Genetic Analysis of Association Between Calcium Signaling and Hippocampal Activation, Memory Performance in the Young and Old, and Risk for Sporadic Alzheimer Disease

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**IMPORTANCE** Human episodic memory performance is linked to the function of specific brain regions, including the hippocampus; declines as a result of increasing age; and is markedly disturbed in Alzheimer disease (AD), an age-associated neurodegenerative disorder that primarily affects the hippocampus. Exploring the molecular underpinnings of human episodic memory is key to the understanding of hippocampus-dependent cognitive physiology and pathophysiology.

**OBJECTIVE** To determine whether biologically defined groups of genes are enriched in episodic memory performance across age, memory encoding–related brain activity, and AD.

**DESIGN, SETTING, AND PARTICIPANTS** In this multicenter collaborative study, which began in August 2008 and is ongoing, gene set enrichment analysis was done by using primary and meta-analysis data from 57 968 participants. The Swiss cohorts consisted of 3043 healthy young adults assessed for episodic memory performance. In a subgroup (n = 1119) of one of these cohorts, functional magnetic resonance imaging was used to identify gene set–dependent differences in brain activity related to episodic memory. The German Study on Aging, Cognition, and Dementia in Primary Care Patients cohort consisted of 763 elderly participants without dementia who were assessed for episodic memory performance. The International Genomics of Alzheimer’s Project case-control sample consisted of 54 162 participants (17 008 patients with sporadic AD and 37 154 control participants). Analyses were conducted between January 2014 and June 2015. Gene set enrichment analysis in all samples was done using genome-wide single-nucleotide polymorphism data.

**MAIN OUTCOMES AND MEASURES** Episodic memory performance in the Swiss cohort and German Study on Aging, Cognition, and Dementia in Primary Care Patients cohort was quantified by picture and verbal delayed free recall tasks. In the functional magnetic resonance imaging experiment, activation of the hippocampus during encoding of pictures served as the phenotype of interest. In the International Genomics of Alzheimer’s Project sample, diagnosis of sporadic AD served as the phenotype of interest.

**RESULTS** In the discovery sample, we detected significant enrichment for genes constituting the calcium signaling pathway, especially those related to the elevation of cytosolic calcium ($P = 2 \times 10^{-4}$). This enrichment was replicated in 2 additional samples of healthy young individuals ($P = .02$ and .04, respectively) and a sample of healthy elderly participants ($P = .004$). Hippocampal activation ($P = 4 \times 10^{-4}$) and the risk for sporadic AD ($P = .01$) were also significantly enriched for genes related to the elevation of cytosolic calcium.

**CONCLUSIONS AND RELEVANCE** By detecting consistent significant enrichment in independent cohorts of young and elderly participants, this study identified that calcium signaling plays a central role in hippocampus-dependent human memory processes in cognitive health and disease, contributing to the understanding and potential treatment of hippocampus-dependent cognitive pathology.

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Episodic memory (EM), the ability to encode and retrieve a particular event along with its contextual information,\(^1\) is a polygenic cognitive trait characterized by large interindividual variability and substantial heritability.\(^2\)\(^-\)\(^5\) As a consequence of physiological aging processes in brain regions, such as the hippocampus and the medial temporal lobe, performance in EM tasks declines with age.\(^6\)\(^-\)\(^9\) Pathological EM impairment is a behavioral hallmark of age-related neurodegenerative conditions, such as Alzheimer disease (AD).\(^10\)\(^,\)\(^21\)

Genome-wide studies using single-marker statistics have been successful in identifying single loci linked to intact and impaired EM.\(^3\)\(^,\)\(^12\)\(^-\)\(^14\) Triggered by statistical approaches for the analysis of gene expression, gene set enrichment analysis (GSEA) has become available. By taking into account prior biological knowledge, GSEA examines whether test statistics for a group of related genes have consistent deviation from chance.\(^15\)\(^,\)\(^16\) As shown in studies on working memory,\(^17\) logical knowledge,\(^18\) GSEA can identify convergent molecular pathways relevant to neuropsychiatry.

