Genome-wide pathway analysis of memory impairment in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort implicates gene candidates, canonical pathways, and networks

Vijay K Ramanan[1,2,3], **Sungeun Kim**[1,4], **Kelly Holohan**[1,2], **Li Shen**[1,4], **Kwangsik Nho**[1,4], **Shannon L. Risacher**[1], **Tatiana M. Foroud**[2,4], **Shubhabrata Mukherjee**[5], **Paul K. Crane**[5], **Paul S. Aisen**[6], **Ronald C. Petersen**[7], **Michael W. Weiner**[8,9], and **Andrew J. Saykin**[1,2,4,*] **for the Alzheimer's Disease Neuroimaging Initiative (ADNI)**[Ω]

[1]Center for Neuroimaging, Department of Radiology and Imaging Sciences, Indiana University School of Medicine, Indianapolis, IN, USA

[2]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA

[3]Medical Scientist Training Program, Indiana University School of Medicine, Indianapolis, IN, USA

[4]Center for Computational Biology and Bioinformatics, Indiana University School of Medicine, Indianapolis, IN, USA

[5]Department of Medicine, University of Washington, Seattle, WA, USA

[6]Department of Neuroscience, University of California, San Diego, San Diego, CA, USA

[7]Department of Neurology, Mayo Clinic Minnesota, Rochester, MN, USA

[8]Center for Imaging of Neurodegenerative Diseases, San Francisco VA Medical Center, San Francisco, CA, USA

[9]Department of Radiology and Biomedical Imaging, University of California, San Francisco, San Francisco, CA, USA

Abstract

Memory deficits are prominent features of mild cognitive impairment (MCI) and Alzheimer's disease (AD). The genetic architecture underlying these memory deficits likely involves the combined effects of multiple genetic variants operative within numerous biological pathways. In order to identify functional pathways associated with memory impairment, we performed a pathway enrichment analysis on genome-wide association data from 742 Alzheimer's Disease Neuroimaging Initiative (ADNI) participants. A composite measure of memory was generated as the phenotype for this analysis by applying modern psychometric theory to item-level data from the ADNI neuropsychological test battery. Using the GSA-SNP software tool, we identified 27 canonical, expertly-curated pathways with enrichment (FDR-corrected p-value < 0.05) against this composite memory score. Processes classically understood to be involved in memory consolidation, such as neurotransmitter receptor-mediated calcium signaling and long-term potentiation, were highly represented among the enriched pathways. In addition, pathways related

Corresponding Author: (asaykin@iupui.edu). Phone (317)278-6947. Fax (317)274-1067. .

to cell adhesion, neuronal differentiation and guided outgrowth, and glucose- and inflammation-related signaling were also enriched. Among genes that were highly-represented in these enriched pathways, we found indications of coordinated relationships, including one large gene set that is subject to regulation by the *SP1* transcription factor, and another set that displays co-localized expression in normal brain tissue along with known AD risk genes. These results 1) demonstrate that psychometrically-derived composite memory scores are an effective phenotype for genetic investigations of memory impairment and 2) highlight the promise of pathway analysis in elucidating key mechanistic targets for future studies and for therapeutic interventions.

Keywords

INTRODUCTION

Memory deficits are prominent features of Alzheimer's disease (AD) and of mild cognitive impairment (MCI), a transitional state with significant risk of progression to clinically-diagnostic AD (Aisen et al. 2010). While genomic studies of MCI and AD susceptibility are frequent and ongoing, surprisingly, there has been less large-scale research on the genetic variants specifically related to memory impairment. Genetic studies, especially of quantitative traits (QT) such as memory, are highly dependent on the quality of the phenotypic data. There are numerous extant metrics for assessing memory in amnestic populations, and these metrics can have differential sensitivities to various deficits (Lezak 2004). As a result, studies often attempt to leverage the relative strengths and weaknesses of these metrics by creating composite memory scores from multiple assessments given to study participants (Barbeau et al. 2011; Sloan et al. 2010). In addition, emerging evidence suggests that applying modern psychometric theory to the creation of these composite scores may yield an optimized measure of memory functioning that serves as a more powerful phenotype for genetic studies (see (Crane et al. 2011) and the other companion papers in this issue for more detail).

In addition, it is clear from human and animal studies that the complex processes of memory consolidation and recall involve numerous and diverse cellular and molecular pathways (Sweatt 2009). While genome-wide association (GWA) studies of complex phenotypes have historically focused on identifying individual susceptibility loci, their efficacy has been confounded by three factors: 1) most common alleles implicated by GWA studies have exhibited modest effect sizes (Chee Seng et al. 2010); 2) gene and gene variant associations do not always indicate therapeutic targets (Penrod et al. 2011); and 3) it is well-understood that genes do not exist in isolation, but instead function as sets within biological pathways and networks (Schadt 2009). As a result, GWA studies of complex phenotypes are increasingly being analyzed through statistical methods designed to identify gene sets with significant relationships to those phenotypes (Hirschhorn 2009; Ramanan et al. 2012).

As a particularly prominent approach, pathway enrichment methods analyze genomic data through gene sets representing biological pathways. Pathway enrichment analysis uses the association signals of genomic markers within a pathway to determine whether that pathway is enriched (i.e., has a statistically significant association) against a phenotype. While many tools and strategies for pathway enrichment analysis have been developed in recent years, to date there is no gold standard approach (Cantor et al. 2010; Wang et al. 2010). Nevertheless, despite using diverse strategies, high-profile investigations of numerous complex disorders, including breast cancer (Li et al. 2011), type 2 diabetes (Zhong et al. 2010), and Multiple

Sclerosis (Sawcer et al. 2011), suggest that pathway analysis of GWA study data can reveal larger effects that are otherwise concealed from standard single locus analysis.

