 0.00028$) (Fig. 1). Despite the consistency of our findings, these results did not reach a GWAS significant $p$ value ($5 \times 10$E-7). This suggests that the role of *NRXN3* in AD could be minor and, thus, the gene or its genetic markers have not been associated to AD in previous GWAS. Studies with larger samples that take into account the dimorphism observed will be necessary to clarify the role of *NRXN3* in AD susceptibility.

Morphological and functional sexual dimorphisms have been well-established in the human brain [43]. These differences are determined by steroid hormone exposure during a perinatal sensitive period that alters subsequent hormonal and non-hormonal responses throughout lifespan [44]. This sexual dimorphism might be associated with differences between men and women in the etiology, incidence, and course of brain disorders, including AD. Several pieces of evidence support this hypothesis. First, some studies have found sexual dimorphism for *ESR1* and *APOE* in AD [45, 46]. Second, the incidence of AD has been widely reported to be higher in women than in men [47]. Finally, a recent meta-analysis performed in AD patients concluded that men modestly but significantly outperform women in several cognitive domains [48], suggesting sex differences in the neuropathology of this disease. These data suggest that the stratification by gender in the GWAS analysis might be a strategy to identify new genetic variants associated to AD susceptibility.

It is not known if the NRXN3 is functionally affected by the sexual dimorphism in humans so far. Interestingly, it has been reported that Nrxn1α heterozygous KO mice showed increased locomotor activity levels in a new environment and enhanced habituation upon subsequent exposures to this environment. However, this effect was mainly observed in male mice [49]. This study shows that Nrxn1α, a member of the NRXN family, is affected by gender in mice.

In summary, our results suggest that *NRXN3* gene might have a role in AD susceptibility in males. The dimorphism observed in this study might explain why *NRXN3* has not been identified as an AD gene in previous GWAS. Further replication studies in larger population samples as well as meta-analysis of the pre-existing data will be necessary to confirm our results.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1657).

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