Independent and epistatic effects of variants in VPS10-d receptors on Alzheimer disease risk and processing of the amyloid precursor protein (APP)

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Genetic variants in the sortilin-related receptor (*SORL1*) and the sortilin-related vacuolar protein sorting 10 (VPS10) domain-containing receptor 1 (*SORCS1*) are associated with increased risk of Alzheimer's disease (AD), declining cognitive function and altered amyloid precursor protein (APP) processing. We explored whether other members of the (VPS10) domain-containing receptor protein family (the sortilin-related VPS10 domain-containing receptors 2 and 3 (*SORCS2* and *SORCS3*) and sortilin (*SORT1*)) would have similar effects either independently or together. We conducted the analyses in a large Caucasian case control data set ($n = 11\,840$ cases, $10\,931$ controls) to determine the associations between single nucleotide polymorphisms (SNPs) in all the five homologous genes and AD risk. Evidence for interactions between SNPs in the five VPS10 domain receptor family genes was determined in epistatic statistical models. We also compared expression levels of *SORCS2*, *SORCS3* and *SORT1* in AD and control brains using microarray gene expression analyses and assessed the effects of these genes on γ -secretase processing of APP. Several SNPs in *SORL1*, *SORCS1*, *SORCS2* and *SORCS3* were associated with AD. In addition, four specific linkage disequilibrium blocks in *SORCS1*, *SORCS2* and *SORCS3* showed additive epistatic effects on the risk of AD ($P \leq 0.0006$). *SORCS3*, but not *SORCS2* or *SORT1*, showed reduced expression in AD compared with control brains, but knockdown of all the three genes using short hairpin RNAs in HEK293 cells caused a significant threefold increase in APP processing (from $P < 0.001$ to $P < 0.05$). These findings indicate that in addition to *SORL1* and *SORCS1*, variants in other members of the VPS10 domain receptor family (that is, *SORCS1*, *SORCS2*, *SORCS3*) are associated with AD risk and alter APP processing. More importantly, the results indicate that variants within these genes have epistatic effects on AD risk.

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Introduction

A central event in the pathogenesis of Alzheimer's disease (AD) is the deposition of amyloid β ($A\beta$) 1–40 and $A\beta$ 1–42 peptides generated by proteolytic cleavage by β - and γ -secretase from a larger membrane-bound protein, the amyloid precursor protein (APP).¹ APP and the secretases are integral transmembrane proteins dynamically sorted through the plasma membrane. Modulation of APP sorting through the membrane or altering APP cleavage by secretase enzymes could affect the regulation of $A\beta$ production or processing.

Variants in two members of the vacuolar protein sorting 10 (VPS10) domain-containing receptor protein family, sortilin-related receptor (*SORL1*) and sortilin-related VPS10 domain-containing receptor 1 (*SORCS1*), are associated with late-onset AD presumably through effects on APP sorting and cleavage.^{2–4} The VPS10 domain-containing receptor protein family contains five type I membrane homologs (*SORL1*, sortilin (*SORT1*), *SorCS1*, *SorCS2* and *SorCS3*),^{5–9} that are expressed in the central nervous system. All contain a single Vps10p-D situated at the N-terminus of their luminal/extracellular moiety. The VPS10 motif functions as a sorting

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receptor in the Golgi compartment required for the intracellular sorting and delivery of proteins, including APP. In SORT1, also known as neurotensin receptor-3, the Vps10p-D makes up the entire luminal extracellular part of the receptor, but the other four receptors have additional modules. In SORL1, the Vps10p-D is followed by five low-density lipoprotein receptor class B repeats flanked by an epidermal growth factor precursor-type repeat, a cluster of 11 low-density lipoprotein receptor class A repeats and 6 fibronectin type-III repeats. The mutually highly homologous SorCS1, SorCS2 and SorCS3 contain a leucine-rich segment between the Vps10p-D and the transmembrane domain. Structure prediction of the leucine-rich segment suggests a beta-sandwich fold and relates the domain to the immunoglobulin-like fold (E-set) superfamily. Following the extracellular and transmembrane segment, each receptor carries a short (40–80 amino acids) cytoplasmic domain comprising typical motifs for interaction with cytosolic adaptor molecules. In genomic DNA, members of this family are large with many exons but the coding sequence lengths are usually <3700 nucleotides. Very large introns (introns 1–2) typically separate the exons encoding the VPS10 domain; the remaining exons are separated by much smaller introns. Exons 1–3 encode the VPS10 domain.

