

Initial Assessment of the Pathogenic Mechanisms of the Recently Identified Alzheimer Risk Loci

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Summary

Recent genome wide association studies have identified *CLU*, *CR1*, *ABCA7*, *BIN1*, *PICALM* and *MS4A6A/MS4A6E* in addition to the long established *APOE*, as loci for Alzheimer's disease. We have systematically examined each of these loci to assess whether common coding variability contributes to the risk of disease. We have also assessed the regional expression of all the genes in the brain and whether there is evidence of an eQTL explaining the risk. In agreement with other studies we find that coding variability may explain the *ABCA7* association, but common coding variability does not explain any of the other loci. We were not able to show that any of the loci had eQTLs within the power of this study. Furthermore the regional expression of each of the loci did not match the pattern of brain regional distribution in Alzheimer pathology. Although these results are mainly negative, they allow us to start defining more realistic alternative approaches to determine the role of all the genetic loci involved in Alzheimer's disease.

Keywords: Alzheimer's disease, genetic risk, GWAS

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Introduction

The recent application of genome wide association studies (GWAS) to the dissection of the risk for late onset Alzheimer's disease (AD) has proved an outstanding success and has led to the identification of many new loci (*CLU*, *PICALM*, *CR1*, *BIN1*, *MS4A6A/MS4A4E*, *CD33*, *CD2AP*, *ABCA7* and *EPHA1*) in addition to the long established apolipoprotein E locus (Harold et al., 2009; Lambert et al., 2009; Hollingworth et al., 2011; Naj et al., 2011). When such loci are identified, they simply appear as single nucleotide polymorphisms (SNPs), which have significantly different frequencies between cases and controls. It is not initially clear whether these risk SNPs are in linkage disequilibrium (LD) with coding changes or have an impact on gene expression. For all traits studied by GWAS only ~12% of the associated SNPs are located in, or occur in high LD with, protein coding regions of genes. The vast majority (~80%) of trait associated SNPs are located in intergenic regions or noncoding introns (Manolio, 2010). AD is no different: taking into account the 21 SNPs reported in the nine new loci by GWAS assessing over 1500 cases and 1500 controls (see Table 1 for details on the SNPs), 10 are located in intergenic regions; 8 in intronic regions; 1 SNP is located in the 3'UTR of *MS4A6A*; and 2 SNPs are located in exons (one SNP is a non-synonymous variant in *ABCA7*—Gly1527Ala and one synonymous variant was found as significant in *PICALM*). These findings clearly indicate that follow up studies should not only examine coding variability, but should also pay close attention to the potential roles of these intronic and intergenic regions in the regulation of gene expression (Myers et al., 2007; Hardy and Singleton, 2009; Manolio, 2010). In fact, for any disease associated SNP, the true variant underlying the phenotype studied may be: (1) the GWAS hit itself; (2) a known common SNP in LD with the identified GWAS hit; (3) an unknown common SNP or rare single nucleotide variant tagged by a haplotype on which the hit occurs; or (4) a linked copy number variant (Hindorf et al., 2009). In general, GWAS follow up studies rely on fine mapping of the associated locus or loci, deep re-sequencing of the associated region(s) in samples of interest (which allows the identification of all possible functional variants) and a variety of bioinformatic approaches to prioritise variants to be further studied (Stranger et al., 2011).

Confirmed functional variants underlying validated GWAS hits are still sparse in the literature, when considering all the diseases and traits studied, but each of these is extremely valuable to the respective research and clinical environments. For example, the *IRF5* locus includes variants that disrupt intron splicing, decrease mRNA transcript stability and delete part of the interferon regulating factor protein (Graham et al., 2007), explaining the independent associations of this locus with three different phenotypes: systemic lupus erythematosus

(Sigurdsson et al., 2005; Graham et al., 2006), inflammatory bowel disease (Dideberg et al., 2007), and rheumatoid arthritis (Stahl et al., 2010). Similarly, allele-specific chromatin remodelling affecting the expression of several genes in the *ORMDL3* locus region (Verlaan et al., 2009) explains its association with asthma (Moffatt et al., 2007), Crohn's disease (Barrett et al., 2008) and type 1 diabetes (Barrett et al., 2009). With this in mind we have undertaken an analysis of the recently identified AD risk loci with three components: (1) we have assessed by sequencing whether there is common coding variability in LD with the associated SNPs (2) we have assessed in a database of control human cerebral cortex samples whether the SNPs are associated with genetic variability in expression (3) we have assessed the regional distribution of expression and splicing of the genes at the risk loci to see whether this distribution is in any way consistent with the distribution of pathology in the disease.

Materials and Methods

Genotyping Analysis

Samples

The 96 DNA samples selected for genotyping were previously used in a GWAS in AD (Corneveaux et al., 2010). These 96 Alzheimer disease samples were diagnosed according to the NINDS-ADRDA diagnostic criteria for Alzheimer disease, consisting of 67 females and 29 males with a mean age of 81 years (range 66–95) and mean age at onset of 71.9 years (ranging from 65 to 85 years).

SNPs studied

The GWAS SNPs studied were those found to be significantly associated with late onset AD (LOAD) by two recent studies: (Corneveaux et al., 2010; Hollingworth et al., 2011). For a complete list of SNPs analysed in the present study please refer to Table 1.

Coding SNPs were chosen based upon their reported minor allele frequency (MAF) or heterozygosity in dbSNP. For this, publicly available data in dbSNP was used and SNPs were chosen based upon the fact that they induced a coding change in the resultant protein and that they had a MAF or heterozygosity greater than 0.05 in the general population. For *CR1*, SNPs were excluded if they were located in highly homologous exons in order to avoid genotyping errors.

Most of the SNPs studied conformed to these specifications, however, there were some that did not and were included in the study because no better proxies were available (such as rs17259045, rs76037557, rs74727972, rs79741566, rs72973581).

Table 1 Features and minor allele frequencies of all SNPs studied here, including the GWAS hits