While GWA-based pathway analysis has been used against complex neurological phenotypes, including brain glutamate levels (Baranzini et al. 2010), cerebrospinal fluid (CSF) amyloid-β42 peptide levels (Han et al. 2010), and information processing speed (Luciano et al. 2011), to our knowledge this strategy has not been previously applied to a quantitative memory phenotype. In this study, we use enrichment analysis of GWA data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort to identify pathways associated with changes in a psychometrically-derived composite episodic memory score. In addition, we identify genes with high representation in these pathways as key targets for future studies of memory deficits. Finally, using transcription factor network analysis and data from a human brain tissue expression atlas, we isolate sets of these gene targets which are co-regulated and/or co-expressed, suggesting that memory-impaired phenotypes are the result of gene variants exerting their effects through complex pathway and network interactions. The collection of enriched pathways, gene targets, and gene networks identified in this analysis 1) represent prime targets for further studies of memory impairment and normal memory processes and 2) further demonstrate the efficacy of pathway-based approaches to analyzing GWA data on complex phenotypes.

METHODS

Alzheimer's Disease Neuroimaging Initiative (ADNI)

Data used for this study were obtained through the ADNI database (http://adni.loni.ucla.edu/). ADNI was launched in 2003 (PI: Michael W. Weiner, MD, VA Medical Center and University of California-San Francisco) with the goal of evaluating biomarkers of AD-related neuropathology in patients with MCI and early AD. This multi-site longitudinal study is supported by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies, and non-profit organizations. Participants in ADNI include older individuals, aged 55-90 years, who were recruited from 59 sites across the United States and Canada. These subjects include approximately 200 cognitively normal patients (CN), 400 patients diagnosed with MCI, and 200 patients diagnosed with early probable AD. As described elsewhere (Jack et al. 2009; Weiner et al. 2010; Aisen et al. 2010), diagnoses of participants were made on a clinical basis (via neuropsychological assessment data and patient and informant reports of cognitive performance and functioning in activities of daily living) at consensus conferences involving neurologists, neuropsychologists, and study coordinators. Written informed consent was obtained for all participants and prior Institutional Review Board approval was obtained at each participating institution. All demographic information, neuropsychological and clinical assessment data, and diagnostic information used in this study were downloaded from the ADNI clinical data repository (http://adni.loni.ucla.edu/). Genotype data used in this study include genome-wide single nucleotide polymorphism (SNP) data obtained from a GWAS on the full ADNI sample (Saykin et al. 2010) and *APOE* ε4 allele status. Further information about ADNI can be found in (Weiner et al. 2010) and at http://www.adni-info.org/.

Participants

Seven hundred and forty-two non-Hispanic Caucasian participants (188 AD, 396 MCI, and 226 CN at baseline) from the ADNI cohort with baseline composite episodic memory scores (see later section) and genotype data passing quality control procedures (see later section) were included in the present analyses (Table 1). Sample characteristics were evaluated using

the IBM SPSS 19.0 statistical software (SPSS, Inc., Chicago, IL). The sample was analyzed for differences across diagnostic groups using a one-way analysis of variance (ANOVA) for continuous variables and a Pearson chi-square test for categorical variables.

Composite Episodic Memory Scores

All ADNI participants (original $N = 818$) were administered an extensive neuropsychological assessment, including several measures of memory, at each study visit. For each subject, a composite score for episodic memory at the baseline visit was calculated by applying modern psychometric theory to item-level data from the ADNI neuropsychological battery. This psychometrically-derived episodic memory score was developed for the ADNI cohort as part of the 2011 Friday Harbor Workshop on Advanced Psychometrics (see (Crane et al. 2011) and the other companion papers in this special issue for details). Briefly, the authors used psychometric theory to select test battery items which could be considered as indicators of episodic memory functioning. An iterative process of confirmatory factor analysis was used to construct the final, optimized model for describing episodic memory performance at baseline. In particular, the following item-level tests were applied to the final model: the memory sub-scores from the Mini-Mental Status Examination (Folstein et al. 1975; Cockrell and Folstein 1988); the immediate and delayed recall and recognition scores on a word list learning task from the Alzheimer's Disease Assessment Scale-cognitive subscale (Mohs et al. 1997); all immediate and delayed recall and recognition scores from the Rey Auditory Verbal Learning Test (Rey 1964); and all immediate and delayed recall scores on Logical Memory prose passages from the Wechsler Memory Scale-Revised (Wechsler 1987). The final model exhibited excellent fit based on standard criteria (Confirmatory Fit Index > 0.95, Tucker Lewis Index > 0.95, and Root Mean Squared Error of Approximation < 0.05) (Reeve et al. 2007). Notably, a composite episodic memory score could not be calculated for eight participants due to incomplete item-level data at baseline.

Genotyping and Quality Control

The acquisition and processing of genotype data for the ADNI sample have been previously described in detail (Saykin et al. 2010). All participants analyzed in this study were genotyped according to the manufacturer's protocol using the Human610-Quad BeadChip (Illumina, Inc., San Diego, CA), which included 620,901 SNPs and copy number variant markers. In addition, given the strong association of its genetic variants with MCI and AD susceptibility (Bertram et al. 2010; Corder et al. 1993; Strittmatter et al. 1993), *APOE* genotype status was also of interest. Since the two *APOE* SNPs (rs429358, rs7412) that characterize the ε2, ε and ε4 risk loci alleles were not directly available on the Human 610-Quad BeadChip, these SNPs were genotyped separately and their data were made available in the ADNI database (Potkin et al. 2009; Saykin et al. 2010).

The ADNI genotype data (original $N = 818$ participants) was subjected to standard quality control procedures as previously described (Shen et al. 2010) using Plink, version 1.07 (Purcell et al. 2007). Genotype markers were excluded if they had a call rate < 90%, Hardy-Weinberg equilibrium test < 10^{-6}, or minor allele frequency < 5%. Samples were excluded if they had a call rate < 90% (1 participant), ambiguous gender identification (2 participants), or failed an identity check (3 participants). In order to select non-Hispanic Caucasian participants, multidimensional scaling analysis was performed on the combined genotype data from ADNI and from reference populations in HapMap phase 3, release 2 (International HapMap 2003); this resulted in the exclusion of 62 participants. Following all quality control procedures, 750 participants and 531,096 SNPs were designated for subsequent analyses and the genotyping rate was > 0.995 among the remaining samples.

