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Calmodulin-binding transcription activator 1 (*CAMTA1*) alleles predispose human episodic memory performance

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Little is known about the genes and proteins involved in the process of human memory. To identify genetic factors related to human episodic memory performance, we conducted an ultra-high-density genome-wide screen at >500 000 single nucleotide polymorphisms (SNPs) in a sample of normal young adults stratified for performance on an episodic recall memory test. Analysis of this data identified SNPs within the calmodulin-binding transcription activator 1 (*CAMTA1*) gene that were significantly associated with memory performance. A follow up study, focused on the *CAMTA1* locus in an independent cohort consisting of cognitively normal young adults, singled out SNP rs4908449 with a *P*-value of 0.0002 as the most significant associated SNP in the region. These validated genetic findings were further supported by the identification of *CAMTA1* transcript enrichment in memory-related human brain regions and through a functional magnetic resonance imaging experiment on individuals matched for memory performance that identified *CAMTA1* allele-specific upregulation of medial temporal lobe brain activity in those individuals harboring the 'at-risk' allele for poorer memory performance. The *CAMTA1* locus encodes a purported transcription factor that interfaces with the calcium-calmodulin system of the cell to alter gene expression patterns. Our validated genomic and functional biological findings described herein suggest a role for *CAMTA1* in human episodic memory.

INTRODUCTION

Human memory is a demonstrated, complex heritable trait, and multiple lines of evidence from twin studies suggest ~50% of phenotypic differences in memory among individuals is because of heritable factors (1). One form of human memory, known as episodic memory, involves in the conscious retrieval of prior experiences (2). Regions of the medial temporal lobe, including the hippocampus, are generally considered to play critical roles in episodic memory formation and retrieval, and pathology in these regions (e.g. in patients with Alzheimer's disease; AD) is typically associated with memory loss (3). Although researchers have gained important insights about the cognitive operations and brain regions associated with episodic memory and other memory systems, relatively little is known about the

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genes and proteins involved in the complex process of memory formation.

Recent candidate-gene association studies have identified variations in multiple genes such as protein kinase C (PKC), protein kinase A (PKA), brain derived neurotrophic factor (BDNF) and serotonin receptor 2A (HTR2A) that have a significant impact on memory aptitude, in a candidate-gene fashion (4–6). Advances in the development of ultra-high-density genotyping platforms (>500 000 single nucleotide polymorphisms; SNPs) now greatly facilitate whole genome association studies for complex diseases (7,8). Recently, we have shown that whole-genome association studies can also be successfully applied to such physiological multigenic traits as human episodic memory (9).

In this report, we provide evidence from genetic, transcriptomic and functional magnetic resonance imaging (fMRI) studies suggesting involvement of common SNPs within the gene encoding calmodulin-binding transcription activator 1 (*CAMTA1*) in the modification of normal episodic human memory performance. These SNPs were identified by further analyzing the data of our recently reported genome-wide screen of 502 627 SNPs (9), which was done in pooled DNA from young adults stratified for episodic memory performance (see Materials and Methods); the SNPs were verified by individual genotyping in the identical training cohort using a different genotyping technology, and were validated subsequently in a *completely independent* cohort of normal subjects.