We studied the enrichment of biologically defined gene sets in EM across age, EM-related brain activity, and an EM-related neurodegenerative disorder (Figure 1). Genome-wide GSEA of EM performance was performed in multiple independent data sets of young and elderly cognitively healthy participants (\(n = 3806\)). We also performed GSEA for the risk of sporadic AD in a large case-control sample (\(n = 54\) 162).

**Methods**

This study started in August 2008 and is still ongoing. The ethics committees of the Cantons of Basel and Zurich approved the study protocol.

**Samples**

**Discovery Sample**

This sample is part of an ongoing continuously recruiting behavioral genetics study in Basel, Switzerland. For the purposes of this study (data lock August 2013), data from 1458 healthy young Swiss adults were available (eTable 1 in the Supplement). Participants had no neurological or psychiatric conditions and did not take medication at the time of the study. All participants provided written informed consent and completed a picture delayed free recall task, which reflected EM performance. For a detailed description of the procedure, see the eAppendix in the Supplement.

**Replication Sample**

This sample is part of an ongoing continuously recruiting imaging genetics study in Basel, Switzerland. For the purposes of this study (data lock August 2013), data from 1176 healthy young Swiss adults were available (eTable 1 in the Supplement). Participants had no neurological or psychiatric conditions and did not take medication at the time of the study. All participants provided written informed consent and while undergoing functional magnetic resonance imaging (FMRI) acquisition completed a similar picture delayed free recall task as in the discovery sample. For a detailed description of the procedure, see the eAppendix in the Supplement.

**Zurich Sample**

We recruited 409 healthy young Swiss adults for a behavioral genetics study in Zurich, Switzerland (eTable 1 in the Supplement). Participants had no neurological or psychiatric conditions and did not take medication at the time of the study. All participants provided written informed consent and completed a picture delayed free recall task. For a detailed description of the procedure, see the eAppendix in the Supplement.

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**Figure 1. Schematic Description of Study Workflow and Included Samples**

<table>
<thead>
<tr>
<th>A</th>
<th><strong>Episodic memory testing in healthy young adults</strong></th>
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</thead>
<tbody>
<tr>
<td><strong>Discovery sample</strong> ((n = 1458))</td>
<td><strong>Replication sample</strong> ((n = 1176))</td>
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<tr>
<td>Delayed free recall of pictures</td>
<td>Delayed free recall of pictures</td>
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<tr>
<th>B</th>
<th><strong>Hippocampal activation in healthy young adults</strong></th>
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<tbody>
<tr>
<td>Functional magnetic resonance imaging sample</td>
<td>Activation during picture encoding</td>
</tr>
<tr>
<td>(subsample of replication sample; (n = 1119))</td>
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<tr>
<th>C</th>
<th><strong>Additional samples with episodic memory data</strong></th>
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</thead>
<tbody>
<tr>
<td><strong>Zurich sample</strong> of healthy young adults ((n = 409))</td>
<td><strong>German Study on Aging, Cognition, and Dementia in Primary Care Patients sample of healthy elderly adults ((n = 763))</strong></td>
</tr>
<tr>
<td>Delayed free recall of pictures</td>
<td>Delayed free recall of words</td>
</tr>
<tr>
<td>butter</td>
<td>shore</td>
</tr>
<tr>
<td>cabin</td>
<td>arrow</td>
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<tr>
<th>D</th>
<th><strong>Alzheimer disease risk</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>International Genomics of Alzheimer’s Project</strong> (case-control sample; (n = 54) 162)</td>
<td>Alzheimer disease diagnosis</td>
</tr>
</tbody>
</table>
Healthy Elderly Adults Sample
This sample consisted of 763 healthy elderly participants of the German Study on Aging, Cognition, and Dementia in Primary Care Patients (n = 3237; eTable 1 in the Supplement). The study on Aging, Cognition, and Dementia in Primary Care Patients is an ongoing primary care–based prospective longitudinal study on the early detection of mild cognitive impairment and dementia established by the German Competence Network Dementia (eAppendix in the Supplement).25

Genetic Heterogeneity
For each of the 4 cognitively healthy samples, the genomic control inflation factor λ was calculated to assess admixture.26 The genomic control inflation factor λ showed a range between 1.0046 and 1.0449, indicating the absence of noteworthy admixture in these samples (eFigures 1, 2, 3, and 4 in the Supplement).