Previously, we demonstrated that SORL1 modulates the translocation and retention of APP in subcellular compartments, which are less favorable for secretase processing, thereby reducing the extent of proteolytic breakdown into both amyloidogenic and non-amyloidogenic products.³ Furthermore, we showed that under-expression of *SORL1* leads to overexpression of A β and an increased risk of AD. Subsequently, we demonstrated that genetic variation in *SORCS1* also influences AD risk, cognitive performance, APP processing and A β 40 and A β 42 levels through an effect on γ -secretase processing of APP.^{2,10} Overexpression of SorCS1 reduced A β 40 and A β 42 levels, whereas suppression of SorCS1 increased γ -secretase processing of APP. The association of *SORL1* with AD has been supported by a meta-analysis of Caucasian and Asian data sets that included a total of 12 464 cases and 17 929 controls¹¹ and has been further validated in various ethnic groups, including African Americans, Israeli Arabs and Caribbean Hispanics, although with some degree of allelic heterogeneity.^{3,11–18} In addition, these data are supported by a study in which overexpression of SorCS1c β -myc in cultured cells caused a significant reduction in A β generation, whereas, conversely, endogenous murine A β 40 and A β 42 levels were increased in the brains of Sorcs1 hypomorphic mice.¹⁹

We hypothesized that variants in other members of the sortilin-related VPS10 domain containing receptor family, namely *SORCS2*, *SORCS3* and *SORT1*, would also be associated with AD risk either independently or through epistatic effects. These homologous genes are expressed in different brain regions with different subcellular localisations,^{20–22} but there are many brain regions, such as the hippocampus, in which these genes are co-expressed albeit at low levels.²⁰

We conducted single-marker association and epistasis analyses of all the five homologous genes in a large Caucasian case-control data set, with sufficient power to detect

modest effect sizes and interactive effects. In addition, we conducted microarray gene expression analyses and γ -secretase assays for *SORCS1*, *SORCS2*, *SORCS3* and *SORT1*.

Participants and methods

Ethics statement. Informed consent was obtained from all the participants using procedures approved by institutional review boards at each of the clinical research centers collecting human subjects for the ADGC project.

Participants. The data set included 11 840 cases and 10 931 controls from the ADGC data set.²³ The clinical characteristics are summarized in Table 1. The diagnoses of 'probable' or 'possible' AD were defined based on the National Institute of Neurological and Communication Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) diagnosis criteria at clinics specializing in memory disorders or in clinical investigations. Persons were classified as 'controls' when they were without cognitive impairment or dementia at last visit.

Genotyping. HapMap2-imputed genotypic data for single nucleotide polymorphisms (SNPs) in *SORCS1* ($n=648$), *SORCS2* ($n=740$), *SORCS3* ($n=742$), *SORL1* ($n=160$) and *SORT1* ($n=40$) was obtained from the previously published genome-wide association study.²³ The SNPs assessed included both intronic and exonic SNPs. The *SORCS1* SNPs were not identical to the SNPs assessed in our previous study, which had been selected based on previous reports.² Details regarding apolipoprotein E (*APOE*) genotyping are described in the Supplementary methods.

Cell culture and transfection. Using HEK293 cell lines, reverse transcriptase-PCR (RT-PCR) and western analysis were used to detect all five VPS10 proteins and to verify the knockdown and specificity of each short hairpin RNA (shRNA) as previously described.² The corresponding shRNA DNA sequences are shown in Supplementary Table 3.

APP-GV Assay. The γ -secretase activity and nuclear translocation of the APP/Fe65/TIP60 protein complex was

Table 1 Characteristics of the study sample

Characteristics	
Number of cases with AD	11 840
Number of controls	10 931
Age at Onset for AD cases (s.d.)	74.55 (6.8)
Age at last exam for controls (s.d.)	76.26 (7.2)
Proportion of females	59.66%
<i>Frequency of APOE e4 +</i>	
Cases	0.38
Controls	0.14
<i>Frequency of APOE e4 –</i>	
Cases	0.62
Controls	0.86

Abbreviation: AD, Alzheimer's disease; APOE, apolipoprotein E.

monitored with the APP-GV assay.²⁴ The APP-GV assay is a luciferase-based assay²⁴ consisting of the APP gene's C-terminus (AICD) fused to a transcription factor composed of the GAL4 DNA-binding domain with VP16 transcriptional activator (GV). In addition, the AICD fragment is fused to the GV domains as a positive control of AICD generation and allows for the evaluation of the AICD-specific contribution to the observed modulation in the APP-GV assay. Briefly, SorCS2 cDNA or SorCS2 shRNAs transiently transfected were evaluated in either the APP-GV or the AICD-GV assay, as previously described²⁴ in the HEK293 cell line. SorCS3 cDNA or SorCS3 shRNAs, and SORT1 cDNA or SORT1 shRNAs, were evaluated in a similar fashion.