Genome-wide Association (GWA) Analysis

Of the 750 subjects who passed sample quality control procedures, 742 subjects were identified as having a composite, psychometrically-defined episodic memory score at baseline. To generate input data for pathway analysis, a GWA analysis against this composite memory score was performed using an additive genetic model with the linear association analysis for quantitative traits in Plink. Demographic factors with known influences on memory or cognition were included as covariates in this analysis: these factors included age at the baseline visit, education, gender, and handedness. The direct and inverse relationships, respectively, of age and education level on memory decline have been well-established (Negash et al. 2011), while putative effects of gender and handedness on cognition are subjects of active exploration (Andreano and Cahill 2009; Gunstad et al. 2007). In addition, *APOE* ε4 allele status (presence vs. absence) was also used as a covariate in the GWA analyses to account for the largest known genetic influence on memory performance in an MCI- and AD-based clinical population (Bookheimer and Burggren 2009). For all SNPs included in the analysis, a *p*-value was generated by the model, representing the nominal association of that genotype marker to the composite memory score. Manhattan and Quantile-Quantile (Q-Q) plots for the GWA analysis were generated using Plink, Haploview (Barrett et al. 2005), and the R statistical package, version 2.15.

Genome-wide Pathway Analysis

Pathway analysis of the GWA results was performed to identify functional gene sets with significant association to the composite memory phenotype. All SNPs included in the GWA analysis were mapped to genes using the NCBI Build 36.1 reference sequence (International Human Genome Sequencing Consortium 2004). A gene mapping window of 20kb was used to account for SNPs belonging to putative regulatory regions; notably, this resulted in some SNPs being mapped to more than one gene. In total, 277,615 SNPs were assigned to 17,456 genes. Pathway annotations, representing gene sets defined by membership in biological pathways, were downloaded from the Molecular Signatures Database (Subramanian et al. 2005), version 3.0 (http://www.broadinstitute.org/gsea/msigdb/index.jsp/). This annotation data comprised a collection of canonical, expertly-curated gene sets from three publically-available pathway databases, BioCarta (http://www.biocarta.com/), the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/), and Reactome (http://www.reactome.org/). In total, 818 gene sets were downloaded, included 217 sets from BioCarta, 186 sets from KEGG, and 430 sets from Reactome. Analysis of these gene sets was restricted to those containing between 10 and 200 genes; as a result, 280 total gene sets were analyzed for enrichment.

The GSA-SNP software (Nam et al. 2010) was used to assess for pathways enriched against the composite memory score. This software uses a competitive enrichment algorithm (Goeman and Bühlmann 2007), where the null hypothesis holds that a pathway-phenotype association is not significantly different from all other pathway-phenotype associations under analysis. Competitive enrichment strategies are robust to the effects of genomic inflation due to population stratification or other confounding factors (Holmans 2010; Fridley and Biernacka 2011). In GSA-SNP, the significance score for each gene under analysis was calculated as the -log of the *k*-th best SNP-level *p*-value in the gene. Corresponding with the authors' recommendation (Nam et al. 2010), we selected $k = 2$ to limit the effects of both single, highly-significant loci and of spurious SNP-level associations on driving pathway enrichment. Each gene set (representing a pathway) was then assessed for phenotype enrichment by the *Z*-statistic method (Kim and Volsky 2005), which incorporates the gene-wide significance scores and the number of genes within each set. In addition, since small pathways can exhibit spurious phenotype associations due to large single locus effects (Holmans 2010), and since large pathways are more likely to

exhibit association by chance alone (Elbers et al. 2009), analysis was restricted to gene sets containing 10-200 genes. To correct for multiple hypothesis testing, the False Discovery Rate (FDR) (Benjamini and Hochberg 1995) was applied to the p-values generated by the enrichment algorithm. For pathways enriched at an FDR-corrected p-value < 0.05, we analyzed their constituent genes to obtain a count of each gene's occurrences in those enriched pathways. Genes that were highly-represented among the enriched pathways (defined as being constituents of > 15%, i.e., 5, of the enriched pathways) were isolated for two follow-up analyses.

Transcription Factor Network Analysis

We further investigated the list of highly-represented genes from our enriched pathways through network analysis using MetaCore (GeneGo, Inc.). In particular, we applied the transcription factor network analysis algorithm to identify subsets of those genes with coordinate regulation by known transcription factors. In addition, *APOE* was also included in these network analyses, given its well-characterized association with MCI and AD and their related memory deficits.

Gene Expression Analysis Using the Allen Human Brain Atlas

We also interrogated the list of highly-represented genes from our enriched pathways for their expression profiles in normal brain tissue using the Allen Human Brain Atlas (Allen Institute for Brain Science, Seattle, WA; available from http://www.brain-map.org/). The Allen Human Brain Atlas (AHBA) includes genome-wide microarray-based expression profiles in postmortem brain tissue from subjects with no known neuropsychiatric or neuropathological history. These expression profiles cover the entire brain through systematic sampling of regional tissue, and are integrated with multi-modal brain imaging and other data for visualization and analysis. Detailed information on the AHBA is available on-line (http://human.brain-map.org/docs.html/). We employed the AHBA to examine genes of interest (including highly-represented genes from our enriched pathways) for patterns in their expression profiles. In particular, we used the heat map tool (which visually displays normalized expression for a gene probe across 25 large neuroanatomic regions) and correlational analysis (which calculates a Pearson correlation coefficient between the expression profiles of two gene probes) to identify a set of key genes with high ($r > 0.7$) co-localization and co-expression.

RESULTS

Demographic characteristics and mean composite memory scores for all diagnostic groups (CN, MCI, and AD) are presented in Table 1. While baseline age and handedness were not significantly different across diagnostic groups, gender exhibited a significant difference (p < 0.05), with males relatively overrepresented among MCI subjects. In addition, as expected, education level and *APOE* ε4 allele status exhibited significant differences across groups (p < 0.001). Also as expected, composite memory scores differed across all diagnostic groups, including all pairwise group comparisons (p < 0.001).