Array-Based SNP Genotyping
Samples were processed as described in the Genome-Wide Human SNP Nsp/Sty 6.0 User Guide (Affymetrix Inc). Single-nucleotide polymorphism calls and array quality control were performed using the command line programs of the Affymetrix Power Tools package, version 1.14.4.1 (Affymetrix Inc). According to the manufacturer's recommendation, contrast quality control was chosen as the quality control metric using the default value of 0.4 or higher. All samples passing quality control criteria were subsequently genotyped using the Birdseed algorithm, version 2 (Affymetrix Inc). The mean call rate for all samples was more than 98.5%. The sex check in PLINK (http://pngu.mgh.harvard.edu/~purcell/plink) led to the exclusion of 1 individual in the discovery sample and 3 individuals in the replication sample. Identity by descent analysis (PLINK) indicated the absence of duplicates within and between samples. For a detailed description of the procedure, see the eAppendix in the Supplement.

Brain Imaging
Preprocessing and data analysis was performed using Statistical Parametric Mapping, version 8 (Wellcome Trust Centre for Neuroimaging; http://www.flim.ion.ucl.ac.uk/spm) implemented in MATLAB R2011b (MathWorks). The functional and structural images were spatially normalized by applying DARTEL, which led to an improved registration between participants.27,28 For a detailed description, see the eAppendix in the Supplement.

fMRI Group Statistics
The first-level contrast parameters were used for behavioral analyses in a random-effects model (second-level analysis). We used a regression model to analyze associations between brain activation differences (meaningful vs scrambled pictures) and the multiallelic score. Age and sex were included as covariates. A 1-sample t test was computed to assess the significance of task-related activation (meaningful vs scrambled pictures) at the group level. The analysis was focused on the left and right hippocampi, which were defined using the template-based hippocampal regions of interest. For a detailed description of the construction of a population-averaged anatomical probabilistic atlas, see the eAppendix in the Supplement.

Statistical Genetic Analysis

Genome-Wide Association Analyses
For each genome-wide analysis (Genome Reference Consortium GRCh37, hg19), P values of effectively genotyped (ie, not imputed) variants were obtained using linear regression analyses as implemented in PLINK (SNPs in the discovery sample, n = 730 540; replication sample, n = 736 286; Zurich sample, n = 737 533; and Study on Aging, Cognition, and Dementia in Primary Care Patients sample, n = 683 072).29 Sex and age were included as covariates. We applied the following quality control criteria: nonsignificant deviation from Hardy-Weinberg equilibrium (P > 1 × 10−4) and a minor allele frequency of more than 0.01. The mean per SNP call rate was more than 99%.

Pathway Analysis
Gene set enrichment analysis was performed using MAGENTA (Broad Institute).30 The method first mapped SNPs onto genes. In this study, only intragenic SNPs were used for SNP to gene mapping (ie, ±0 kb of the annotated gene according to Genome Reference Consortium GRCh37, hg19). We chose the ±0 kb threshold to avoid potentially significant biases, which are introduced by overlapping signals in especially gene-rich regions. After SNP to gene mapping, MAGENTA assigned each gene a SNP association score (ie, the maximum SNP P value). The analysis was corrected for gene size, number of SNPs, number of independent SNPs, number of recombination hot spots, linkage disequilibrium, and genetic distance. Lastly, a gene set enrichment–like statistical test was applied to determine if a gene set was enriched for highly ranked P values compared with a gene set of identical size randomly drawn from the genome. The false discovery rate based on the 75th percentile of associated P values from all genes was used for multiple testing correction. As recommended, we used the 75th percentile cutoff because it yields optimal power for weak genetic effects that are expected for highly polygenic traits (eg, EM performance).31 The gene sets we used were extracted and curated from the MSigDB, version 3.1, database (Broad Institute, http://www.broadinstitute.org/gsea/msigdb, downloaded in February 2013), including gene sets from different online databases (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/; Gene Ontology, http://geneontology.org/; BioCarta, http://www.biocarta.com/; and Reactome, http://www.reactome.org/).31,32 We used a gene set size ranging between 20 and 200 genes to avoid both overly narrow and broad functional gene set categories, resulting in 1411 gene sets to be analyzed.