SNP	Gene/locus	Chr	Chr position— genome build 37.1	Chr position— genome build 36.3	Category	mRNA accession number	SNP class	Ancestral allele	MAF in our study	1000 genomes MAF
rs4844600	<i>CR1</i>	1	207679307	205745930	Targeted	NM_000651.4	Triallelic SNP: cds-synonymous (Glu60Glu) or missense (Glu60Asp)	G	A = 0.23	A = 0.209/263
rs6656401	<i>CR1</i>	1	207692049	205758672	AD Risk - C	NM_000651.4	Intronic	G	A = 0.18	A = 0.107/135
rs3737002	<i>CR1</i>	1	207760773	205827396	Targeted	NM_000651.4	Missense (Thr1858Met)	C	T = 0.28	T = 0.268/337
rs17259045	<i>CR1</i>	1	207782707	205849330	Targeted	NM_000651.4	Missense (Asn1990Ser)	A	G = 0.09	G = 0.041/51
rs17047661	<i>CR1</i>	1	207782889	205849512	Targeted	NM_000651.4	Missense (Arg2051Gly)	A	G = 0	G = 0.242/219
rs4844609	<i>CR1</i>	1	207782916	205849539	Found	NM_000651.4	Missense (Thr2060Ser)	T	A = 0.02	A = 0.010/12
rs6691117	<i>CR1</i>	1	207782931	205849554	Targeted	NM_000651.4	Missense (Ile2065Val)	G	G = 0.22	G = 0.382/481
rs3818361	<i>CR1</i>	1	207784968	205851591	AD Risk - H	NM_000651.4	Intronic	C	A = 0.01	A = 0.301/379
rs3811381	<i>CR1</i>	1	207790088	205856711	Targeted	NM_000651.4	Missense (Pro2277Arg)	G	G = 0.21	G = 0.165/208
rs2296160	<i>CR1</i>	1	207795320	205861943	Targeted	NM_000651.4	Triallelic SNP: missense Thr2419Ala or Thr2419Ser	C	A = 0.2	A = 0.233/293
rs76037557	<i>BIN1</i>	2	127815174	127531644	Targeted	NM_139351.1	Intronic	G	0	NA
rs744373	<i>BIN1</i>	2	127894615	127611085	AD Risk - H	NA	5' near BIN1	T	G = 0.38	G = 0.380/478
rs79741566	<i>MS4A2</i>	11	59857929	59614505	Targeted	NM_001142303.1	Missense (Irp103Gly)	T	0	NA
rs335630	<i>MS4A2</i>	11	59861532	59618108	Targeted	NM_000139.3	Missense (Asn211Lys)	G	A = 0	A = 0.086/30
rs610932	<i>MS4A6A</i>	11	59939307	59695883	AD Risk - H	NM_152852.1	3'UTR	A	T = 0.39	T = 0.455/572
rs7232	<i>MS4A6A</i>	11	59940599	59697175	Targeted	NM_152852.1	Missense (Thr185Ser)	T	A = 0.33	A = 0.203/255
rs670139	<i>MS4A4E</i>	11	59971795	59728371	AD Risk - H	NA	3' near MS4A4E region	C	T = 0.46	T = 0.387/487
rs10750931	<i>MS4A4A</i>	11	60059810	59816386	Targeted	NM_148975.1	Missense (Lys52Glu)	A	G = 0	G = 0.139/303
rs6591561	<i>MS4A4A</i>	11	60070176	59826752	Targeted	NM_148975.1	Missense (Met178Val)	A	G = 0.28	G = 0.323/705
rs74727972	<i>PICALM</i>	11	85723420	85401068	Targeted	NM_007166.2	Missense (Leu188Ile)	G	0	NA
rs541458	<i>PICALM</i>	11	85788351	85465999	AD Risk - C	NA	5' of PICALM region	T	C = 0.29	C = 0.376/473
rs3764645	<i>ABC A7</i>	19	1042809	993809	Targeted	NM_019112.3	Missense (Glu188Gly)	A	G = 0.46	G = 0.370/466
rs72973581	<i>ABC A7</i>	19	1043103	994103	Targeted	NM_019112.3	Missense (Gly215Ser)	G	A = 0.04	A = 0.020/25
rs3752232	<i>ABC A7</i>	19	1043748	994748	Targeted	NM_019112.3	Missense (Thr319Ala)	G	G = 0.01	G = 0.103/130
rs4622634	<i>ABC A7</i>	19	1043864	994864	Found	NM_019112.3	Intronic	G	C = 0.47	C = 0.336/423
rs3764647	<i>ABC A7</i>	19	1044712	995712	Targeted	NM_019112.3	Missense (His395Arg)	G	G = 0.03	G = 0.099/125
rs3764648	<i>ABC A7</i>	19	1044753	995753	Found	NM_019112.3	Intronic	C	T = 0.35	T = 0.358/450
rs3752233	<i>ABC A7</i>	19	1045173	996173	Targeted	NM_019112.3	Missense (Arg463His)	G	A = 0.03	A = 0.080/74
rs3764650	<i>ABC A7</i>	19	1046520	997520	AD Risk - H	NM_019112.3	Intronic	T	G = 0.09	G = 0.207/261
rs3745842	<i>ABC A7</i>	19	1055191	1006191	Targeted	NM_019112.3	Missense (Arg1349Gln)	C	A = 0.41	A = 0.401/504
rs3752246	<i>ABC A7</i>	19	1056492	1007492	AD Risk - H	NM_019112.3	Missense (Gly1527Ala)	C	G = 0.19	G = 0.188/236
rs4147918	<i>ABC A7</i>	19	1058176	1009176	Targeted	NM_019112.3	Missense (Gln1686Arg)	A	G = 0.03	G = 0.053/67
rs4147934	<i>ABC A7</i>	19	1065018	1016018	Targeted	NM_019112.3	Missense (Ala2045Ser)	G	G = 0.27	G = 0.387/487
rs4147935	<i>ABC A7</i>	19	1065044	1016044	Found	NM_019112.3	Synonymous (Gly2053Gly)	C	T = 0.36	T = 0.201/253

Chr, chromosome; AD Risk-C; this SNP was identified as a risk marker for AD in the GWAS study published by Corneveaux et al. (2010); Risk-H; this SNP was identified as a risk marker for AD in the GWAS study published by Hollingworth et al. (2011); Targeted; this SNP was identified as a common coding SNP in a gene containing an AD risk SNP from the previously mentioned GWASs; Found; this SNP was found when genotyping targeted SNPs because it was located near one of the targeted SNPs; and showed up during the sequencing of the targeted SNP region.

DNA sequencing and data analysis

The genotypes of the coding SNPs used to establish the LD structure were determined by Sanger sequencing. The exon in which the SNP is located was targeted for amplification, or, in the case of intronic GWAS SNPs, the sequence 150 bases upstream and 150 bases downstream was amplified. For the PCR reactions, AmpliTaq Gold[®] 360 MasterMix (Applied Biosystems, Foster City, CA, USA) was used together with specific primers designed using ExonPrimer (<http://ihg.gsf.de/ihg/ExonPrimer.html>). For the SNPs rs3752239, rs4147934 and rs4147935, DMSO was included in the PCR protocol. Each purified PCR product was sequenced using Applied Biosystems BigDye terminator v3.1 sequencing chemistry and ran on an ABI3730xl (Applied Biosystems, CA) genetic analyser as per manufacturer's instructions. The sequences were analysed with Sequencher software, version 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

DNA Methylation and mRNA Expression in the Human Brain

Tissue samples

Frozen samples from the frontal cerebral cortex and cerebellum were obtained from 387 Caucasian subjects without neurological disease in lifetime (Gibbs et al., 2010; Hernandez et al., 2011). Genomic DNA was extracted using phenol-chloroform and quantified on a Nanodrop1000 spectrophotometer before genotyping or bisulfite conversion for DNA methylation analysis.

CpG methylation

Bisulfite conversion of genomic DNA was performed using Zymo EZ-96 DNA Methylation kits according to the manufacturer's protocol, using 1 μ g of DNA input. The CpG methylation status of DNA at >27,000 sites was determined using Illumina Infinium HumanMethylation27 BeadChips (Illumina Inc., San Diego, CA, USA). Samples were included in the analysis if the threshold call rate for inclusion of was >95% in the tissue. As a second quality control, we compared reported genders with methylation levels of CpG sites on the X chromosome. After these steps, 292 samples with data at 27,465 CpG sites in the frontal cortex tissue samples and 27,419 sites in the cerebellum tissue samples were used for further analysis.

mRNA expression

Messenger RNA (mRNA) expression was analysed using Illumina HumanHT-12 v3 Expression Beadchips. Individual probes were excluded from analyses if the *P* value for de-

tection was >0.01 and samples were excluded if <95% of probes were detected. Intensity values for each probe were normalised using cubic spline and transformed using log₂ prior to statistical analyses. Probes were annotated using ReMOAT tool to exclude individual probes that are known to have problems in design or with ambiguous mapping. We also removed all probes that included any known SNP. After these quality control steps, data was available for 399 samples at 9814 probes from the frontal cortex, and 9587 probes in cerebellum.