Microarray gene expression and quantitative RT-PCR.

Expression profiling was performed separately for the cerebellum, parietal-occipital neocortex and amygdala regions from 19 AD and 10 control brains from the New York Brain Bank (www.nybb.hs.columbia.edu). This three-region approach allowed us to enhance the signal-to-noise ratio²⁵ and to determine those changes in expression that are specific for late-onset AD and consistent with the distribution of AD pathology. For the expression profiling of AD and control brains, the Affymetrix GeneChip Human Exon 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) were used. Frozen brain tissue was ground over liquid nitrogen and stored at -80°C until use. Total RNA was extracted and purified using the TRIzol Plus RNA purification kit (Invitrogen, Life Technologies, Grand Island, NY, USA). All RNA preparations were analyzed using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA; RNA 6000 nano-kit) to determine RNA quantity/quality and only samples with RNA integrity number >8 were used in the subsequent RNA amplification and hybridization steps. The Genechip expression two-cycle target labeling kit (Affymetrix) was used for all samples according to Affymetrix's protocols. Briefly, the procedure consists of an initial ribosomal RNA reduction step and two cycles of reverse transcription followed by *in vitro* transcription. For each sample, 1 μg of total RNA is initially subjected to removal of ribosomal RNA using the RiboMinus Transcriptome Isolation Kit (Invitrogen) and spiked with Eukaryotic PolyA RNA controls (Affymetrix). The ribosomal RNA-depleted fraction was used for cDNA synthesis by reverse transcription primed with T7-random hexamer primers, followed by second strand synthesis. This cDNA served as the template for *in vitro* transcription to obtain amplified antisense cRNA. Subsequently, cRNA from the first round was reverse transcribed using random primers to obtain single-stranded sense DNA. In this second reverse transcription, dUTP (2'-deoxyuridine, 5'-triphosphate) is incorporated into the DNA to allow for subsequent enzymatic fragmentation using a combination of UDG (uracil-DNA glycosylase) and APE1 (apyrimidinic endonuclease 1). All reverse and *in vitro* transcription steps were performed using the GeneChip WT cDNA synthesis and amplification Kit (Affymetrix). The resulting fragmented DNA was labeled with Affymetrix DNA Labeling Reagent. Labeled fragmented DNA was hybridized to Affymetrix Human Exon 1.0 ST arrays, washed and stained using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix).

Fluorescent images were recorded on a Genechip scanner 3000 and analyzed with the GeneChip operating software.

Significant results obtained from the microarray study were validated by quantitative RT-PCR using the same set of AD and control samples. Total RNA (1 μg) from of the amygdala region was used to generate cDNAs using the AffinityScript first-strand synthesis kit (Agilent Stratagene, CA, USA). RT-PCR primers were designed for three randomly selected exons of SorCS3 (10, 17 and 21). The housekeeping gene, TBP (TATA-binding protein), was used as the endogenous control; and samples were analyzed in triplicate. The primers used in the quantitative RT-PCR are available from Supplementary Table 1. Real-time RT-PCR was done using SYBR Green reagent (TaKaRa Mirus Bio, Madison, WI, USA) on an ABI7500 system (Applied Biosystems, Foster City, CA, USA).

Statistical methods. Extensive quality review of SNPs and samples were previously completed.²³ Then multivariate logistic regression analyses in PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) were used to assess additive genotypic and allelic associations with AD risk in the case-control data sets, and generalized estimating equation models were used for family-based data sets. All models were first adjusted for age at examination, sex and population stratification and subsequently for *APOE-ε4* (additive effect) as well. For adjustment for population stratification, the first two, three or four estimated principal components were used, as described previously.²³ Logistic generalized estimating equation models^{26,27} were used to evaluate association in the family-based data sets, using the same adjustments. Then, a meta-analysis of the individual study results was performed using inverse variance weights for the effect estimates as implemented in METAL (<http://www.sph.umich.edu/csg/abecasis/Metal/>). In order to take linkage disequilibrium (LD) between the markers into account, the *P*-value threshold for multiple testing correction was, in both single-marker and epistasis analyses, determined by applying the algorithm by Li and Ji.²⁸ As this was a candidate gene study with the *a priori* hypothesis of an association between each of the explored genes and AD, the calculation was done separately for each gene.