We also applied INRICH (http://atgu.mgh.harvard.edu/inrich/), a software tool that examines the enrichment of association signals for genetic gene sets.33 Unlike MAGENTA, which uses the lowest P value of each gene-phenotype association to quantify gene set enrichment, INRICH defines linkage disequilibrium–independent genomic regions (ie, intervals) that contain the top genome-wide association study hits. These intervals are then tested for overlaps with gene targets. A permutation-based resampling method was used to
obtain the null distribution by random data shuffling and by calculating the overlap between random test intervals of equal size as the observed and target gene sets.

**Multilocus Genetic Score Calculation**

To capture the multiallelic effect of the calcium signaling pathway gene set, we generated an individual multilocus genetic score.\(^{17}\) The score comprised variants of the calcium signaling pathway geneset (1 SNP per gene) of the discovery sample that remained significant (\(P < .05\)) after correcting for the number of independent SNPs per gene, gene size, genetic distance, and recombination hotspots (eTable 2 in the Supplement). The algorithm calculated the score by summing the individual number of reference alleles across all SNPs and calculating the mean score by the number of nonmissing SNPs. We weighted each SNP by the direction of effect on EM performance (ie, with 1 [the reference allele enhanced performance] or –1 [the reference allele decreased performance]). This procedure harmonized the single-variant effects and avoided bias owing to the overestimation of accidentally large SNP effects.

**Results**

**GSEA of EM in Young Healthy Adults**

**Discovery Sample (n = 1458)**

Gene set enrichment analysis was performed with MAGENTA.\(^{30}\) Among the 1411 database-derived gene sets, MAGENTA identified significant enrichment (false discovery rate < .05, multiple testing-corrected) for 1 gene set, the calcium signaling pathway gene set (Kyoto Encyclopedia of Genes and Genomes, entry hsa04020; Table 1). The calcium signaling pathway gene set was also significant when applying INRICH,\(^{33}\) an alternative GSEA method. Of 864 independent intervals that contained the best genome-wide association signals, INRICH identified 26 intervals overlapping with the target genes of the calcium signaling pathway gene set. Subsequent permutation analysis was done by generating a null distribution through the calculation of multiple random intervals of the same size as the observed intervals and computing the overlap with the target gene sets. This analysis revealed significant enrichment for the calcium signaling pathway gene set (empirical \(P = .02\)).

**Replication Sample (n = 1176)**

Next, GSEA of the identical task (picture free recall task) was performed in an independently recruited replication sample. The calcium signaling pathway gene set was enriched significantly (\(P = .02\)).

**Calcium Signaling Pathway Allelic Load Correlates With Hippocampal Activation**

In an additional experiment conducted in a subgroup of the replication sample (n = 1119), fMRI was used to identify gene set–dependent differences in brain activity related to EM. We focused our search on the hippocampus because the calcium signaling pathway gene set was associated with EM, which depends on the hippocampus.\(^{34-37}\) and genes of this gene set are part of the signaling cascade involved in the formation of hip-

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**Table 1. GSEA Results in the Discovery Sample**