In order to assess SNP associations to the composite memory scores in this sample, we performed a GWA analysis with the addition of five covariates. The GWA analysis failed to identify any SNPs with significant association to the composite memory score at a Bonferroni-determined threshold p-value of 9.42×10^{-8} (i.e., 0.05/531,096). The peak SNP in the association analysis was rs9890008 (Chr 17), which has not been mapped to a known gene and which exhibited an unadjusted p-value of 2.21×10^{-6}. Overall, 25,960 SNPs showed nominal p-values < 0.05 (unadjusted). Manhattan (Figure 1) and Q-Q (Figure 2) plots are displayed for the GWA analysis. Notably, when *APOE* ε4 allele status was

removed as a covariate, one SNP (rs2075650) residing in the *TOMM40* gene did exhibit significant association ($p = 2.19 \times 10^{-9}$) to the composite memory score. This result was probable, given that the *TOMM40* gene is adjacent to, and often considered as one locus with, *APOE* on chromosome 19. In addition, *TOMM40* variants have been associated with late-onset AD (Roses et al. 2010; Wijsman et al. 2011; Potkin et al. 2009) as well as with structural brain and cognitive function changes suggestive of presymptomatic late-onset AD (Johnson et al. 2011).

Next, the *p*-value output from the GWA analysis was used as input for pathway enrichment analysis. Using the GSA-SNP software tool, we identified 27 canonical pathways with enrichment (FDR-corrected *p*-value < 0.05) against the composite memory score (Table 2). Following these analyses, we examined the enriched pathways in detail in order to better characterize their biological import in memory deficits. First, we used existing knowledge and insight to conceptually categorize the 27 enriched pathways into 4 broader realms of biology (Figure 3). In particular, 11 enriched pathways represented classical cellular and molecular processes essential in normal memory consolidation signaling (Sweatt 2009). These pathways included functions of neurotransmitter receptor activation, downstream calcium-mediated signaling, and long-lasting potentiation of synaptic strength, among other processes. In addition, six pathways related to cell adhesion were enriched, including focal adhesion pathways from both the Reactome and KEGG databases, and interactions involving neuronal cell adhesion molecule 1 (NCAM1). Finally, four enriched pathways were related to neuronal differentiation and guided axonal growth, while a further six enriched pathways were involved in inflammation or other complex signaling processes. Notably, while we restricted analysis to pathways containing 10-200 genes, the enrichment results were nearly identical when upper limits of 300 or 400 genes were used: in those cases, two additional Reactome pathways exhibited enrichment (transmembrane transport of small molecules, FDR-corrected *p*-value = 0.029; adherens junctions interactions, FDR-corrected *p*-value = 0.049).

As further follow-up, we identified 44 genes that were highly-represented across the 27 enriched pathways (Table 3). Half (22) of these 44 genes were constituents of 6 or more enriched pathways, suggesting that variants in those genes can have wide-ranging roles in mediating memory impairment due to their diverse functions. We also assessed for underlying transcriptional relationships among these highly-represented genes and *APOE*, given the latter's singular association with MCI and AD and their related memory deficits. Using network analysis in MetaCore, we discovered that 14 of the 22 most-represented genes from our analyses were part of a transcriptional regulation network driven by the specificity protein 1 (*SP1*) transcription factor and involving *APOE* and the *APOE* receptor-2 (Figure 4).

Finally, we used data from the Allen Human Brain Atlas to evaluate if the identified genes of interest exhibited co-expression in normal brain tissue. Through heat map visualization and correlational analysis, we identified a set of 10 key genes with strong co-expression (Pearson *r* > 0.7) across the major neuroanatomic regions of the brain (Figure 5a). In particular, 6 of these genes (*CAMK2A*, *CACNB1*, *CALM1*, *CALM3*, *GRIN2A*, and *MAPK1*) were highly-represented among our enriched pathways, while the other 4 genes (*CDK5*, *GSK3B*, *GRIN2B*, and *PRNP*) were constituents of our enriched pathways that were also known AD susceptibility genes found in the AlzGene database (http://www.alzgene.org/) (Bertram et al. 2007). An example of the cortical and subcortical expression patterns common to this gene set was also generated for one of these genes, *CAMK2A* (Figure 5b). These findings suggest further underlying functional relationships between MCI and AD disease risk and the pathogenesis of memory impairment.

DISCUSSION

In this study, we used a psychometrically-optimized composite measure of overall episodic memory performance as a phenotype for GWAS in a sample of MCI and AD patients and controls. Then, through a genome-wide pathway analysis, we identified 27 canonical pathways with enrichment against this composite memory phenotype. These enriched pathways suggest that the genetic architecture of memory impairment in this sample spans both processes classically understood to be involved in normal memory consolidation as well as processes with broader roles in cognition and aging, such as those involving neuronal cell adhesion and inflammation.

Moreover, the results of this pathway enrichment study have valuable implications for the future. First, these results illuminate prime cellular, molecular, and genetic targets for future studies of normal and impaired memory states. Indeed, it should be emphasized that pathway-based approaches analyze genetic data in the context of its operative functional groups; as a result, pathway analysis findings are uniquely and naturally connected to the functional biology underlying complex phenotypes. This insight is vital for future investigations, given that pathway mechanisms are principal sources for developing strategies to diagnose, treat, and prevent complex disorders. It is also important to note that our analysis elucidated pathways with robust enrichment despite using GWA input data that included a relatively modest distribution of SNP-level phenotype associations. This insight affirms the potential of pathway-based analytical approaches to detect significant relationships that are otherwise concealed within single-SNP or single-gene analysis. Finally, the use of genome-wide pathway analysis in this study facilitated the detection of unexpected relationships with memory performance, including pathways not classically related to memory signaling, and subsequently, interesting transcriptional and expression networks. While targeted "candidate pathway" approaches have advantages, these unexpected relationships would not have been easily predicted as candidates for analysis based on prior knowledge.