Genotyping and imputation of control brains in epigenetic analyses

The same tissue samples were genotyped using Illumina HumanHap550 v3, Human610-Quad v1 or Human660W-Quad v1 Infinium Beadchips and shared SNPs were extracted for each sample. We excluded samples where the reported sex did not match X chromosome heterozygosity from genotype data or if the per sample genome-wide call rate was less than 95%. Individual SNPs were excluded if there was a <95% genotyping success rate per SNP, if MAF < 0.01 or if Hardy-Weinberg equilibrium (HWE) $P < 1 \times 10^{-7}$. Multi-dimensional scaling was used to cluster samples after merging SNPs common to CEU, JPT, CHB and YRI samples from Phase II of HapMap. Outliers >3 standard deviations from the mean component vector estimates for C1 or C2 for CEU samples were then removed, as were samples sharing greater than a proportion of 0.15 alleles. Genotypes for all European ancestry participants were imputed using MACHv1.0.16 with haplotypes derived from sequencing of 112 European ancestry samples present in the August 2009 release of phased data from the 1000 Genomes Project (available at <http://www.sph.umich.edu/csg/abecasis/MACH/download/1000G-Sanger-0908.html>). Data was imputed by first generating error and crossover maps as parameter estimates for the imputation on a randomly selected set of 200 samples over 100 iterations of the initial statistical model. These parameter estimates were then used to generate maximum likelihood allele dosages per SNP based on reference haplotypes for the entire study cohorts. We excluded SNPs with R^2 quality estimates <0.30, resulting in ~5.1 million SNPs available for analysis.

methQTL and eQTL mapping

SNPs with a fixed-effects $P < 1 \times 10^{-5}$ from the AD meta-analysis (Stage 1 + 2) were considered candidate quantitative trait loci (QTL; Naj et al., 2011). For each SNP, CpG sites and expression probes within ± 1 MB were used for linear regression modelling using MACH2QTLv1.08. We estimated the association between the allelic dosage of each SNP against gene expression or methylation levels using linear regression

models adjusted for covariates of gender and age at death, the first two component vectors from multi-dimensional scaling, post-mortem interval (PMI), brain bank and batch in which preparation or hybridisation were performed. SNPs with less than three minor homozygotes detected were excluded from analyses. We tested probes within 1 MB of each of 224 candidate probes in the expression datasets and 220 in the methylation datasets, resulting in 2542 associations for expression-QTLs and 11,522 associations for methylation-QTLs in the frontal cortex samples and 2395 expression-QTLs and 11,510 methylation-QTLs in the cerebellum. The resulting *P* values were corrected for multiple testing using the Bonferroni method after removing SNPs having $r^2 > 0.5$ with SNPs in adjacent sliding windows of 50 SNPs that moved two SNPs per iteration. After these filters, the analyses used 152 mRNA probes and 603 CpG sites.

Regional Brain Expression and Splicing Analysis

Human post-mortem brain tissue collection and mRNA extraction

A detailed description of the samples used in the study, tissue processing and dissection is provided in Trabzuni et al. (2011). In brief, brain and CNS tissue originating from 137 control individuals was collected by the Medical Research Council (MRC) Sudden Death Brain and Tissue Bank, Edinburgh, UK (Millar et al., 2007), and the Sun Health Research Institute (SHRI) an affiliate of Sun Health Corporation, USA (Beach et al., 2008). All samples had fully informed consent for retrieval and were authorised for ethically approved scientific investigation (Research Ethics Committee number 10/H0716/3).

Total RNA was isolated from human post-mortem brain tissues using the miRNeasy 96 kit (Qiagen), processed with the Ambion® WT Expression Kit and Affymetrix GeneChip Whole Transcript Sense Target Labelling Assay, and hybridised to the Affymetrix Exon 1.0 ST Arrays following the manufacturers' protocols. Hybridised arrays were scanned on an Affymetrix GeneChip® Scanner 3000 7G and visually inspected for hybridisation artifacts.

Exon array data analysis

All arrays were pre-processed using Robust Multiarray Averaging (RMA; Irizarry et al., 2003) with quantile normalisation and GC background correction in Partek's Genomics Suite v6.6 (Partek Incorporated, St. Louis, MO, USA). In order to filter out low expression signals, detection above background (DABG) *P* values of exon probe sets were calculated using Affymetrix Power Tools v1.14.3 (APT, http://www.affymetrix.com/partners_programs/programs/

developer/tools/powertools.affx). After re-mapping the Affymetrix probe sets onto human genome build 19 (GRCh37) as documented in the Netaffx annotation file (HuEx-1_0-st-v2 Probeset Annotations, Release 31), we restricted analysis to 174,328 probe sets that had gene annotation, contained at least three probes with unique hybridisation and had DABG *P* < 0.001 in 50% of male or female individuals. We defined an expressed gene as any gene containing ≥ 1 exon with a median DABG *P* < 0.001 in at least 50% of male or female individuals in at least one brain region. The gene-level expression was calculated for 19,597 genes by calculating the Winsorised mean value (winsorizing the data below 10% and above 90%) of all probe set signals annotated to a single gene. Region-specific expression and splicing was investigated using Partek's mixed-model ANOVA and alternative splice ANOVA (Partek Genomics Suite v6.6). In all types of analysis, the date of array hybridisation, brain bank and gender were included as co-factors. All *P* values were corrected for multiple comparisons using Bonferroni correction.

Results and Discussion

GWAS are able to identify associations between phenotypes and genetic loci. Since only tagging SNPs and not all genetic variants are assessed in these studies it is not possible, solely from GWAS results, to accurately pinpoint the associated variant(s) or even gene(s). In this study, we have chosen to study, for each associated region, the gene considered as the most likely to be associated by either having been reported as such in the original GWAS or because it was considered as the most interesting gene in the region from a functional perspective in regards to AD pathobiology.

AD Loci

CR1—complement component (3b/4b) receptor 1; chromosome 1q32

GWAS have consistently identified intronic SNPs in *CR1* (rs6701713, rs1408077, rs3818361 and rs6656401) to be associated with an increased risk of AD onset (Fig. 1). The LD between these intronic SNPs and common coding variants in the same gene was investigated.

The genotyping of eight common coding SNPs in *CR1* in 96 pathologically confirmed late-onset AD cases revealed that the GWAS associated SNP rs6656401 was in LD with two coding SNPs: rs4844600 (p.Glu60Asp, NP_000642.3) and rs2296160 (p.Thr2419Ala, NP_000642.3), located in exons 2 and 44 respectively. The GWAS associated SNP rs3818361