RESULTS

Cohort 1 memory testing and genotyping

Cognitively and neurologically normal young adults from Switzerland ($n = 351$) were recruited for the initial pooled genetic analysis. To control for genetic background, structured association and genomic control analyses were performed on the recruited individuals through the individual genotyping of 318 SNPs. Ten individuals were identified as outliers (with a probability of allocation to the study population <25%) and were excluded subsequently from the association study. The remaining population ($n = 341$) was stratified into four groups according to their performance on a verbal episodic memory task quantifying their 5 min recall success from a list of 30 semantically unrelated nouns. Equimolar pools of genomic DNA from individuals in each quartile were genotyped at 502 627 SNPs on the Affymetrix GeneChip Mapping EA 500K SNP array chipset. Poorly performing SNPs were discarded, and a single-point statistical approach was employed to select SNPs associated with memory performance. A limited number of studies have been conducted that demonstrate it is possible to accurately predict the allelic frequencies of a SNP from a pooled sample on a microarray and, in fact, identify quantitative trait loci (10–13). In this study, we focused on the genomic region harboring *CAMTA1* (see Materials and Methods for a detailed description of the SNP selection criteria). A *CAMTA1* SNP (rs7547519 at chr1:7250402) yielded a high significance value of allelic association with memory performance ($P = 7 \times 10^{-9}$) at the pooling phase. In addition, an adjacent SNP (rs7549382 at chr1:7255169) also yielded significant results ($P = 0.0001$). The significance value of rs7547519 survives a Bonferroni-corrected significance level (corrected $\alpha = 0.05/502\,627$); a correction widely considered to be too extreme for genome-wide association studies. The pooled analysis was undertaken as an approach to rapidly screen the genome for plausible associated regions; therefore, individual genotyping was employed to confirm these results in both the training population and in an entirely new population. The raw, pooled SNP data for the *CAMTA1* locus are available at http://www.tgen.org/neurogenomics/data.

Individual genotyping was performed on 97 tagSNPs covering 446 888 bp of the genome surrounding the locus highlighted in the pooled analysis in the same individuals from Swiss cohort 1 that contributed to the above detailed pools. The genotype chi-square *P*-values are illustrated in Figure 1 (in black). Two of the SNPs probed on the Affymetrix 500K array during the pooled analysis, rs7547519 and rs7549382, were individually genotyped in the tag SNP analysis and their significances were found to be 0.004 and 0.0005, respectively. Significant SNPs within *CAMTA1*, especially rs7549382 and rs947678, yielded significance levels either below or just above the Bonferroni-corrected (for 97 tests) *P*-value of 0.0005.

Cohort 2 memory testing and genotyping

A new sample of 472 cognitively and neurologically normal young adults from Switzerland (Swiss cohort 2) was tested for visual episodic memory performance. It is important to note that the method of episodic memory testing deliberately differed from the first Swiss cohort, in that the individuals were asked to recall pictures instead of words. The individual SNP significance levels of the same 97 tag SNP panel in this new cohort are also illustrated in Figure 1 (in red). This analysis yielded two different SNPs with significance levels below the Bonferroni-corrected *P*-value, rs4908449 ($P = 0.00002$) and rs705677 ($P = 0.0002$). Note that this correction was for the entire panel of tag SNPs ($n = 97$), which is clearly an overcorrection as a large percentage of the tag SNPs is in linkage disequilibrium (LD) with one another and are therefore not considered to be independent tests. The individual significances in each population are indicated for these select SNPs in Table 1. Significances for each individually genotyped SNP within the locus are provided in Supplementary Material, Table S1.

There were no allele-dependent differences in the outcomes of the d2 cancellation test and the digit span test. The d2 cancellation test reliably quantifies attention and concentration, whereas the digit span test quantifies working memory capacity. Thus, performance in these tests mirrors the engagement of brain structures different than those engaged in episodic memory. We therefore conclude that rs4908449 was not associated with attention, concentration or working memory in this population, but specifically with episodic memory performance.

The rs4908449 SNP is a common $C \rightarrow T$ polymorphism located in an intron of the *CAMTA1* gene (NM_015215). Haplotyping analysis (using the D′ statistic) revealed 24 different blocks in this locus, and SNP rs4908449 was in greatest