Epistasis. Using only the SNPs that were associated with AD in the single-marker analyses ($P \leq 0.05$), we tested for an interaction between SNPs in the five homologous genes. The analysis was carried out using PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) adjusting for population stratification. The model based on generalized estimating equations yields a list of SNP-by-SNP comparisons with beta coefficients and *P*-values. Based on the number of independent interactions tested, we accepted a *P*-value of ≤ 0.0001 as statistically significant. As described above, LD between the markers was taken into account applying the algorithm by Li and Ji.²⁸

Statistical analysis for the gene expression and quantitative RT-PCR data. To determine in which genes expression levels differ between affected and unaffected brain regions, as well as between AD and control brains,

(a)

(b)	CHR	BP	Allele 1	Allele 2	Freq1	β	s.e.	P ^c	NUMOBS	Location ^d	Splice site distance
	10	rs12258738	t	g	0.9754	-0.1415	0.0665	0.0328	23821	Intron 3	21495
	10	rs12248379	t	g	0.1844	0.0761	0.0257	0.003058	23821	Intron 3	25558
	10	rs17121613	t	g	0.8766	-0.0773	0.0304	0.01106	23821	Intron 3	26216
	10	rs4917491	t	c	0.5952	-0.0405	0.0202	0.04463	23821	Intron 2	60743
	10	rs7076579	t	c	0.5951	-0.04	0.0202	0.04719	23821	Intron 2	63104
	10	rs7096260	a	g	0.5949	-0.0396	0.0201	0.04944	23821	Intron 2	62788
	10	rs12356136	t	c	0.4006	0.0403	0.0203	0.04731	23821	Intron 2	59091
	10	rs7895881	a	t	0.4002	0.0406	0.0204	0.04674	23821	Intron 2	57674
	10	rs1004921	a	g	0.4	0.041	0.0204	0.04501	23821	Intron 2	57223
	10	rs10884389	t	g	0.4129	-0.0388	0.0197	0.04886	23821	Intron 2	20494
	10	rs10786997	a	c	0.3673	0.0401	0.0196	0.04094	23821	Intron 2	11724
	10	rs11193127	a	g	0.4125	-0.0399	0.0196	0.04169	23821	Intron 2	10249
	10	rs11193128	t	c	0.5876	0.0397	0.0196	0.04273	23821	Intron 2	10073
	10	rs108709366	a	g	0.5877	0.0399	0.0196	0.04167	23821	Intron2	6905
	10	rs10884391	a	c	0.4124	-0.0406	0.0196	0.03801	23821	Intron 2	6379
	10	rs10786998	a	c	0.4124	-0.0408	0.0196	0.03737	23821	Intron 2	6144
	10	rs12245675	t	c	0.4116	-0.0413	0.0196	0.03543	23821	Intron 2	4038
	10	rs17278802	t	c	0.4117	-0.0414	0.0196	0.03474	23821	Intron 2	3873
	10	rs2149197	c	g	0.5884	0.0435	0.0196	0.0268	23821	Intron 1	446
	10	rs1718454	t	c	0.5875	0.0435	0.0196	0.02677	23821	Intron 1	2116
	10	rs4918282	a	g	0.4074	0.0651	0.0263	0.01134	20952	Intron 1	60986
	10	rs10787010	a	g	0.6392	-0.061	0.0303	0.04421	17630	Intron 1	60767
	10	rs11193209	t	c	0.9776	-0.1544	0.0746	0.03843	23821	Intron 1	33591

(c)	SNP	CHR	BP	Allele 1	Allele 2	Freq1	β	s.e.	P ^c	NUMOBS	Location ^d	Splice site distance
4	rs11722747	4	7 314 043	a	g	0.1431	0.0554	0.0281	0.04836	23821	Intron 1	83972
4	rs4689707	4	7 326 199	t	c	0.1329	0.0663	0.0328	0.0434	19918	Intron 1	17 816
4	rs3842403	4	7 328 416	a	g	0.6996	-0.0554	0.0265	0.03639	19918	Intron 1	69599
4	rs7665496	4	7 328 734	t	c	0.9063	-0.1045	0.0415	0.01184	19918	Intron 1	69281
4	rs7661158	4	7 329 065	a	g	0.6996	-0.0497	0.0235	0.03715	19918	Intron 1	68950
4	rs6840423	4	7 329 324	a	g	0.121	0.0849	0.0356	0.01723	19918	Intron 1	68691
4	rs3864202	4	7 329 426	a	g	0.7018	-0.049	0.0233	0.03506	19918	Intron 1	68589
4	rs16840053	4	7 330 676	a	g	0.1462	0.0778	0.0315	0.0136	19918	Intron 1	67 339
4	rs13110208	4	7 353 052	c	c	0.5296	-0.0626	0.0241	0.009441	17630	Intron 1	44 963
4	rs4689720	4	7 390 442	t	c	0.1019	-0.1178	0.0531	0.02643	16732	Intron 1	7573
4	rs7684383	4	7 417 241	t	c	0.0403	-0.1429	0.069	0.03841	19633	Intron 2	19 159
4	rs4234804	4	7 417 632	a	g	0.0396	-0.1509	0.02505	0.02674	20990	Intron 2	19 550
4	rs6837589	4	7 419 276	t	c	0.1688	-0.0589	0.0277	0.03358	22439	Intron 2	21 194
4	rs13105690	4	7 420 184	c	c	0.2742	-0.0599	0.0248	0.01552	19918	Intron 2	22 102
4	rs4292336	4	7 420 785	a	g	0.8316	0.0546	0.0277	0.04904	22439	Intron 2	22 703