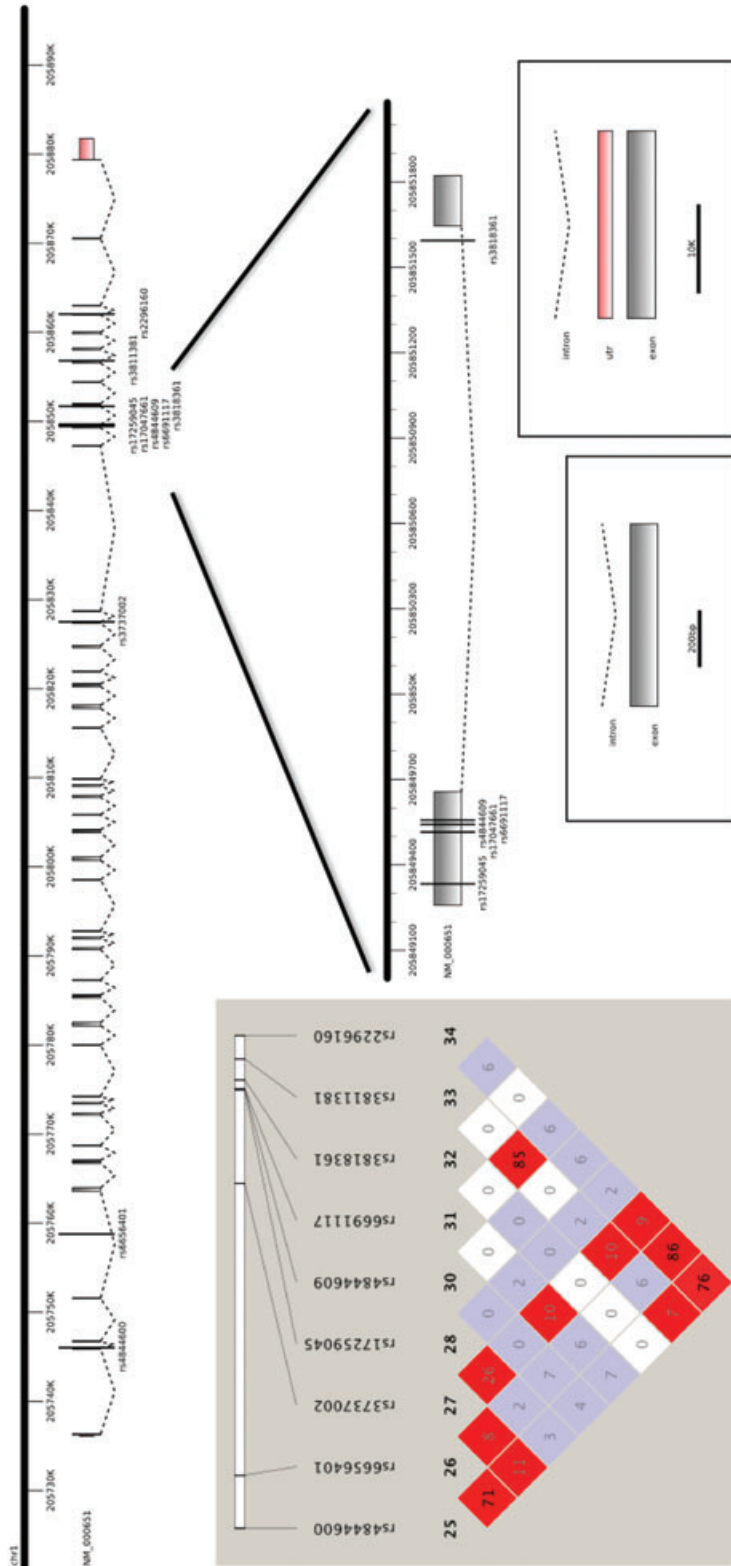


Figure 1 Representation of the *CR1* gene with the localization of the SNPs found as significant by GWAS as well as the SNPs studied here. On the left, linkage disequilibrium plot for the *CR1* SNPs found to be significant in GWAS and the common coding variants genotyped in this study.

was not in LD with any of the common coding SNPs studied here (Fig. 1).

The structure of the human *CR1* gene is complex. The gene is composed of tandem long homologous repeating segments that encode binding sites for C3b or C4b. Four *CR1* alleles differing in the total numbers of repeating segments are known and thought to have arisen through an homologous recombination with unequal crossover mechanism. The encoded protein is made up of four structurally significant domains. These are the signal peptide, extracellular, transmembrane and cytoplasmic domains (Wong, 1990). Three of these domains are homologous in each of the four allotypes but they differ in the lengths of the extracellular domain. This region is made up of short consensus repeats (SCRs) that are also known as complement-control-protein repeats (CCPs) (Klickstein et al., 1988). These repeats are highly conserved and are characterised by the presence of three cysteine residues and one tryptophan. In addition, there is a high degree of homology between every eighth SCR, thus grouping the SCRs into sevens. Each group of seven SCRs is termed a long homologous repeat (LHR) (Klickstein et al., 1988). The smallest allotype is rare and is termed either CR1-C or CR1-F', it has three LHR regions. The most common allotype is called CR1-A or CR1-F and has four LHR regions. The CR1-B or CR1-S allotype contains five LHR regions and the very rare CR1-D allotype contains six LHR regions (Holers et al., 1987). The extracellular LHR regions contain the binding sites for the protein, including the binding sites for the complement fragments C3b and C4b, thus individuals with different *CR1* alleles have different numbers of binding sites for these complement fragments and it is likely that this results in a different ability in the clearance of these fragments. There still remains some uncertainty about this process, mainly because it is currently poorly understood as to whether a decreased ability to clear complement fragments is beneficial or harmful in terms of AD aetiology. It had previously been thought that an increased ability to clear the complement fragments would be beneficial as it would decrease the activation of the complement system, generally thought of as pathogenic. However, it has been shown that individuals presenting CR1-B or CR1-S genotypes (the largest common allotype) actually have a raised risk of AD (Brouwers et al., 2012), indicating that an increased ability to clear the complement fragments C3b and C4b may be pathogenic in the situation of AD. Additionally, common variation at the *CR1* locus, more specifically rs6656401 has been shown to have a broad impact on cognition. This effect was shown to be largely mediated by an individual's amyloid plaque burden (Chibnik et al., 2011).

Using microarrays, the only brain regions in which CR1 could be detected were white matter and cerebellum and the expression levels in both regions were low. This would suggest that the role of CR1 in AD may be related to its function at

a systemic level or in relation to the brain vasculature. Taken together, our results suggest that *CR1* genetic variability does not act through different splicing variants or through differences in expression. The most probable scenario is that the variants found to be significant in GWAS and the coding variants found here to be in LD with these GWAS hits are tagging the structural variants known to exist in the *CR1* gene.

BIN1—bridging integrator 1; chromosome 2q14

Three SNPs in the *BIN1* locus (rs744373 (Hollingworth et al., 2011; Hu et al., 2011), rs7561528 (Hu et al., 2011; Naj et al., 2011) and rs12989701 (Hu et al., 2011) have been identified by GWAS as associated with LOAD. These three SNPs lie in a noncoding region upstream of *BIN1* and downstream of *CYP27C1*. In this locus, *BIN1* (encoding the 'bridging integrator 1' protein) appears to be the most likely functional candidate. Neither of the GWAS associated SNPs lie in regulatory regions, CpG islands or in microRNA target sites. Rs744373 is in a reported recombination hotspot (in HapMap) and all SNPs are in predicted transcription binding sites. There are only four non-synonymous SNPs published (NCBI SNP database, version 132) in *BIN1* and for these, MAFs are described for rs112318500 and only for African populations. We attempted to genotype two SNPs in *BIN1* in our cohort, rs76037557 and rs112318500, and were unable to identify the presence of the minor allele in any of the cases studied.

Although no significant eQTLs could be found for *BIN1* in our samples, it is interesting to note the regional expression differences in its expression within the CNS. On the basis of the microarray results, *BIN1* had the highest expression in white matter, with the mean gene expression being 5.1 times higher in white matter as compared to cerebellum (Bonferroni corrected $P < 1.0 \times 10^{-30}$). In addition, we found evidence of alternative splicing by brain region with one or more of three isoforms (NM_139343, NM_139344 and NM_139345, Bonferroni corrected alternative splicing $P < 1.0 \times 10^{-30}$) being lower in white matter as compared to all other CNS regions (Fig. 2).