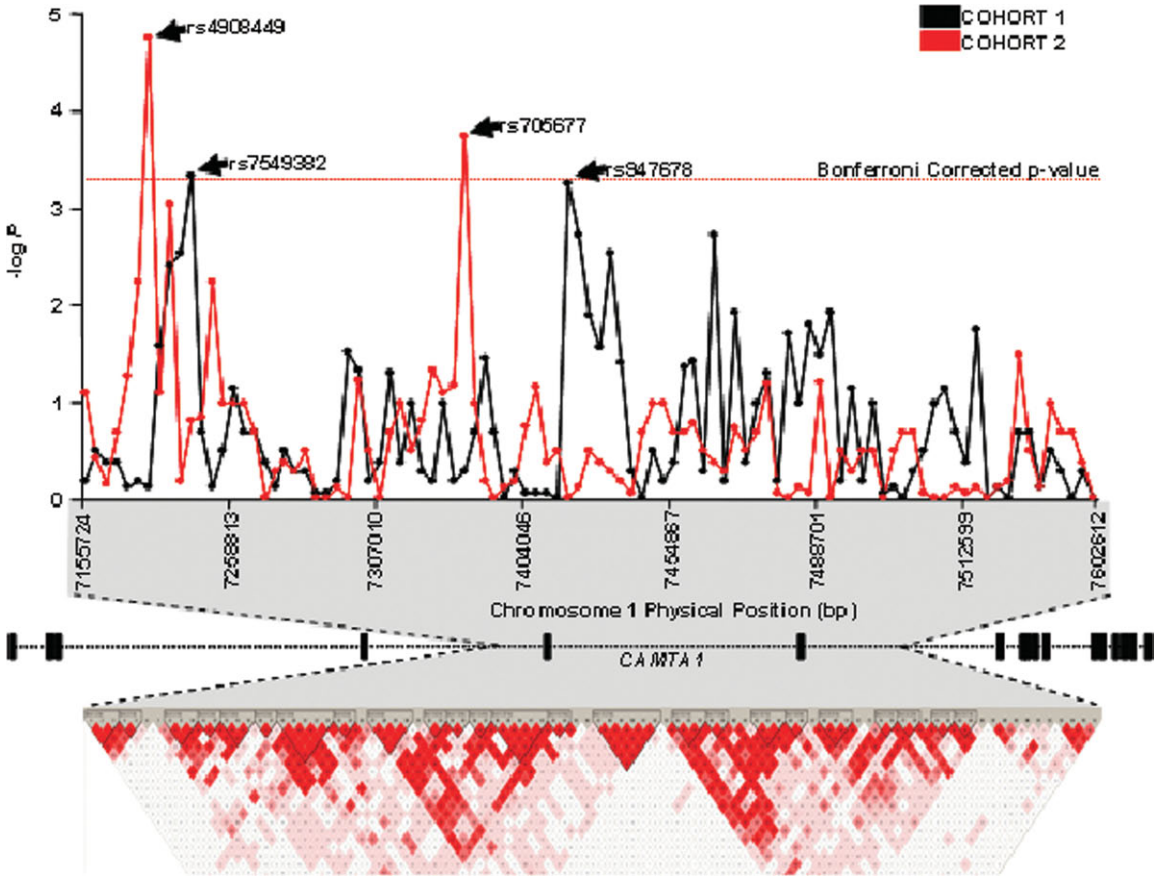

Figure 1. Individual significance levels of tag SNPs in training and validation cohorts. Filled circles indicate the significance level of individual SNPs for each representative population. A Bonferroni-corrected (for 97 tests) significance level of $P = 0.0005$ is indicated by the red horizontal line. Swiss cohort one (black) consists of individuals included during the pooled analysis training, Swiss cohort 2 (red) is individual results from the independent validation cohort. SNPs highlighted in this study are denoted with arrowheads. The relative genomic structure of the *CAMTA1* gene is illustrated in the middle portion of the panel. Filled boxes delineate predicted *CAMTA1* coding exons. Note the region of interest is indicated by dashed connecting lines and spans exons 5–6 of *CAMTA1*. The lower portion of the figure illustrates the haplotype structure of the region as assessed by Haploview 3.2. Red squares represent regions of high linkage disequilibrium (high D′ values).

Table 1. Key associated SNPs and their *P*-values in the Swiss population (cohorts 1 and 2)

SNP	Chromosome 1 location	*P*-value	
		Cohort 1	Cohort 2
rs4908449	7226966	0.7	0.00002
rs7547519	7250402	0.004	0.001
rs7549382	7255169	0.0005	0.15
rs705677	7344176	0.5	0.0002
rs947678	7426882	0.0006	0.9

linkage disequilibrium with haplotype block 5 (Fig. 1, bottom) firmly located within the *CAMTA1* locus, therefore, our observed association is likely unrelated to linkage disequilibrium with any adjacent genes.