At a functional level, the enriched pathways identified in this study present interesting biological implications in relation to memory impairment. In a sense, it might be expected that cellular and molecular processes classically understood as mediating memory consolidation would constitute a major part of the genetic architecture of memory impairment. However, the processes underlying memory consolidation are numerous and diverse, and to date it has not been clear which specific pathways are essential objects of the impact of genetic variants. The results of our enrichment analysis suggest prime potential components of this genetic architecture. In particular, we observed significant enrichment of pathways related to neurotransmitter receptor activation and downstream signaling. These pathways and their resultant calcium-mediated signaling are vital in converting short-term memories, which exist as axonal firing patterns, into long-lasting changes in synaptic strength (Sweatt 2011). As a parallel, it makes sense that a composite long-term potentiation pathway (comprising multiple processes leading to long-lasting increases in synaptic strength) would include genetic determinants related to memory performance. Finally, our results indicate the need for further exploration of long-term depression as a substrate for memory impairment, particularly given its proposed roles in mediating the cognitive effects of acute stress and the elimination of synapses in neurodegenerative diseases (Collingridge et al. 2010).

Meanwhile, other pathways enriched with regard to memory impairment in this study have previously been implicated in other roles involving neuronal development and cognition. There is an extensive literature on the roles of neuronal cell adhesion molecules (NCAMs) in susceptibility for schizophrenia, bipolar disorder, and autism-spectrum disorders (Corvin

2010). Genetic variants of NCAMs have also been associated with CSF biomarkers for AD (Han et al. 2010), and cell adhesion molecule pathways have exhibited enrichment in a GWA-based pathway analysis of AD susceptibility (Liu et al. 2012). In addition, expression of NCAMs in cholinergic neurons appears to increase susceptibility to AD-related neurodegeneration (Aisa et al. 2010), and there is emerging evidence of interactions among NCAMs, the MAPK pathway, and amyloid precursor protein (Chen and Dou 2012). More broadly, these findings suggest a prominent role for cell adhesion pathways in maintaining the processes of synaptic plasticity that are believed to underlie learning and memory (Ho et al. 2011).

Finally, it is interesting that pathways on axon guidance, including those involving functions of ephrins, semaphorins, and rho GTPases, were enriched in this study. Axon guidance pathways are key in forming guided neuronal network connections, and have been previously implicated in early neuronal development and associated genetic conditions (Izzi and Charron 2011). Together with these enrichment results, the proposed interaction between vascular and neuronal factors related to axon guidance (Arese et al. 2011) in relation to memory may be an important direction for further studies. In addition, given the complex interactions among brain cells and immune system functions, the immune-related pathways enriched in this study suggest additional candidates for modulation of memory and synaptic plasticity (Yirmiya and Goshen 2011). It may be particularly fruitful to examine immune mediators of memory dysfunction that exert influences independent from amyloid-related activation of microglia in AD (Emilio 2010).

A related perspective – and additional interesting targets for future investigations – can be achieved by examining the set of genes that were highly-represented across the enriched pathways in this study. Prominent groups of gene products represented in this set are particularly important in memory consolidation. For example, integrins, cadherins, and alpha-actinin are known to regulate neuronal cytoskeletal structure to mediate synaptic plasticity; further, these molecules are proposed to signal through MAPK cascades for localized protein synthesis at the specific dendrites being activated to precisely potentiate their synaptic connections (Sweatt 2009). Another important group of gene products is related to the calcium influx that follows neurotransmitter receptor activation at synapses: this calcium influx leads to activation of a signaling axis involving calmodulin, protein kinases (PKA, PKC-α, CAMKII subtypes, and CAMKIV), and transcription factors (CREB subtypes), among other molecules (Sweatt 2009). Overall, since the highly-represented genes from our data act in numerous pathways, our results demonstrate the importance of studying their gene variants within a pathway-based framework: in this context, variants of moderate individual effect sizes can nevertheless be identified as exerting strong and wide-ranging effects when juxtaposed with other meaningful variants in the same functional unit (Ramanan et al. 2012). Extensions of this pathway-based analytic framework will be extremely valuable in identifying localized effects of specific pathways on particular brain regions, particularly given that imaging correlates have been identified for loci with known effects on memory, such as the impact of *KIBRA* gene variants on hippocampal activation (Pawlowski and Huentelman 2011). Notably, innovative voxelwise SNP- (Stein et al. 2010a; Stein et al. 2010b) and gene-based (Hibar et al. 2011) imaging genetics approaches have been successfully employed in studies of AD, as has a novel method for generating multivariate "genetic components" for imaging analysis (Meda et al. 2012). These strategies will serve as rich foundations for future pathway-based imaging genetics analyses.

In addition, the network analyses in this study reinforce the notion that key genes related to memory impairment function coordinately. We found that a preponderance of the most highly-represented genes in our enriched pathways were constituents of a transcriptional regulation network driven by the *SP1* transcription factor (Figure 4). The *SP1* transcription

factor has known binding regions in the promoters of genes related to beta-amyloid precursor protein (Yu et al. 2010; Rossner et al. 2006), tau protein (Santpere et al. 2006), and *APOE*. In particular, *SP1* has been proposed as a regulator of *APOE* promoter activity in relation to two promoter polymorphisms with significant association to AD (Maloney et al. 2010). Given that networks of common regulation represent prime targets for identifying common functions, further investigation of the transcriptional network that we have identified may elucidate the as-yet-unknown mechanistic connections among *APOE* and other susceptibility loci, AD pathogenesis, and MCI- and AD-related memory impairment.