Results

Epistasis analysis. Upon testing for epistatic effects between the SNPs that were associated in the single-marker analyses (Table 3a), 34 pairs of SNPs showed epistatic effects at a P -value of <0.01 . The vast majority ($n=26$ pairs) included a specific LD block in *SORCS3* with two specific LD blocks in *SORCS2* (Table 3, Figures 1a and b). Consistent with the single-marker analyses, the interacting SNPs were located in introns 1 and 2 (Table 3, Figure 1), adjacent to the exons coding for the VPS10 domain. The epistasis β for these *SORCS2/SORCS3* interactions ranged from -0.94 to 0.94 , reflecting larger effects (additive) than the single-marker effects ($-0.15 < \beta < 0.20$).

Eight pairs resembled additional epistatic effects between *SORCS1/SORCS3* and between *SORCS1/SORCS2* (Table 3b). Of note, the single *SORCS3* SNP (rs1670036) interacting with *SORCS1* is located in the specific LD block also showing interaction with *SORCS2* (Figure 1a), and again all SNPs constituting the LD block in *SORCS1* are located in

c)											
CHR	SNP	BP	Allele 1	Allele 2	Freq1	β	s.e.	P ^c	NUMOBS	Location ^d	Splice site distance
4	rs17465564	7 622 347	a	g	0.9136	0.0861	0.04	0.03151	22439	Intron 3	17708
4	rs2214459	7 667 486	t	c	0.6948	-0.0519	0.0234	0.02645	21275	Intron 7	1288
4	rs12233824	7 733 843	a	g	0.45	-0.04	0.0203	0.04877	23821	Intron 23	1206
d)											
CHR	SNP	BP	Allele 1	Allele 2	Freq1	β	s.e.	P ^e	NUMOBS	Location ^d	Splice site distance
10	rs12249460	106 605 440	a	g	0.0361	0.136	0.0585	0.01999	23821	Intron 2	2823
10	rs6584629	106 608 435	a	g	0.0475	0.1038	0.0457	0.02303	23821	Intron 2	5818
10	rs12259189	106 615 387	t	c	0.0474	0.0972	0.0458	0.03358	23821	Intron 2	12770
10	rs3976793	106 616 736	a	g	0.0474	0.0969	0.0458	0.03433	23821	Intron 2	14119
10	rs12262245	106 621 704	c	g	0.9529	-0.0941	0.046	0.04088	23821	Intron 2	19087
10	rs7086583	106 622 009	a	c	0.9529	-0.0929	0.046	0.04342	23821	Intron 2	19392
10	rs1670036	106 807 189	a	c	0.978	0.2082	0.0867	0.0164	23821	Intron 5	4303
10	rs749304	106 990 399	a	g	0.7655	-0.0469	0.0227	0.0391	23821	Intron 20	7392
10	rs12263804	106 993 770	t	c	0.2348	0.0474	0.0227	0.03711	23821	Intron 20	10763
10	rs7920533	107 013 252	a	g	0.3247	0.0546	0.0221	0.01354	23821	Intron 23	588
10	rs750261	107 023 390	t	c	0.2396	0.045	0.0226	0.04653	23821	UTR-3	
10	rs10884126	107 025 028	a	g	0.2397	0.0451	0.0226	0.04627	23821	NearGene-3	

Abbreviations: BP, base pair position; β , beta coefficient; CHR, chromosome; location, single nucleotide polymorphism (SNP) location; NUMOBS, number of subjects; s.e., standard error of beta coefficient; SORCS1, sortilin-related VPS10 domain-containing receptor 1; SORL1, sortilin-related receptor 1; UTR, untranslated region.

^aBased on the number of tests performed, a *P*-value of 0.0002 can be considered statistically significant. ^bExons 1–16 encode the VPS10 domain. ^cBased on the number of tests performed, a *P*-value of 0.0009 can be considered statistically significant. ^dExons 1–18 encode the VPS10 domain. ^eBased on the number of tests performed, a *P*-value of 0.0006 can be considered statistically significant. ^fExons 1–16 encode the VPS10 domain. ^gBased on the number of tests performed, a *P*-value of 0.0002 can be considered statistically significant.