Expression analysis of *CAMTA1* in the human and mouse brains

Expression levels of *CAMTA1* in memory-related human brain regions were determined using quantitative reverse transcriptase polymerase chain reaction (RT-PCR). *CAMTA1* mRNA levels were high in the temporal cortex and entorhinal cortex, key regions in human memory processes, and in the cerebellum (Fig. 2). Furthermore, *in situ* hybridization studies in mice show that *CAMTA1* expression is highest in the dentate gyrus and the CA1 regions of the hippocampal formation (Fig. 2, inset), the two brain regions thought to play critical roles in aspects of episodic and spatial memory. Additionally, immunohistochemical staining of CAMTA1 in human middle temporal gyrus sections indicates that its expression is detectable in neurons (Fig. 3). Therefore, *CAMTA1* expression patterns in both the human and mouse brains are consistent with a role in memory performance.

fMRI analysis of the effect of *CAMTA1* alleles on brain activity

fMRI was used to study the relation between *CAMTA1* genotype and human memory-related brain functions. We postulated that the medial temporal brain regions of persons carrying the allele associated with poorer memory perform-

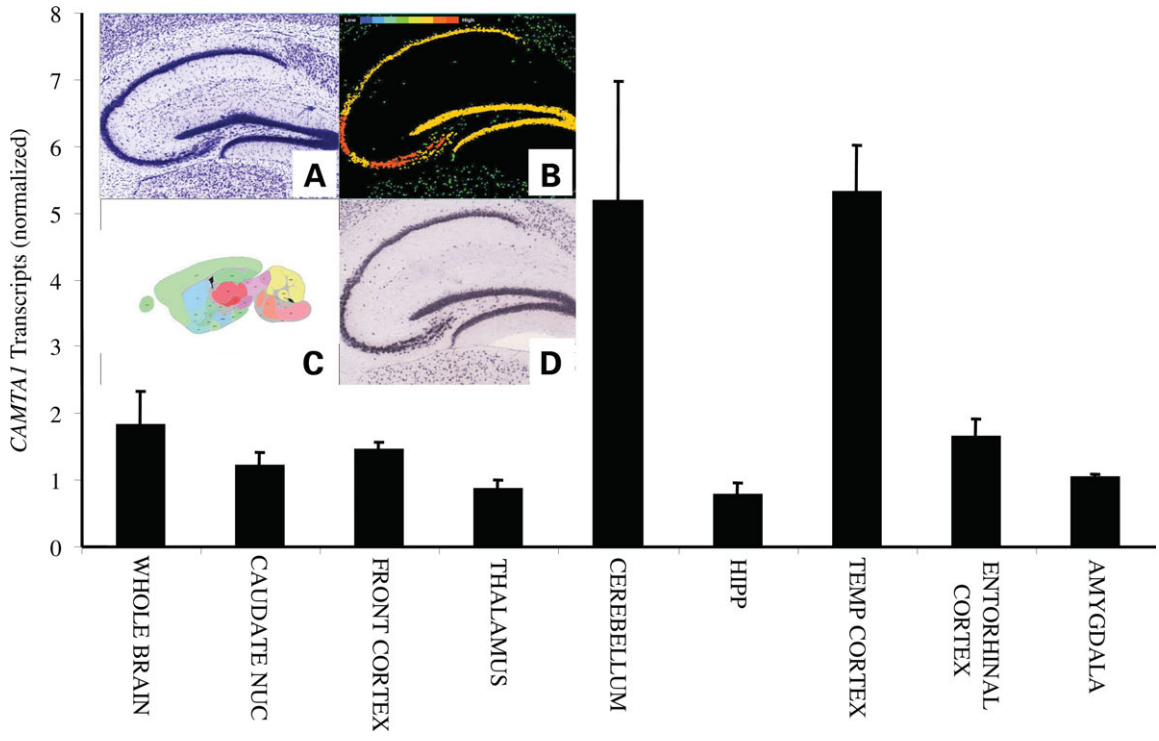

Figure 2. *CAMTA1* expression in human and murine brain. Expression levels of *CAMTA1* were measured by qRT-PCR with data normalized to glyceraldehydr-3-phophate dehydrogenase (GAPDH) expression levels. The inset shows the expression of *CAMTA1* in the murine brain as detected by *in situ* hybridization (Allen Institute for Brain Science, www.brainatlas.com). (**A**) Nissl-stained reference brain section for (**B**), which illustrates a gene expression filter for *CAMTA1* (with red indicating the highest regions of expression). (**C**) Illustration of the relative atlas region and (**D**) magnified view of the raw *in situ* hybridization data for *CAMTA1* in the hippocampal formation, the dark purple staining indicates areas of highest expression.

ance would have to 'work harder' to perform memory tasks as well as non-carriers of this allele. Thirty-five subjects from first Swiss sample (22 rs4908449 *T* allele carriers versus 13 non-carriers of the *T* allele) underwent fMRI. The allelic groups were matched for sex, education, age and 5 min delayed recall performance (all $P > 0.3$) in order to minimize potentially confounding effects of demographic differences or differential task performance when we compared memory-task dependent increases in regional brain activity in the two