<table>
<thead>
<tr>
<th>Database</th>
<th>Gene Set Identification</th>
<th>Gene Set Name</th>
<th>Genes, No.</th>
<th>Significant Genes, No.</th>
<th>(P) Value</th>
<th>(q) Value (FDR)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEGG</td>
<td>hsa04020</td>
<td>Calcium signaling pathway</td>
<td>178</td>
<td>56</td>
<td>(2 \times 10^{-4})</td>
<td>.02</td>
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<td>Gene Ontology</td>
<td>GO:0015837</td>
<td>Amine transport</td>
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<td>17</td>
<td>.001</td>
<td>.11</td>
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<td>Gene Ontology</td>
<td>GO:00015171</td>
<td>Amine transmembrane transporter activity</td>
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<td>18</td>
<td>(6 \times 10^{-4})</td>
<td>.12</td>
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<td>21</td>
<td>.002</td>
<td>.14</td>
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<td>Gene Ontology</td>
<td>GO:0008271</td>
<td>Active transmembrane transporter activity</td>
<td>122</td>
<td>48</td>
<td>(7 \times 10^{-4})</td>
<td>.14</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0045935</td>
<td>Positive regulation of nucleobase–nucleoside–nucleotide and nucleic acid metabolic process</td>
<td>154</td>
<td>38</td>
<td>.002</td>
<td>.15</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0045893</td>
<td>Positive regulation of transcription–DNA dependent</td>
<td>118</td>
<td>24</td>
<td>.003</td>
<td>.18</td>
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<tr>
<td>Gene Ontology</td>
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<td>38</td>
<td>.001</td>
<td>.18</td>
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<td>GO:0051252</td>
<td>Positive regulation of RNA metabolic process</td>
<td>120</td>
<td>48</td>
<td>.004</td>
<td>.19</td>
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<td>G protein coupled receptor activity</td>
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<td>16</td>
<td>.004</td>
<td>.19</td>
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<td>Gene Ontology</td>
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<td>Serine hydrolase activity</td>
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<td>16</td>
<td>.006</td>
<td>.20</td>
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<tr>
<td>Gene Ontology</td>
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<td>Serine-type peptidase activity</td>
<td>45</td>
<td>52</td>
<td>.006</td>
<td>.20</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0008233</td>
<td>Peptidase activity</td>
<td>176</td>
<td>10</td>
<td>.003</td>
<td>.20</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0015082</td>
<td>Di_tri_valent inorganic cation transmembrane transporter activity</td>
<td>22</td>
<td>17</td>
<td>.01</td>
<td>.20</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0008081</td>
<td>Phosphoric diester hydrolase activity</td>
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<td>17</td>
<td>.005</td>
<td>.21</td>
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<tr>
<td>Gene Ontology</td>
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<td>Microsome</td>
<td>42</td>
<td>17</td>
<td>.005</td>
<td>.21</td>
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<tr>
<td>Gene Ontology</td>
<td>GO:0042598</td>
<td>Vesicular fraction</td>
<td>44</td>
<td>16</td>
<td>.01</td>
<td>.25</td>
</tr>
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</table>

Abbreviations: FDR, false discovery rate; GO, gene ontology; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.\(^a\) \(q < .25\).
pocampus-dependent memory in vertebrates. Thus, the left and right hippocampi served as regions of interest. Independent of the allelic load, we detected highly robust picture encoding-related activation (contrast was meaningful vs scrambled pictures) in the hippocampus (eFigure 5 in the Supplement). To capture the multiallelic effect of the calcium signaling pathway gene set on hippocampal activity, we generated an individual multilocus genetic score. The multilocus score for this sample was calculated by using only SNPs (including the respective directions of effect) that proved significant in the independent discovery sample. This procedure prevented model overfitting.

Genetic score–dependent analysis revealed a significant positive correlation between genetic score values and activation in the right hippocampus (peak at 33, −16.5, −16; t = 3.35; uncorrected P = 4 × 10−4; small volume–corrected P < .05). Activations were overlaid on coronal (upper left), sagittal (upper right), and axial (lower left) sections of the study-specific group template displayed at uncorrected P = .001 using color-coded t values.