Table 3 Epistasis between two specific LD blocks in (a) SORCS2 and SORCS3, and (b) SORCS1/SORCS2 and SORCS1/SORCS3

(a)

SORCS3										SORCS2									
	CHR1	BP1	GENE1	Location ^b	CHR2	BP2	GENE2	Location ^b											
	10	106990399	SORCS3	Intron 20	4	7328734	SORCS2	Intron 1	-	-	-	-	?	-	-	-	-	Intron 1	
	10	106993770	SORCS3	Intron 20	4	7328734	SORCS2	Intron 1	-	-	-	-	?	-	-	-	-	Intron 1	
	10	107025390	SORCS3	UTR-3	4	7328734	SORCS2	Intron 1	-	-	-	-	?	-	-	-	-	Intron 1	
	10	107025028	SORCS3	Near gene-3	4	7328734	SORCS2	Intron 1	+	+	+	+	?	+	+	+	+	Intron 1	
	10	106990399	SORCS3	Intron 20	4	7329324	SORCS2	Intron 1	+	-	+	+	?	+	+	+	+	Intron 1	
	10	106993770	SORCS3	Intron 20	4	7329324	SORCS2	Intron 1	+	-	+	+	?	+	+	+	+	Intron 1	
	10	106990399	SORCS3	Intron 20	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106993770	SORCS3	Intron 20	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	107025028	SORCS3	NearGene-3	4	7417241	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	107023390	SORCS3	UTR-3	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106621704	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106622009	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106616736	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106615387	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106608435	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106605440	SORCS3	Intron 2	4	7417241	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106990399	SORCS3	Intron 20	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	107025028	SORCS3	NearGene-3	4	7417632	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106993770	SORCS3	Intron 20	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	107023390	SORCS3	UTR-3	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106621704	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106622009	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106616736	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	
	10	106615387	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106608435	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	+	+	+	+	?	+	+	+	+	Intron 2	
	10	106605440	SORCS3	Intron 2	4	7417632	SORCS2	Intron 2	-	-	-	-	?	-	-	-	-	Intron 2	

(b)

SORCS1										SORCS3/SORCS2									
	CHR1	BP1	GENE1	Location ^b	CHR2	BP2	GENE2	Location ^b											
	10	108710127	SORCS1	Intron 2	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108709892	SORCS1	Intron 2	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108718454	SORCS1	Intron 1	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108712233	SORCS1	Intron 2	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108712398	SORCS1	Intron 2	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108695777	SORCS1	Intron 2	10	106807189	SORCS3	Intron 4	?	+	+	+	?	+	+	+	+	Intron 4	
	10	108890136	SORCS1	Intron 1	4	7667486	SORCS2	Intron 7	+	+	+	?	+	+	+	+	+	Intron 7	
	10	108563116	SORCS1	Intron 3	4	7326199	SORCS2	Intron 1	-	-	-	-	?	+	+	+	+	Intron 1	

Abbreviations: A1, Allele 1; A2, Allele 2; Beta, beta coefficient; CHR, chromosome; DIR, direction of effect; location, single nucleotide polymorphism (SNP) LD, linkage disequilibrium; location; s.e., standard error of beta coefficient; SORCS1, sortilin-related VPS10 domain-containing receptor 1.

^a*P*-value cutoff for significance after correction for multiple testing: *P* = 0.0001. ^bExons 1–18 encode the VPS10 domain.

Figure 1 Black arrows: (a) The linkage disequilibrium (LD) block in sortilin-related VPS10 domain-containing receptor 3 (*SORCS3*) showing significant interaction with (b) two specific LD blocks in *SORCS2* and (c) one specific LD block in *SORCS1*. Red arrows: Additional single nucleotide polymorphisms (SNPs) showing epistasis between *SORCS2* and *SORCS3*. (a) The single LD block in *SORCS3* showing epistasis with two specific LD blocks in *SORCS2* (Figure 3b) and one specific LD block in *SORCS1* (Figure 3c). (b) The two specific two LD blocks in *SORCS2* (black arrows) showing epistasis with *SORCS3*, and SNPs showing in addition epistasis with *SORCS1* (red arrows). (c) The specific LD block in *SORCS1* (black arrows) showing epistasis with the specific LD block in *SORCS3*, and SNPs showing epistasis with SNPs in *SORCS2* (red arrows).