subject groups. Furthermore, because *CAMTA1* was associated with human episodic memory that depends on the function of the hippocampus, we hypothesized that *CAMTA1* genotypes might affect episodic memory-related information processing in the human hippocampus (14). As neuroimaging studies have found that the hippocampus is especially activated by associative episodic memory tasks, the impact of the *CAMTA1* genotype on hippocampal activations in a face-profession association task was tested (15). During memory retrieval, carriers of the *T* allele showed significantly increased brain activations compared with non-carriers in the medial temporal lobe (local maximum in the right hippocampus at coordinate position [24, −10, −16], $t = 4.24$, $P < 0.001$, coordinates according to the Montreal Neurological Institute; Fig. 4). There were no additional increased cortical activations in carriers of the *T* allele in this episodic memory task. Additionally, in a working memory, task carriers of the *T* allele failed to show significantly greater increases in the brain activity than the non-carriers of this allele during the working memory task, suggesting that allele-related differences in memory performance may be specifically related to some of the cognitive operations and brain regions involved in episodic memory. There were no greater task-related cortical activations in the non-carriers of the *T* allele group compared with carriers of the *T* allele and, furthermore, no allele-dependent differences in cortical brain activations during encoding were found, suggesting that the genotype did not affect episodic memory during that early stage of memory formation. There were no allele-dependent differences in volumes of the hippocampus, suggesting that functional imaging results were not biased by morphological differences.

DISCUSSION

Currently, little is known regarding the molecular machinery involved in the intricate processes that enable the formation and retrieval of memories and the corresponding genetic polymorphisms in the human population that lead to differential memory performance. However, it is crucial that we begin to investigate and understand the genetics of memory to aid in the prediction of amnestic disease risk and also to uncover the associated molecular pathways that may lead to better intelligent design of memory sparing or enhancing pharmaceuticals. It is estimated that ∼30 million Americans suffer from some type of memory disorder (16). Of these, AD is the most common, afflicting ∼10% of persons over the age