Isoforms that are expressed in the central nervous system are thought to be involved in synaptic vesicle endocytosis and may interact with dynamin, synaptojanin, endophilin and clathrin. More specifically, BIN1 has been shown to be involved in both dynamin-mediated (Wigge and McMahon, 1998) and clathrin-mediated endocytosis (CME) (Pant et al., 2009). CME is thought to mediate the internalisation of Amyloid Precursor Protein (APP; MIM 104760) from the cell surface (Nordstedt et al., 1993), after which the A β peptide can be cleaved from the APP. Presence of the A β peptide in turn inhibits CME (Kelly and Ferreira, 2007) and therefore stops excess APP from entering the cell by a mechanism of

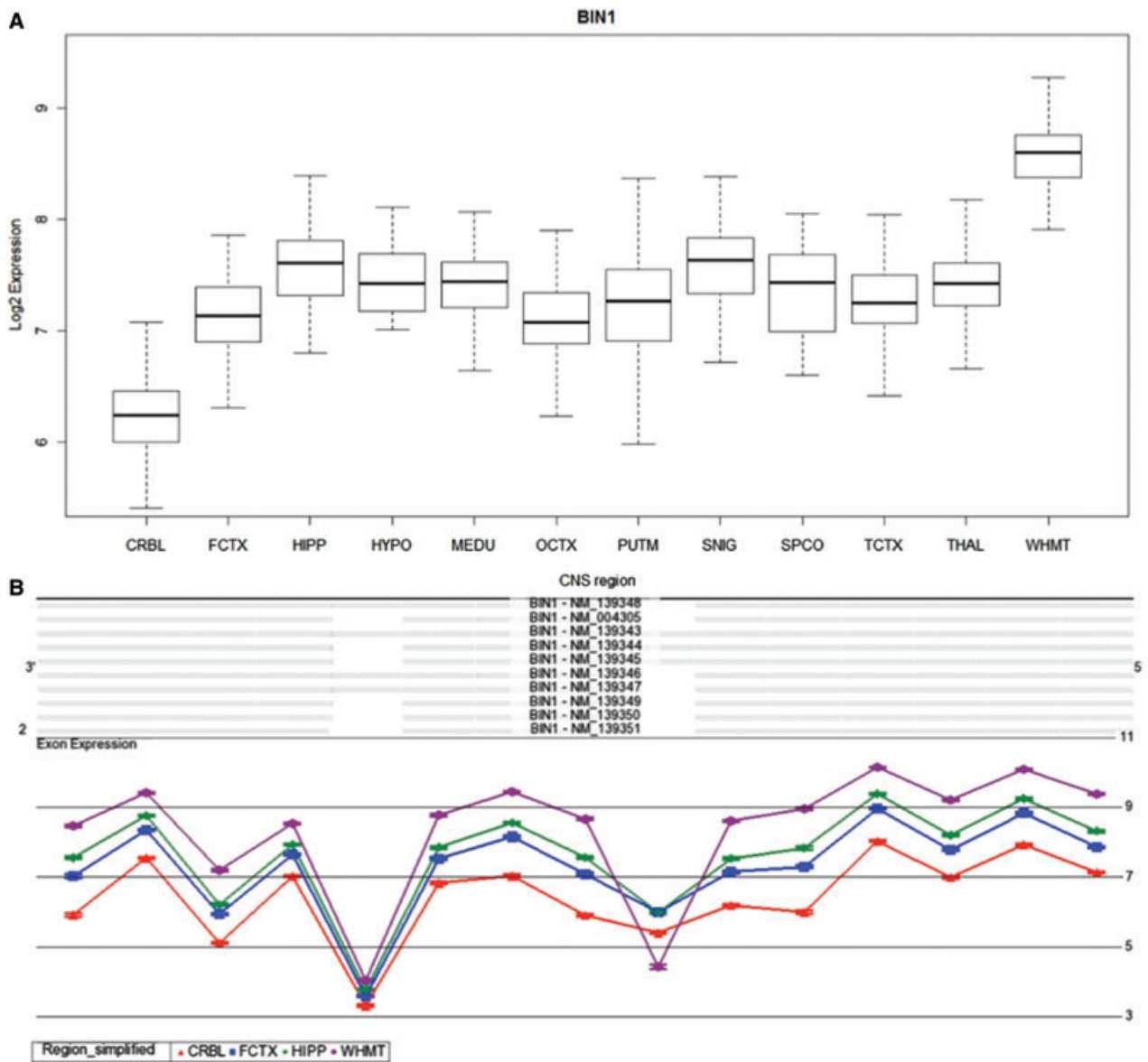


Figure 2 *BIN1* expression and splicing across the human CNS. (A) Boxplot showing *BIN1* expression across 12 human CNS regions, as measured using Affymetrix exon arrays in 137 neuropathologically normal individuals. (B) Plot of expression levels (y axis) for each probe set (x axis) for *BIN1* in cerebellum, frontal cortex, hippocampus and white matter, showing a statistically significant interaction between probe set expression (“exon usage”) and brain region. Non-parallel probeset expression levels (highlighted in the boxed region) indicate region-dependent differential splicing of the corresponding exon. Plots are adapted from Partek Genomics suite auto-generated output.

auto-inhibition. The *BIN1* protein may also have an important endocytic role being involved in synaptic vesicle recycling at the synaptic terminal (Di Paolo et al., 2002; Pant et al., 2009). Aberrant splice variants have been described to be expressed in tumor cell lines and misregulated alternative splicing of *BIN1* has been shown to be associated with T tubule alterations and muscle weakness in myotonic dystro-

phy (Fugier et al., 2011), which leads us to hypothesise a similar situation for the CNS isoforms.

Both *BIN1* and *PICALM* are part of endocytic pathways (Guerreiro and Hardy, 2011; Olgiati et al., 2011). The results we present here (Figs 2 and 3) show both higher expression of *BIN1* and a different complement of *BIN1* isoforms in white matter. Along with the different balance of isoforms for