Figure 2 (a) View of sortilin-related VPS10 domain-containing receptor 3 (*SORCS3*) exon expression profile in 19 Alzheimer's disease (AD; red triangles) and 10 control (CTRL; blue squares) amygdala tissue. Each triangle dot represents least squares mean expression of an exon in AD tissue; each square dot represents least squares mean expression of an exon in control tissue. The mean gene expression intensity of AD vs controls was 4.17 ± 0.43 vs 5.03 ± 0.49 ($P = 5.1E-5$) across all exons. (b) View of *SORCS2* exon expression profile in 19 AD (red triangles) and 10 control (blue squares) amygdala tissue. Each triangle dot represents least squares mean expression of an exon in AD tissue; each square dot represents least squares mean expression of an exon in control tissue. The mean gene expression intensity of AD vs controls was 5.29 ± 0.33 vs 5.08 ± 0.32 ($P = 0.12$) across all exons. (c) View of sortilin 1 (*SORT1*) exon expression profile in 19 AD (red triangles) and 10 control (blue squares) amygdala tissue. Each triangle dot represents least squares mean expression of an exon in AD tissue; each square dot represents least squares mean expression of an exon in control tissue. The mean gene expression intensity of AD vs controls was 9.43 ± 0.38 vs 9.21 ± 0.39 ($P = 0.17$) across all exons.

introns 1 and 2 (Figure 1c) adjacent to the exons encoding the VPS10 domain. The epistasis β for these *SORCS1/SORCS3* and *SORCS1/SORCS2* interactions ranged from -0.39 to 0.40 , again reflecting larger effects (additive) than the corresponding single-marker analyses ($-0.15 < \beta < 0.20$). Although the *P*-values for epistatic effects just missed the multiple testing threshold of 0.0001 , the number of significant interactions was clearly higher than expected by chance (expected *SORCS2/SORCS3* interactions: 10.8). Forest plots for the SNPs with epistatic effects or strongest individual associations with AD (rs7665496, rs6840423, rs7684383, rs4234804, rs1670036, rs1792124, rs12248379, rs13110208) are shown in Supplementary Figure S2.

Microarray gene expression and quantitative RT-PCR analyses. Microarray expression analyses showed lower expression of *SorCS3* in AD brains compared with control brains (mean gene expression intensity: 4.17 ± 0.43 vs 5.03 ± 0.49 ($P = 5.1E - 5$; Figure 2a), in line with what we had previously observed in *SORL1* and *SORCS1*.^{2,3} To validate the significant results of the Affymetrix array, we conducted a quantitative RT-PCR for the *SORCS3* gene, using brain tissue from the amygdala region. Calculation of the fold change rate with the Relative Quantitation method of DataAssist software confirmed the results of the expression array for all the three investigated *SORCS3* exons. Compared with the control samples, the AD samples showed significantly reduced expression of exons 10, 17 and 21 of *SORCS3*; 87% ($P = 0.012$), 74% ($P = 0.003$) and 83% ($P = 0.003$), respectively (Supplementary Figure S3). Notably, these findings were also validated by comparison with publicly available gene expression results (188 cases, 176 controls, $P < 0.0001$, <http://labs.med.miami.edu/myers/>).³⁰ We did not find a significant difference between AD and controls in the expression levels of *SORCS2* or *SORT1* (Figures 2b and c). There was no significant difference in the expression levels between AD and control brains in brain tissue from regions unaffected by the disease process (occipital lobe, cerebellum) for any of the homologs.

Cell culture and transfection. cDNA transfection of Vps10 family members in Hek 293 cells demonstrated a significant ($0.01 \leq P \leq 0.05$) decrease of γ -secretase (APP-GV), whereas there was no effect on the AICD-GV translocation assay (Figure 3).

γ -Secretase processing. In HEK293 cells (Figure 4), 4/4 *SorCS2*-shRNAs, 3/3 *SorCS3*-shRNAs and 2/3 *SORT1*-shRNAs caused a significant increase greater than threefold in APP processing (from $P < 0.001$ to $P < 0.05$) as compared with the result with the scrambled shRNA (analysis of variance with Bonferroni correction) while not affecting the nuclear translocation of the control AICD-GV only-fragment.

Discussion

Taken together with previous studies,^{2,3} the findings here indicate that variants in *SORL1*, *SORCS1*, *SORCS2* and *SORCS3* of the VPS10-d receptor family are associated with AD risk. The results are consistent with previous studies

Figure 3 cDNA overexpression in transfected HEK293 cells. AICD, APP gene's C-terminus; APP, amyloid precursor protein; GV, GAL4 DNA-binding domain with VP16 transcriptional activator; SORCS, sortilin-related VPS10 domain-containing receptor; SORLA, sortilin-related receptor.

showing associations between the SNPs in *SORL1* and *SORCS1* with AD^{2,3,11,13–16,18} and with cognitive performance.¹⁰ Similar to previous reports, the associated SNPs in *SORCS1*, *SORCS2* and *SORCS3* were mostly located in introns 1–3, implicating the VPS10 domain.