Finally, expression analysis using the Allen Human Brain Atlas revealed additional functional relationships among key genes. Since strong co-expression of a set of 10 key genes in the brain may indicate common modes of function, further study of this and other similar sets may be of great value. In addition, the co-expression of highly-represented genes from the enriched pathways in this study with known AD susceptibility genes suggests the possibility of significant crosstalk between AD pathogenesis and basic memory processes. While the data in the Allen Human Brain Atlas has several limitations, including a small number of subjects and the inclusion of only postmortem brain tissue from neuropsychologically- and neuropathologically-normal subjects, at present it is the only available resource which integrates multi-modal brain imaging data with whole-brain genome-wide expression data. As such, this and other functional annotation resources will be vital for identifying mechanistic connections between AD pathogenesis and memory impairment, including future efforts to quantitatively assess the significance of overlap between memory pathways and AD pathways. There are some notable limitations to the current study. First, a pathway analysis is only as good as the functional information underlying its pathway definitions. Importantly, some intragenic SNPs may not affect the function or expression of their assigned gene, while other SNPs may functionally impact distant genes or even multiple genes (Kapranov et al. 2007; Portin 2009). As functional annotation of the genome becomes more extensive, the power of pathway analyses will heighten. For this study, we used a collection of canonical pathways curated through expert review. While these pathway annotations are expected to have high accuracy, differences across pathway databases can lead to divergent enrichment analysis results (Elbers et al. 2009). For example, similarly-named pathways can have vastly different gene constituents, while distinctly-named pathways can nevertheless include significant gene overlap. As a result, an early discussion of pathway analysis methods recommended the use of multiple databases for each analysis (Cantor et al. 2010). While we have followed this recommendation for this analysis, future studies may benefit from formally assessing the relationships in biological coverage among the diverse pathways tested. In addition, at this time there is no gold standard for pathway-based study design. Indeed, different enrichment algorithms and different parameters, such as those guiding SNP-to-gene mapping, can impact analytical results (Gui et al. 2011). As such, pathway enrichment results benefit from further study using independent replication data sets and using alternative enrichment strategies. While differences across annotation resources, data sets, and analytical strategies may impact SNP- or gene-level statistics (Luo et al. 2010), legitimately-associated pathways will likely exhibit significant enrichment or strongly-trending signal across a healthy percentage of studies. Finally, while it is beyond the scope of this study, future efforts will benefit from examining key memory-implicated genes and gene sets for epistatic (gene-gene) interactions with each other and with *APOE*.

In addition, there are several caveats about the clinical setting for this study. First, the ADNI cohort represents a clinical trial population and is not a sample of the general population. As a result, the extent to which the present findings can be extended to account for episodic memory impairment in the general population remains to be determined. In addition, it is probable that the memory deficits in this study's MCI and AD participants are at least

partially driven by AD-related pathology. While using *APOE* ε4 allele status as a covariate in these analyses likely attenuated this effect, a better understanding of the pathways underlying normal memory and other pathologies than AD may be achieved through studies of normal cohorts and other memory-impaired populations without AD-related pathology. In particular, further exploration of the relationships among *APOE* genotype status, amyloid-β load and pathology, and cognition in normal adults (Kantarci et al. 2012; Buchman and Bennett 2012) may be especially fruitful. Additionally, meta-analytic approaches to achieve larger study sample sizes may reveal greater SNP-level phenotype associations which could impact the pathway enrichment results. Finally, while this study used a composite episodic memory score optimized on the basis of modern psychometric theory, similar pathway-based studies using other quantitative memory phenotypes may provide different sensitivity and specificity to fine-grained memory deficits and would potentially serve as a validation for the discoveries of pathways enriched against the phenotype used in this study.

Nevertheless, the present results provide several new insights into key functional pathways associated with memory deficits in older adults with MCI or AD and controls. Importantly, these results highlight numerous candidates for further explorations of the SNPs, genes, and gene sets underlying normal memory processes and memory impairment. Overall, these findings encourage further use of pathway-based genetic analyses of quantitative memory phenotypes as statistically-powerful vehicles for discovery and as bridges to underlying biological mechanisms.

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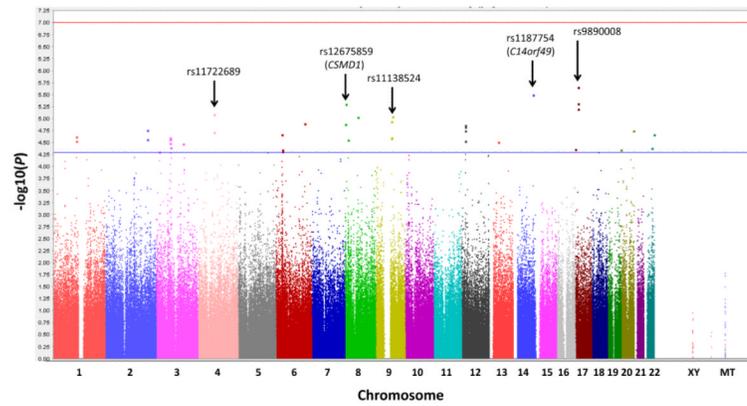
Fig. 1.
Manhattan plot for the GWA analysis of episodic memory. The x-axis refers to positions along the genome (separated by chromosome) for each SNP (represented by a dot) included in the analysis. The y-axis refers to the negative logarithm of the p-value for the test of association between each SNP and the quantitative memory phenotype. No SNPs exhibited genome-wide significant association (red line) to the composite episodic memory score, while 40 SNPs exhibited suggestive association ($p < 5 \times 10^{-5}$, blue line). The 5 most significant, independent ($r^2 < 0.2$) SNPs are labeled along with their corresponding genes (if known).
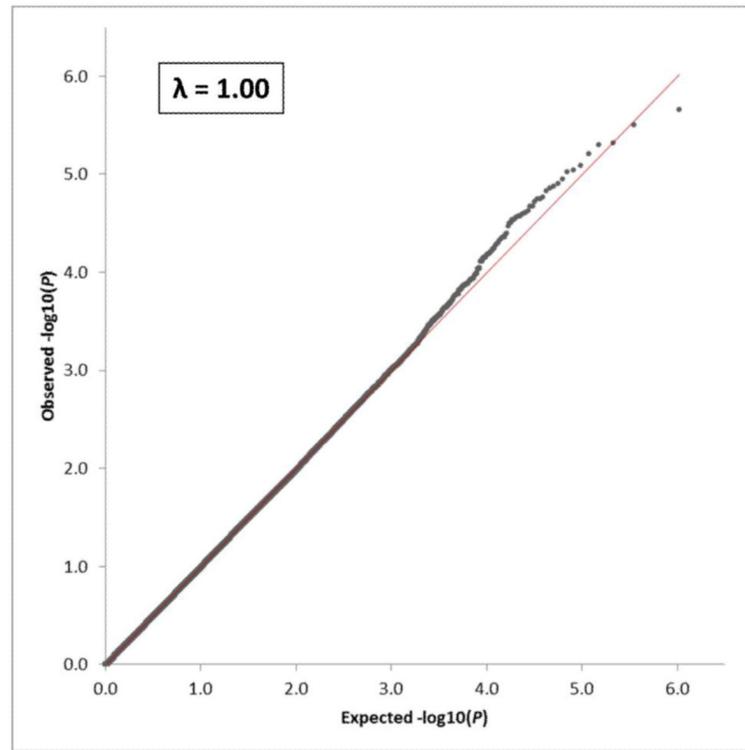
Fig. 2.
Quantile-Quantile (Q-Q) plot for the GWA analysis of episodic memory. The genomic inflation factor (λ) for the analysis was 1.