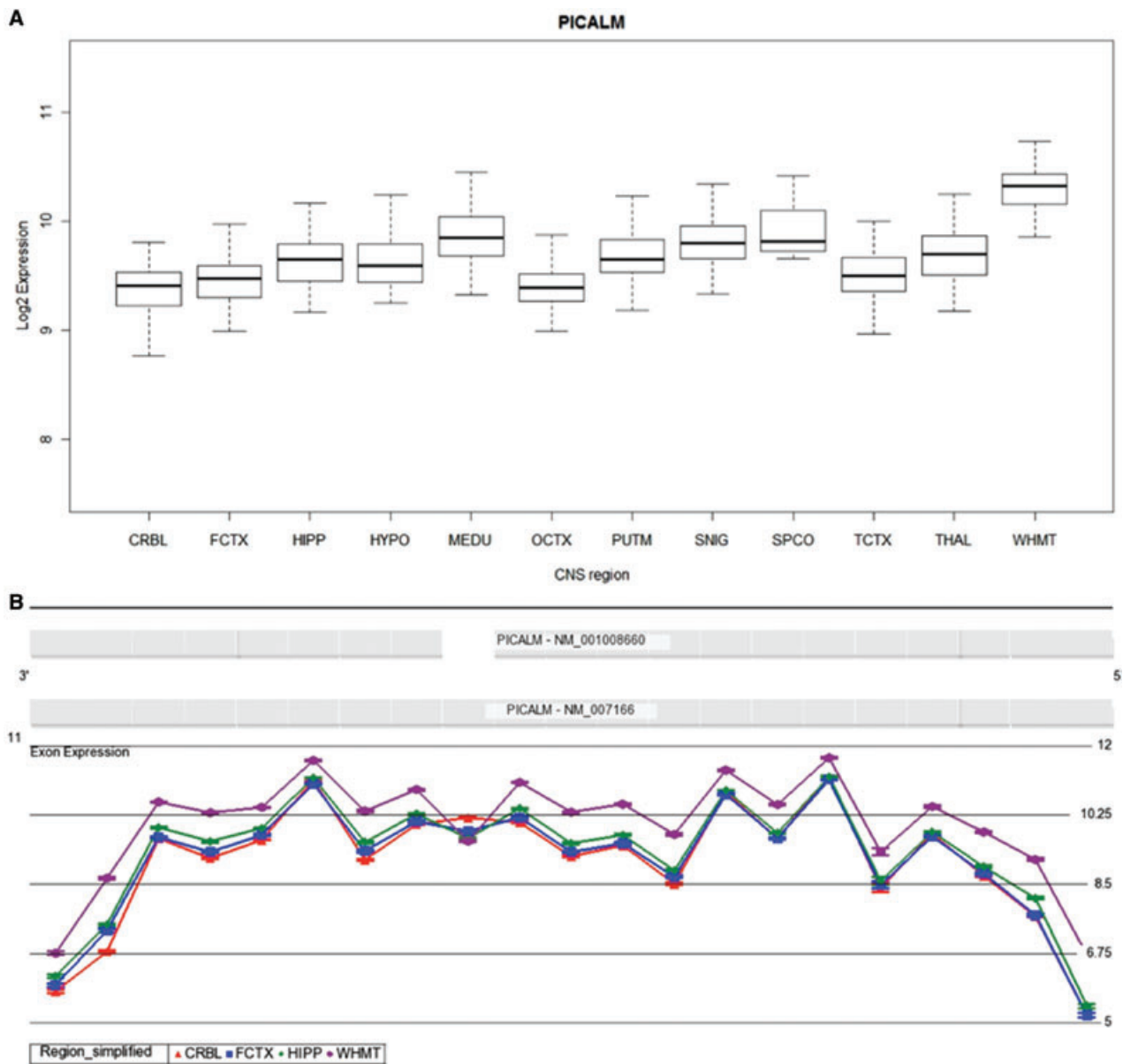


Figure 3 *PICALM* expression and splicing across the human CNS. (A) Boxplot showing *PICALM* expression across 12 human CNS regions, as measured using Affymetrix exon arrays in 137 neuropathologically normal individuals. (B) Plot of expression levels (y axis) for each probe set (x axis) for *PICALM* in cerebellum, frontal cortex, hippocampus and white matter, showing a statistically significant interaction between probe set expression (“exon usage”) and brain region. Non-parallel probeset expression levels (highlighted in the boxed region) indicate region-dependent differential splicing of the corresponding exon. Plots are adapted from Partek Genomics suite auto-generated output.

PICALM in white matter (for more details see next section) we speculate that endocytic pathways function in a distinct manner in white matter as compared to other brain regions.

AD has historically been characterised by neuronal loss and grey matter atrophy. More recently the involvement of white matter in the disease has started to be considered (Hua et al.,

2008). These results seem even more interesting in light of the association obtained by Braskie et al. (2011) between Clusterin rs11136000 and white matter microstructure in young adults. Although no replication dataset was available in this study, white matter disturbances in AD seem to be a common factor worthy of further analyses.

PICALM—phosphatidylinositol binding clathrin assembly protein; chromosome 11q14

There are four SNPs associated with the *PICALM* gene that have been identified in GWAS. These are rs592297 (Harold et al., 2009), rs561655 (Harold et al., 2009; Naj et al., 2011), rs541458 (Lambert et al., 2009; Corneveaux et al., 2010; Harold et al., 2009) and rs3851179 (Harold et al., 2009; Seshadri et al., 2010). The first of these SNPs is a synonymous variant (p.Gln174Gln) located in exon 5 of *PICALM* which may influence the activity of a sequence predicted to be an exon splicing enhancer. The other three SNPs are located upstream of the gene. rs561655 is found within a region that is thought to be a transcription factor binding site (Harold et al., 2009). It is possible that the change at this locus may increase or decrease the affinity of this region of the *PICALM* gene to transcription factors, thus changing the expression levels of the gene. rs541458 is located 8kb 5' of *PICALM* and has been shown to be in LD with rs3851179, located 88.5kb 5' of the gene (Harold et al., 2009). It is possible that the presence of both these SNPs in the 5' region outside the gene may have an effect on the expression of the gene.

Eleven non-synonymous SNPs have been described in the *PICALM* gene (NCBI SNP database, version 132). From these, two were reported to have an established heterozygosity >0.05 (rs118027183 and rs74727972). Genotyping these SNPs in our cohort did not reveal any case with the minor allele, thus it is not likely that the association seen in the GWAS is due to common coding variability in this gene.

Similarly to BIN1, *PICALM* is involved in CME (Dreyling et al., 1996; Tebar et al., 1999; Yao et al., 2005) and has been shown to have a particular influence on the activity of VAMP-2 (Harel et al., 2008), a SNARE protein responsible for directing neurotransmitter vesicles to the presynaptic membrane. This role of the protein, allied to the observation of a reduced synaptic density in the brains of AD patients, suggests that the activity of this protein is potentially important in disease aetiology.

The mean gene expression of *PICALM* was found to be 1.9 times higher in white matter as compared to cerebellum (Bonferroni corrected $P < 1.0 \times 10^{-30}$). Two *PICALM* isoforms are detected by the exon array: full-length *PICALM* and a shorter form that lacks exon 13. Our results show that the short form of *PICALM* is expressed at lower levels in white matter as compared to the other CNS regions, in particular cerebellum (Fig. 3, Bonferroni corrected alternative splicing $P < 1.0 \times 10^{-30}$).

Several methQTL associations were significant after multiple test correction. In cerebellum, these include associations with CpG sites in *APOC1*, *BCL3* and *CBLC* on chromosome 19, as well as *ME3*, *MGC34732* and *MS4A6E* on chromosome 11. In frontal cortex, six significant associations with

a CpG site mapping to *APOE*, as well as one association at *APOC2* were identified. From these, only the association observed between rs10751134 in *PICALM* and a CpG site in *ME3* (a gene adjacent to *PICALM*, Fig. 4) is in moderate LD with the GWAS SNPs studied here (r^2 between rs10751134 and rs561655, rs3851179, rs541458 in HapMap populations varies between 0.5 and 0.6) and may contribute to the genome-wide association signal found for *PICALM*.

MS4A6A/MS4A4E—membrane-spanning 4-domains, subfamily A, members 6A and 4E; chromosome 11q12.1

There are three SNPs in the *MS4A* gene cluster that have been associated with an increased risk of LOAD. These are rs4938933 in *MS4A4A* (Hollingworth et al., 2011; Naj et al., 2011), rs670139 in *MS4A4E* (Hollingworth et al., 2011; Naj et al., 2011) and rs610932 in *MS4A6A* (Hollingworth et al., 2011). Several SNPs are described in these genes. In order to have a comprehensive view of the locus we targeted non-synonymous coding SNPs in *MS4A6A*, *MS4A4E* and *MS4A4A* (Table 1). As expected the SNPs shown to be significantly associated with AD in the previous GWAS were in LD with each other. No significant LD was observed between these AD risk SNPs and the targeted coding SNPs studied here (highest $r^2 = 0.5$ between rs610932 and rs7232), indicating that non-synonymous common genetic variability in the *MS4A4* locus probably does not explain the associations established in the GWAS.