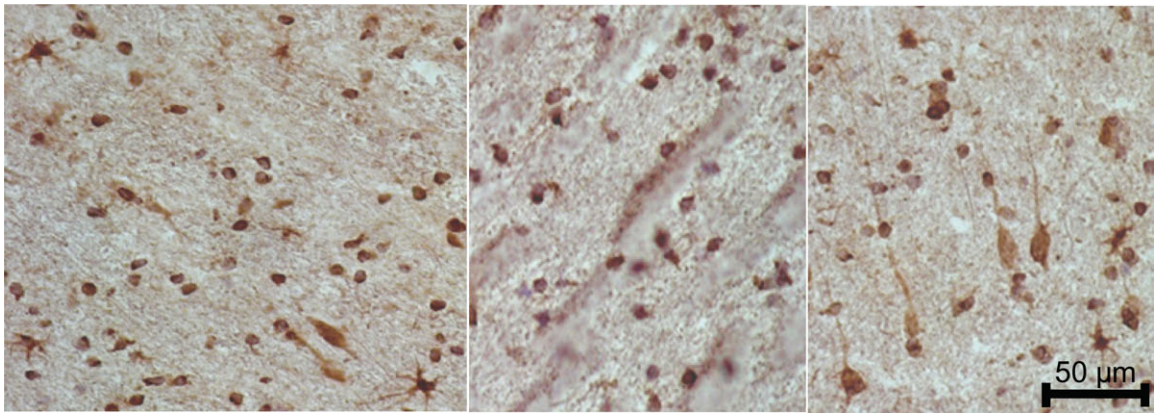

Figure 3. *CAMTA1* expression in middle temporal gyrus human brain sections. *CAMTA1* staining was performed in frozen middle temporal gyrus sections from neurological normal adults. Sections exposed only to secondary antibody were investigated as controls (not shown). Note CAMTA1-positive cells (brown staining) of varying morphologies. The included scale bar is representative for all of the panels shown.