Memory-Related Signaling Pathways	Cell Adhesion Pathways	Neuronal Differentiation and Outgrowth Pathways	Inflammation and other Signaling Pathways
• Transmission across chemical synapses • Calcium signaling • Neurotransmitter receptor binding and downstream signaling events • Long-term depression • Long-term potentiation • Activation of the NMDA receptor • Tryptophan metabolism • Depolarization of the presynaptic terminal • Ionotropic activity of kainate receptors • PLCβ signaling • PLCλ1 signaling	• Focal adhesion • Adherens junction • NCAM1 interactions • Cell adhesion molecules (CAMs) • ECM (extracellular matrix) receptor interactions	• Axon guidance • Semaphorin interactions • SEMA3A plexin repulsion signaling by inhibiting integrin adhesion • CRMPs (collapsin response mediator proteins) in SEMA3A signaling	• Type I diabetes mellitus • Arrhythmogenic right ventricular cardiomyopathy • Glucose and other sugar SLC (solute carrier) transporters • SLC-mediated membrane transport • Viral myocarditis • Allograft rejection

Fig. 3.
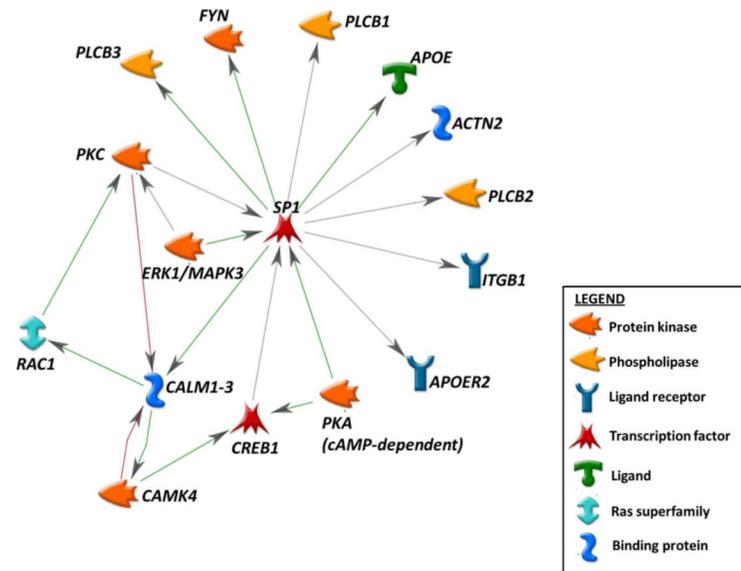Conceptual classification of 27 pathways enriched in the setting of memory impairment

Fig. 4.
Transcriptional regulation network centered on *SP1* involves many genes of interest from pathways enriched in memory impairment. Green arrows indicate positive regulatory effects, red arrows indicate negative regulatory effects, and gray arrows indicate unspecified regulatory effects.
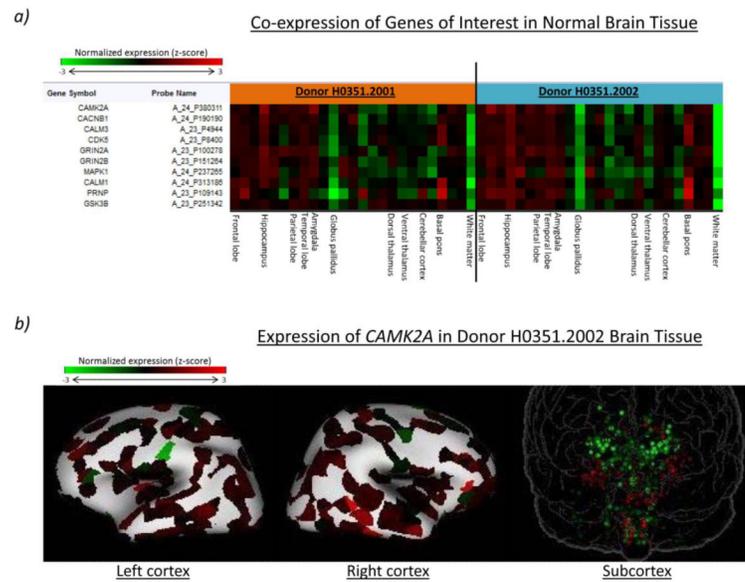*Primary image generated with the MetaCore software (GeneGo, Inc.)

Fig. 5.
Genes of interest in memory impairment exhibit similar expression profiles in postmortem normal brain tissue. a) Normalized, microarray-based expression profiles across 25 major neuroanatomic regions of the brain are provided for 10 key genes of interest. Moving from left to right on the heat map is analogous to moving from anterior to posterior regions first in the cortex, followed by subcortical areas and then the cerebellum and brainstem. The genes represented exhibit strong co-expression (Pearson $r > 0.7$) across the brain in data from two subjects. b) For the co-expressed gene set, a representative cortical and subcortical expression profile is shown for the *CAMK2A* gene.
*Images derived from the Allen Human Brain Atlas.

Table 1

Characteristics for participants included in the analyses

	CN (N = 207)	MCI (N = 362)	AD (N = 173)	p-value[*]
Age at baseline	76.1 ± 5.0	74.9 ± 7.4	75.6 ± 7.5	0.139
Gender (male/female)	113/94	234/128	92/81	0.012
Years of education	16.2 ± 2.7	15.7 ± 3.0	14.9 ± 3.0	< 0.001
Handedness (right/left)	191/16	328/34	161/12	0.586
APOE ε4 allele (absent/present)	152/55	165/197	61/112	< 0.001
Composite memory score[ϕ]	1.05 ± 0.59	−0.14 ± 0.64	−1.01 ± 0.63	< 0.001

Values represented are mean ± SD

[*] For categorical variables, p-values were computed using the Pearson chi-square tests; for continuous variables, p-values were computed using a one-way analysis of variance.