The role of the genes located in the *MS4A* cluster is so far poorly understood. The cluster is found on chromosome 11 and is made up of at least 16 genes (Liang and Tedder, 2001). It has been suggested that the proteins encoded by the cluster may be ion channels or adaptor proteins (Liang et al., 2001; Zuccolo et al., 2010). It is likely that the genes in the cluster all have a similar role due to their high homology (Liang et al., 2001). However, until more is known of their function it is impossible to speculate as to the potential role of polymorphisms in these genes in AD aetiology. Whereas *MS4A6A* was detected in all brain regions, using microarrays, we were unable to confidently detect *MS4A4A* in cerebellum, frontal cortex or occipital cortex.

ABCA7—ATP-binding cassette, subfamily A (ABC1), member 7; chromosome 19p13.3

Two SNPs in *ABCA7* have been associated with LOAD: rs3752246 and rs3764650 (Hollingworth et al., 2011; Naj et al., 2011). rs3752246, in exon 32 of the gene, leads to a protein change (p.Gly1527Ala) which was the only coding non-synonymous change to be identified by GWAS. In an attempt to identify an associated functional variant at the *ABCA7* locus, Hollingworth et al. chose to genotype rs3752246 in an

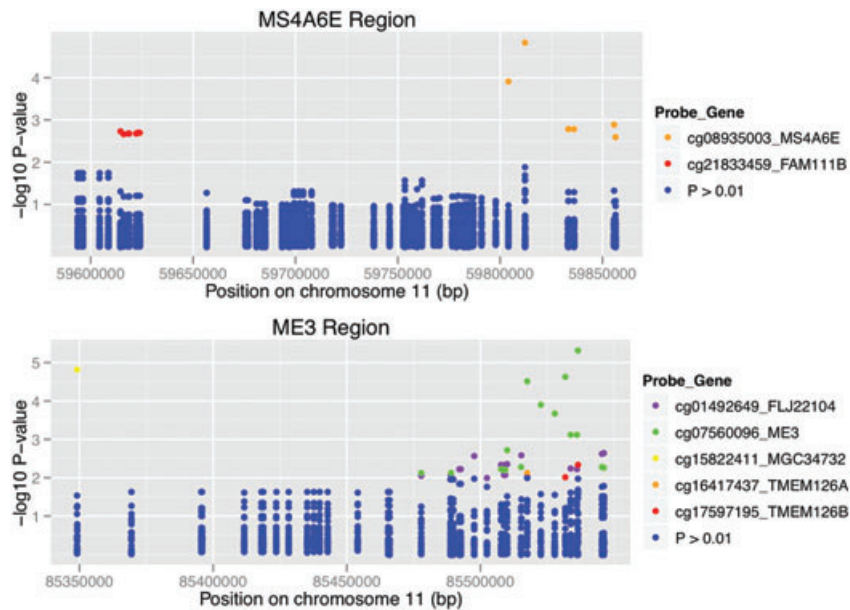


Figure 4 Association between genotypes and CpG sites in cerebellum. Here the results are shown as log-transformed P values colour-coded to match the CpG probe in the loci. The regions associated in chromosome 11 are represented: in the top panel the *MS4A6E* locus and in the bottom panel the *ME3* region. From these, only rs10897024 in *PICALM* (associated with a P value of 4.65×10^{-6} with the probe cg07560096 in *ME3*, represented in green) is in moderate LD with the GWAS hits for *PICALM* (rs561655, rs3851179 and rs541458).

additional cohort because this was a non-synonymous SNP with the highest LD with rs3764650 of all HapMap *ABCA7*-coding variants based on r^2 values ($r^2 = 0.36$, $D' = 0.89$; Hollingworth et al., 2011). Although, in our study, we established a lower degree of LD ($r^2 = 0.1$ between rs3764650 and rs3752246), this was also the highest LD found between rs3764650 and all the coding SNPs studied here (Fig. 5). This suggests that the risk of AD conferred by the presence of rs3764650 is not due to the presence of multiple other common coding SNPs in the gene. rs3764650 SNP is found at position 115 of the intron between exons 13 and 14. There is no evidence to suggest that rs3764650 has an effect on the expression of the gene (Hollingworth et al., 2011). rs3752246 is predicted *in silico* to be a benign variant (Polyphen-2 score = 0). This variant is in moderate LD ($r^2 = 0.6$) with rs4147934, which is also predicted to be non-pathogenic. Nonetheless, rs3752246 was the only missense change to be identified by GWAS and functional studies of the real impact of these two variants at the protein level should further elucidate if any of these are the real risk associated variants in *ABCA7*.

ABCA7 had low, but detectable expression in all CNS regions with no striking regional differences in gene expression patterns (data not shown).

The *ABCA7* gene is a member of a large family of ATP-binding cassette genes divided into the subfamilies A–G based upon sequence homology (Kim et al., 2008). The transporters of the A and G subfamilies are particularly responsible for the movement of lipids such as sterols, phospholipids and bile acids across membranes against the concentration gradient of the substrate (Schmitz et al., 2000; Schmitz and Kaminski, 2001; Kusuhara and Sugiyama, 2007). *ABCA1* is known to have a role in the transport of cholesterol to lipid-free acceptors such as apoA-I and apoE. The high homology between *ABCA1* and *ABCA7* suggests that the two proteins should share a similar role. The high lipid content of the CNS means that lipid homeostasis is essential and so, changes in the ability to transport cholesterol, are potentially pathogenic. It has been shown that the levels of cholesterol influence the processing of the APP protein: during times of high intracellular content, the activity of α -secretase is inhibited, whilst the activity of β - and γ -secretases is enhanced (Bodovitz and Klein, 1996; Tun et al., 2002; Kalvodova et al., 2005; Vetrivel et al., 2005). The role of *ABCA7* in this situation is currently unclear as there is conflicting evidence regarding its ability to transport cholesterol: work with *ABCA7*^{-/-} mice has shown that cholesterol efflux is not dependent upon *ABCA7* (Kim et al., 2005);