The effect sizes of the associated SNPs were small (β : -0.45 to 0.36), but this is consistent with previous observations for the homologous genes *SORL1* and *SORCS1*^{2,3,18} as well as all recently detected novel AD susceptibility loci identified by large genome-wide association studies.^{23,31–34}

The epistasis models of SNPs significant in single-marker analyses further revealed pairwise SNP associations between specific LD blocks in the highly homologous *SORCS1*, *SORCS2* and *SORCS3*. One single LD block in *SORCS3* showed epistasis with both a single LD block in *SORCS1* and two specific LD blocks in *SORCS2*. In addition, the same two regions of *SORCS2* and *SORCS3* interacted. Of note, the epistasis β ranging from -0.94 to 0.94 reflected larger effects (additive) than the corresponding single-marker analyses ($-0.15 < \beta < 0.20$), and the interacting SNPs are almost exclusively located in introns 1 and 2, adjacent to the exons encoding the VPS10 domain. This region has also been demonstrated to include the majority of disease-associated SNPs for both *SORCS1* and *SORL1*.^{2,3,10,18,20} Our findings indicate that there are sequences within these specific LD blocks that are biologically important and that are interacting. The mechanism underlying these interactions is presently unclear. It could arise from direct interaction between the homologs, interactions with a mutual binding partner or interactions with a common substrate, such as APP or APP-CTF. However, it could also result from quite remote interactions that do not require a first- or second-order interaction between these proteins.

Suppression of *SORCS2*, *SORCS3* or *SORT1* increased γ -secretase processing of APP, findings consistent with reported effects by *SORL1* and *SORCS1* on γ -secretase processing of APP and changes in A β 40 and A β 42 levels.^{2,3} Although *SorCS3* and *SorCS1* do not convey trans-Golgi network to late endosome sorting,^{35,36} *SORT1* is—similar to *SORL1*—also capable of mediating sorting of ligands from the trans-Golgi network to late endosomes or lysosomes.²²

Figure 4 γ -Secretase activity and nuclear translocation of amyloid precursor protein (APP) assays with sortilin-related VPS10 domain-containing receptor 2 (*SORCS2*), *SORCS3* or *SORT1* short hairpin RNAs (shRNAs). Both the APP-GV (GAL4 DNA-binding domain with VP16 transcriptional activator) and APP gene's C-terminus (AICD)-GV assay were performed in HEK293 cells. The data from *SorCS2*, *SorCS3* or sortilin 1 (*SORT1*) shRNA was normalized to either APP-GV only or AICD-GV with the scrambled sequence shRNA (shRNA-scrambled), which was included as a negative control. The data are representative for the assays, were performed in ≥ 3 experiments in replicates of eight samples per condition (96-well format, s.d. bars are shown, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with APP-GV only (analysis of variance, Bonferroni's correction)).

Interestingly, sortilin-mediated endocytosis has been shown to determine levels of progranulin involved in frontotemporal dementia.³⁷ Results from the current genome-wide association studies suggest that other genes *PICALM*, *BIN1* and *CD2AP*, modulate intracellular trafficking of cell surface proteins. Thus, it appears that in addition to their effect on γ -secretase processing of APP, some members of the VPS10-d receptor family exert their effect on AD through modulation of APP trafficking.

Reduced expression of SORCS3 may be a secondary effect of the disease, but it is consistent with the γ -secretase assays, which indicated that suppression of SORCS3 activates A β production. If correct, this would provide a potential explanation for how downregulation of SORCS3 might increase risk for AD. We are unable to see any difference in the expression of *SORCS2* and *SORT1*. However, we cannot yet exclude the possibility that this was the result of the small sample size or chosen phenotype. In a previous study by Mufson *et al.*,³⁸ SORT1 levels were—consistent with our findings—not associated with clinical diagnosis or antemortem cognitive test scores. However, there was an association with severity of neuropathology by Braak and NIA-Reagan diagnoses.

The significant strengths of this study are the large sample size, allowing us to detect small effects and explore epistasis. Limitations include that the SNPs assessed were derived from available genome-wide arrays. Thus, they do not cover the complete genetic variation in these genes, and it is possible that there are additional disease-associated markers that have not been genotyped. It is also possible that we lacked the power to detect additional disease-associated markers or interactions of SNPs with lower allele frequencies or effect sizes.

Taken together, our results indicate that in addition to *SORL1* and *SORCS1*, the variants in other members of the VPS 10-D receptor family (*SORCS2*, *SORCS3* and *SORT1*) are associated with AD either independently or through epistatic mechanisms.

Conflict of interest

The authors declare no conflict of interest.

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