[ϕ] Scores for individual participants were represented as z-scores with a defined mean of 0, and standard deviation of 1, based on the 810 participants with complete item-level memory data at baseline.

Table 2

Canonical pathways enriched against the composite memory score

Pathway (Gene Set) Name	Set Size[1]	*p*-value	FDR *p*-value
[r]Transmission across chemical synapses	136 (122)	2.14×10^{-7}	1.77×10^{-4}
[k]Calcium signaling pathway	184 (165)	2.82×10^{-7}	1.17×10^{-4}
[k]Type I diabetes mellitus	50 (41)	7.90×10^{-6}	0.002
[r]Neurotransmitter receptor binding and downstream transmission	90 (78)	2.84×10^{-5}	0.006
[k]Arrhythmogenic right ventricular cardiomyopathy	82 (72)	3.11×10^{-5}	0.005
[r]SLC-mediated membrane transport	175 (162)	3.87×10^{-5}	0.005
[k]Focal adhesion	207 (187)	4.68×10^{-5}	0.006
[k]Axon guidance	135 (123)	4.68×10^{-5}	0.005
[k]Long-term depression	76 (65)	9.02×10^{-5}	0.008
[r]Axon guidance	167 (154)	9.58×10^{-5}	0.008
[k]Adherens junction	81 (71)	1.04×10^{-4}	0.008
[r]Other semaphorin interactions	22 (15)	1.20×10^{-4}	0.008
[r]NCAM1 interactions	50 (42)	1.37×10^{-4}	0.009
[k]Long-term potentiation	76 (65)	3.14×10^{-4}	0.019
[r]Activation of glutamate NMDA receptor and post-synaptic events	42 (32)	5.02×10^{-4}	0.028
[k]Cell adhesion molecules (CAMs)	140 (123)	5.60×10^{-4}	0.029
[r]SEMA3A plexin repulsion signaling by inhibiting integrin adhesion	20 (13)	6.06×10^{-4}	0.030
[k]Tryptophan metabolism	46 (35)	6.53×10^{-4}	0.030
[r]Depolarization of the presynaptic terminal triggers the opening of calcium channels	18 (12)	6.71×10^{-4}	0.029
[r]PLCβ-mediated events	44 (35)	6.91×10^{-4}	0.029
[k]Viral myocarditis	79 (67)	9.98×10^{-4}	0.039
[k]Allograft rejection	44 (34)	0.001	0.039
[r]Glucose and other sugar SLC transporters	88 (80)	0.001	0.039
[r]Ionotropic activity of kainate receptors	18 (11)	0.001	0.042
[k]ECM receptor interaction	90 (83)	0.001	0.041
[r]PLCγ1 signaling	41 (32)	0.001	0.044
[r]CRMPs in SEMA3A signaling	22 (15)	0.001	0.045

[1] Entries are displayed as: number of genes in the set (number of genes from the GWA data)

[r] Reactome pathway

[k] KEGG pathway

Table 3

Highly-represented genes among the 27 canonical pathways enriched against the composite memory score

Occurrences in Enriched Pathways	Gene ID	Gene Name
9	MAPK1	mitogen-activated protein kinase 1
8	CALM1	calmodulin 1 (phosphorylase kinase, delta)
8	CALM2	calmodulin 2 (phosphorylase kinase, delta)
8	CALM3	calmodulin 3 (phosphorylase kinase, delta)
8	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
7	ADCY1	adenylate cyclase 1 (brain)
7	ADCY8	adenylate cyclase 8 (brain)
7	CAMK4	calcium/calmodulin-dependent protein kinase IV
7	FYN	FYN oncogene related to SRC, FGR, YES
7	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29)
7	PRKACB	protein kinase A, cAMP-dependent, catalytic, beta
7	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
6	ACTN2	actinin, alpha 2
6	ADCY3	adenylate cyclase 3
6	CREB1	cAMP responsive element binding protein 1
6	MAPK3	mitogen-activated protein kinase 3
6	PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)
6	PLCB2	phospholipase C, beta 2
6	PLCB3	phospholipase C, beta 3 (phosphatidylinositol-specific)
6	PRKCA	protein kinase C, alpha
6	PRKCG	protein kinase C, gamma
6	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, GTP binding protein)
5	CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit
5	CACNB1	calcium channel, voltage-dependent, beta 1 subunit
5	CACNB2	calcium channel, voltage-dependent, beta 2 subunit
5	CACNB3	calcium channel, voltage-dependent, beta 3 subunit
5	CACNB4	calcium channel, voltage-dependent, beta 4 subunit
5	CAMK2A	calcium/calmodulin-dependent protein kinase II alpha
5	CAMK2B	calcium/calmodulin-dependent protein kinase II beta
5	CAMK2D	calcium/calmodulin-dependent protein kinase II delta
5	CAMK2G	calcium/calmodulin-dependent protein kinase II gamma
5	GRIA1	glutamate receptor, ionotropic, AMPA 1
5	GRIA2	glutamate receptor, ionotropic, AMPA 2
5	GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1
5	GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A
5	GRIN2C	glutamate receptor, ionotropic, N-methyl D-aspartate 2C
5	GRIN2D	glutamate receptor, ionotropic, N-methyl D-aspartate 2D
5	ITGA1	integrin, alpha 1
5	ITPR1	inositol 1,4,5-trisphosphate receptor, type 1

Occurrences in Enriched Pathways	Gene ID	Gene Name
5	*ITPR2*	inositol 1,4,5-trisphosphate receptor, type 2
5	*ITPR3*	inositol 1,4,5-trisphosphate receptor, type 3
5	*PLXNA1*	plexin A1
5	*PLXNA2*	plexin A2
5	*RAC2*	ras-related C3 botulinum toxin substrate 2 (rho family, GTP binding protein)