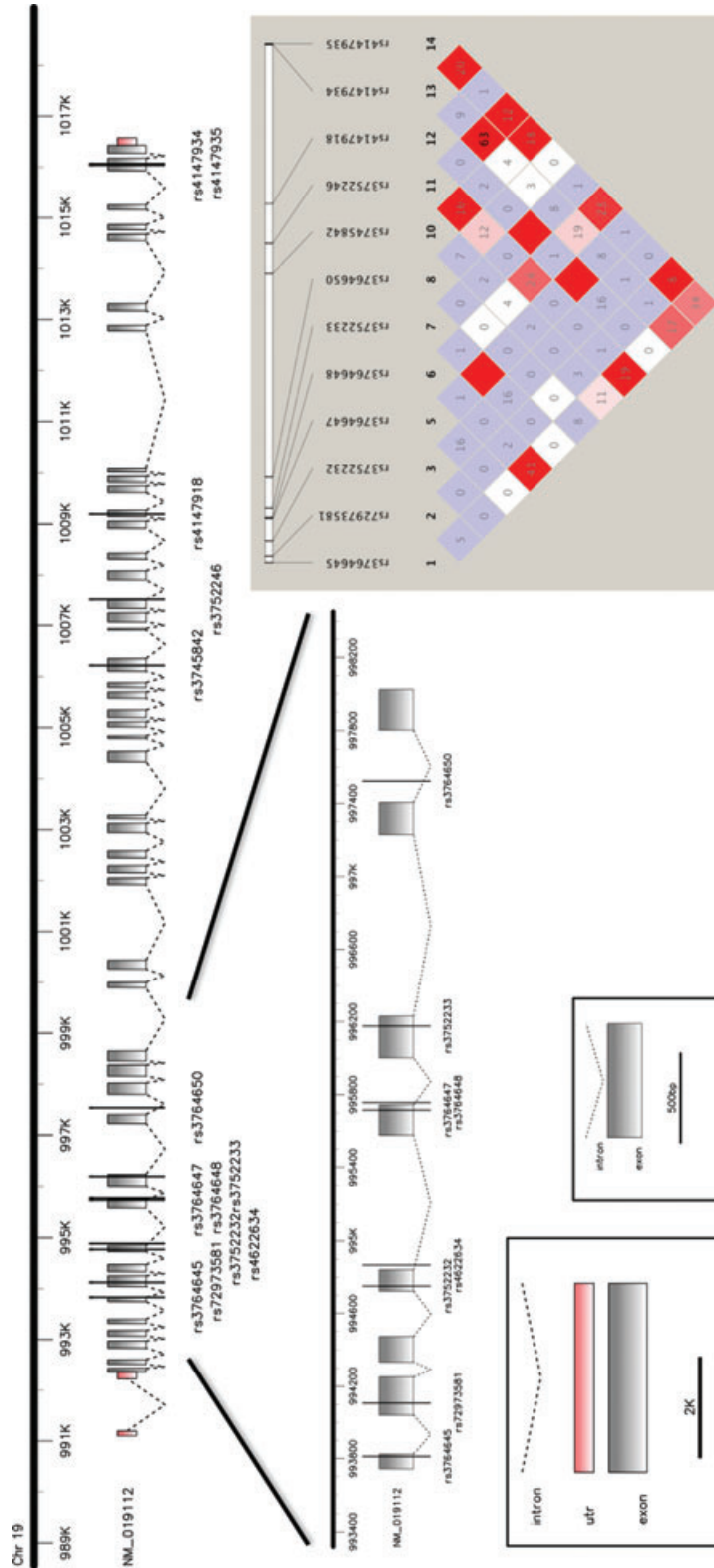


Figure 5 Representation of *ABCA7* with the localisation of the SNPs found as significant by GWAS as well as the SNPs studied here. On the right, linkage disequilibrium plot for the *ABCA7* SNPs found to be significant in GWAS and the common coding variants genotyped in this study.

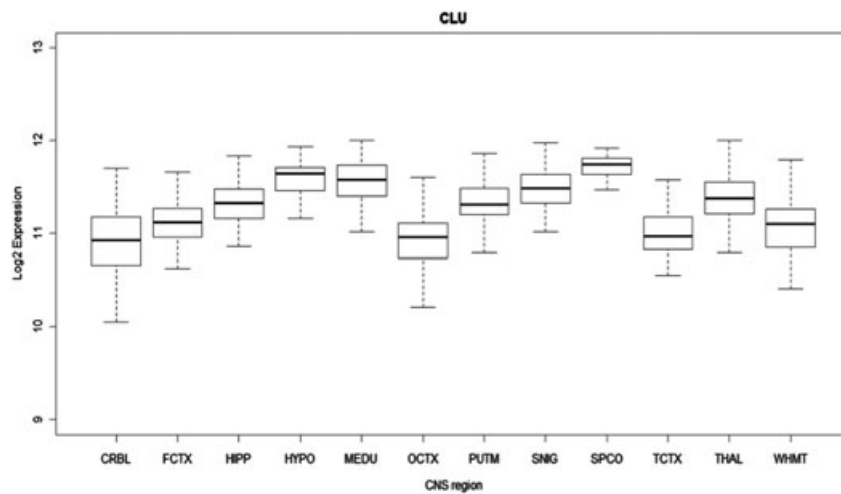


Figure 6 Boxplot showing *CLU* expression across 12 human CNS regions, as measured using Affymetrix exon arrays in 137 neuropathologically normal individuals.

however work with human embryonic kidney (HEK)293 cells has shown that transfection with *ABCA7* cDNA leads to 1.7–2 times increase in cholesterol efflux (Chan et al., 2008). It is likely therefore that *ABCA7* plays a role in cholesterol transport, but that this is a process that also involves other proteins.

CLU—clusterin; chromosome 8p21-p12

The *CLU* gene encodes the clusterin or apolipoprotein J protein, expressed ubiquitously but with a known higher prevalence in the brain, ovary, testis and liver (de Silva et al., 1990). Levels of clusterin are shown to be elevated in the cortex and hippocampus areas of the brains of AD patients (Oda et al., 1994). Although we found high expression of *CLU* throughout the control CNS, we were unable to demonstrate that this gene was more highly expressed in cortex or hippocampus relative to other CNS regions (Fig. 6). Clusterin binds to $A\beta$ plaques in the cerebrospinal fluid, forming a complex that is able to cross the blood–brain barrier (Zlokovic, 1996). Levels of clusterin in the plasma are positively correlated with the risk of AD (Schrijvers et al., 2011). It is therefore possible that the increased risk of AD induced by the SNPs described may be due to an increased level of expression.

We previously sequenced the exonic regions of *CLU* in 495 AD cases and 330 healthy controls and found a total of 24 variants in both cases and controls with similar frequencies between groups, indicating that common coding variability in this gene does not underlie the association seen with the intronic SNPs (Guerreiro et al., 2010). In order to determine if common variants at the *CLU* locus effect expression of nearby (*cis*) mRNA transcripts, an eQTL analysis was also performed. No significant eQTL associations were observed for the SNPs previously associated with AD, which led us

to conclude that the most likely mechanism underpinning the association is either small effects of genetic variability on resting gene expression, or effects on damage induced expression of the protein. These conclusions are also supported by the absence of significant differences in the expression of the gene between several brain regions and in the gene splicing (Fig. 6). More recently, rare coding variants in *CLU* have been associated with the risk for AD (Bettens et al., 2012). However, this variability was found to be independent of the common association signal identified by the GWAS. Small studies have also reported an association between rs9331888 and alternative splicing of *CLU* (Szymanski et al., 2011) and blood clusterin levels (Xing et al., 2012).

Conclusion

Clearly, developing an understanding of the nature and mechanism of loci for AD (and other neurological diseases), which are identified by GWAS and are not coding changes is going to be a considerable challenge (Table 2). This study shows that simple eQTL studies in control brain tissue may not identify effects in many cases. There remain several options which are not mutually exclusive: (1) the study is underpowered (although larger than most previous studies) (2) genetic variability in splicing is an important consideration (3) genetic variability in other RNA species at the locus is important besides the obvious mRNA (4) genetic variability in damage induced expression, and not in resting expression, is the important factor.

The first three possibilities can be gradually overcome by either more samples or by improvements (for example) in sequencing technologies, which would allow transcript QTLs

Table 2 Initial loci identified by GWAS in AD and predicted type of causal variants in each locus.

GWAS identified loci	
APOE ¹	Both amino acid and eQTL effects
CLU	Not common amino acid change/Possible alternative splicing
CR1	Exon insertion polymorphism
BIN1	Not common amino acid change
PICALM	Not common amino acid change/Possible methQTL
ABCA7	Probably amino acid change
MS4A6A/MS4A4E	Not common amino acid change

¹Locus not studied here.

to be assessed as well as QTLs for other RNA species. Understanding QTLs in damage-induced expression is inherently difficult (Webster et al., 2009). Measuring changes in damage induced expression in tissue with changing cell populations and developing rigorous algorithms to interpret such data is problematic, but may be necessary, especially for understanding the aetiology of late onset neurodegenerative diseases.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 SNPs found associated with LOAD by GWAS assessing >1500 cases and >1500 controls.

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