Comparing the significant components of the calcium signaling pathway gene set between the discovery and replication samples. The overlap was significant (P = .007, exact hypergeometric probability). Of the 144 calcium signaling pathway genes, 26 genes contributed to gene set significance in both samples whereas 66 genes did not contribute to gene set significance in either sample. The remaining 52 genes contributed to gene set significance in 1 of the 2 samples. Thus, the former group of 26 genes was defined as the replicated EM core gene set (Table 2, Figure 3, and eTables 3 and 4 in the Supplement). Further exploratory analysis revealed that the EM core gene set was highly significantly enriched with genes involved in the elevation of cytosolic calcium (42.3% of the genes, P = 8.9 × 10−18). In comparison, the enrichment of the group of 66 noncontributing genes with molecules involved in the elevation of cytosolic calcium (10.6% of the genes, P = 2.7 × 10−7) was 10 orders of magnitude weaker.

**GSEA in Additional Samples**

**Zurich Sample (n = 409)**
Participants performed a picture free recall task similar to the task used in the discovery and replication samples. The EM core gene set was significantly enriched (P = .04, Figure 3). No significant enrichment (P = .70, Figure 3) was found for the set of 66 genes, which did not contribute to the significance of the calcium signaling pathway gene set in any of the discovery and replication samples.

**GSEA in Elderly Participants Without Dementia From the Study on Aging, Cognition, and Dementia in Primary Care Patients Sample (n = 763)**
This sample of cognitively healthy elderly individuals was included to investigate whether the observed association of the EM core gene set with EM performance could also be observed in older adults. Similar to the discovery, replication, and Zurich samples, genome-wide P values for the association with...
EM performance (delayed verbal free recall) under the additive genetic model were used for GSEA. The MAGENTA software revealed significant enrichment ($P = .004$, Figure 3) of the EM core gene set. In addition, no significant enrichment in this sample ($P = .28$, Figure 3) was found for the set of 66 genes, which did not contribute to the significance of the calcium signaling pathway gene set.

**GSEA in AD**

Episodic memory deficits represent a behavioral hallmark of AD and are observed early in the disease. We investigated the enrichment of the EM core gene set in a large AD case-control sample (for a detailed study description, see the eAppendix in the Supplement).

International Genomics of Alzheimer’s Project Case-Control Sample

There were 7,036,050 autosomal SNP $P$ values associated with sporadic AD that served as input for MAGENTA. The MAGENTA software was run with identical parameters as in the studies of cognitively healthy participants. The EM core gene set was significantly enriched ($P = .01$, Figure 3). Also, in this sample, no significant enrichment ($P = .38$, Figure 3) was found for the set of 66 genes, which did not contribute to the significance of the calcium signaling pathway gene set in the EM samples. No significant enrichment was observed for the whole calcium signaling pathway gene set ($P = .16$).

**Discussion**

We detected consistent and robust associations between calcium signaling pathway genes and human EM performance. In particular, a core gene set comprising 26 genes was significantly enriched in 4 independent cohorts of young and elderly cognitively healthy individuals ($n = 3806$). This finding is compatible with the critical role of calcium signaling in molecular processes underlying memory as shown in model organisms and in vitro studies. For example, increases in intracellular calcium are causally related to the induction of long-term potentiation and long-term depression. Two cellular correlates of learning and memory. The results of the present study suggest that genes involved in calcium signaling are also related to human EM throughout adulthood.

Moreover, FMRI data revealed that the individual calcium signaling-related allelic load correlated with hippocampal activity measured during memory encoding. Animal studies have amply demonstrated that calcium signaling genes are crucial for the formation of hippocampus-dependent memory. Our findings suggest that calcium signaling genes are also related to the formation of hippocampus-dependent memory in humans.

The EM core gene set was also significantly enriched in a large case-control study of sporadic AD. Calcium signaling dysregulation has been repeatedly observed in cell cultures and animal models of AD. Our results propose a role for calcium signaling genes in AD and support published studies that identified significant enrichment of the calcium signaling pathway in AD.
Conclusions

By showing robust and consistently significant enrichment in independent cohorts of young and elderly participants, our study identified that calcium signaling plays a central role in hippocampus-dependent human memory processes, both in cognitive health and disease and, therefore, contributes to the under standing and potential treatment of hippocampus-dependent cognitive pathology.

ARTICLE INFORMATION

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Author Contributions: Dr Heck had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Calcium Signaling in Hippocampus-Dependent Memory and Alzheimer Disease