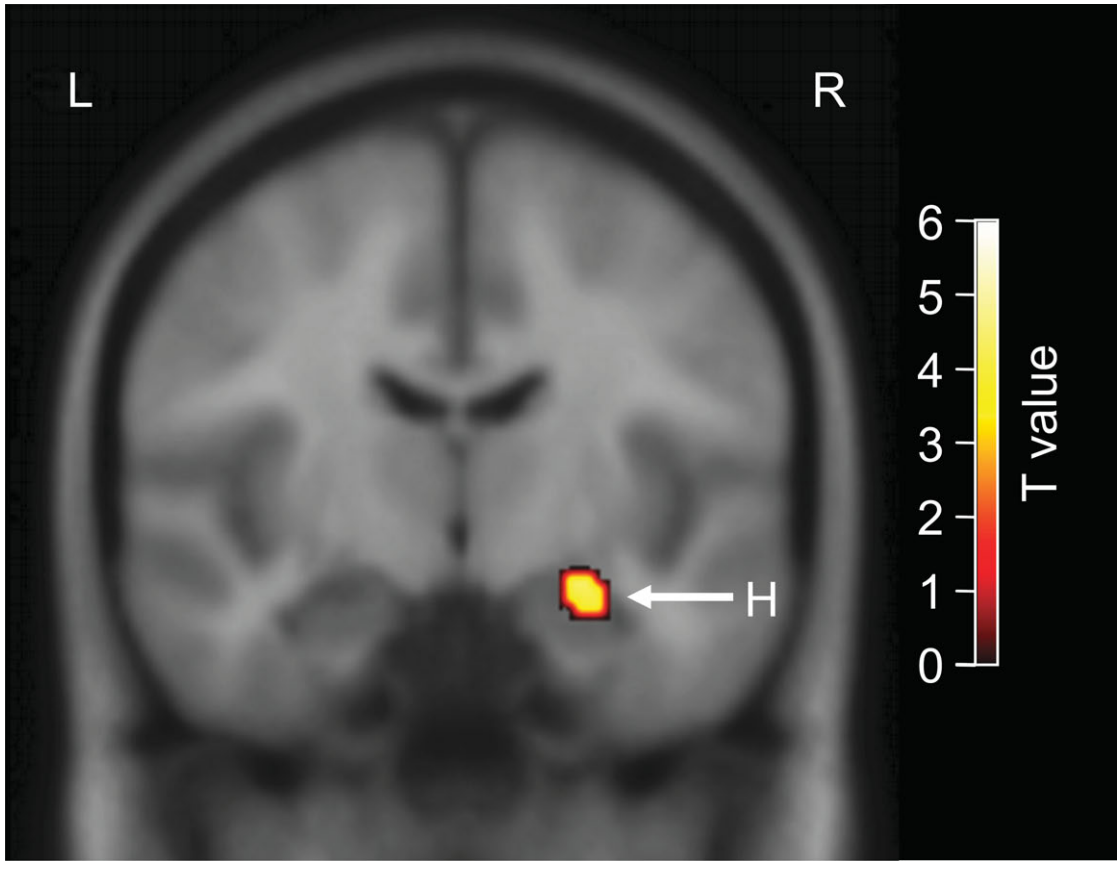

Figure 4. *CAMTA1* allele-dependent differences in episodic-memory-related hippocampal activation as measured with fMRI. The figure illustrates significantly greater activations in the right hippocampus of carriers of the *T* allele ($n = 22$) than in non-carriers of the *T* allele ($n = 13$) of SNP rs4908449 during a face-profession associative memory task. Activations were overlaid on a coronal section of a T1-weighted MR image of SPM2 and displayed in color-coded *t*-values (see bar). Threshold: $P < .001$. H: hippocampus; L/R: left/right side of brain.

of 65 years and almost half of those >85 years (17). Other diseases with an amnestic component include Down's syndrome, schizophrenia, depression, Parkinson's disease and epilepsy (18–23). This is all in addition to the normal memory decline associated with the aging process. With all of this said, there is currently only one major class of drugs approved for use to combat memory impairment. However, it is well known that if one could delay the onset of AD by 5 years, presumably through the use of memory-sparing pharmaceuticals, the incidence of the disease would, in turn,

decrease by 50% (24). All of this points to a growing need to better understand the genes and proteins participating in the normal intact process of memory.

To begin to address this need, we performed a pooling-based whole-genome association study in cognitively normal individuals stratified by episodic memory performance. The use of a pooled association study design was undertaken, because it affords a rapid, cost-effective approach for the identification of the top, several, significantly associated regions (11,13,25,26). However, the use of such an experimental design is admittedly not ideal for genome-wide association since by its nature all individual haplotyping ability is lost by the pooled approach. In any case the important aspect to realize is that the pooled approach is an effective prioritization strategy that requires subsequent individual genotyping validation and replication of any associated SNP(s). In this study, we performed individual genotyping in two independent populations to identify the significantly associated SNPs. *CAMTA1* association through the use of the same training cohort was detailed in our previous study, highlighting the association of *KIBRA* with episodic memory (9). Of note, *CAMTA1* SNPs were significantly associated with human episodic memory performance irrespective of modality of the task (i.e. verbal or visual episodic memory). We also report here the identification of significant SNPs within the same genetic locus that differ between our two seemingly genetically homogeneous cohorts. This is a common observation in association studies of polygenic phenotypes and diseases, as was also shown in a recent study on the association of SORL1 with AD (27).

The associated gene, *CAMTA1*, is a member of the family of calmodulin-binding transcriptional activators (CAMTAs), shown to be conserved across a wide range of multicellular eukaryotes including *Arabidopsis*, *Drosophila* and *Caenorhabditis* in addition to humans (28,29). Human CAMTA family members encode for four main protein domains, including a transcription factor immunoglobulin (TIG)-like DNA-binding domain, calmodulin-binding IQ motifs, ankyrin repeats and the family-specific DNA-binding domain termed as CG-1 that contains a bipartite nuclear localization signal (29). The majority of the published literature on *CAMTA1* in humans has focused on its purported role as a tumor suppressor gene in neuroblastoma patients where its genomic deletion and lowered expression were found to be significantly associated with poor clinical outcome (30–33). Other *CAMTA1* biology in humans is still poorly understood; however, in *Arabidopsis* it was shown that CAMTAs are able to bind double-stranded DNA and calmodulin, are primarily localized to the nucleus and could activate transcription (29). Calcium-responsive proteins including CREB, calcineurin, CAMKII and calmodulin have an established role in neuronal plasticity, LTP, and learning and memory across multiple organisms (34–39). Polymorphisms in a calcium-responsive transcriptional activator, as described herein, suggest a mechanism involving differential regulation of genes that may participate in the downstream more lasting changes involved in long-term memory. Our findings illustrated here demonstrate an association between a *CAMTA1* allele and a differential performance in normal episodic memory, and raise the possibility of adding *CAMTA1* to the list of calcium-responsive proteins that are responsible for the process of memory in humans. Additional studies are needed to confirm its role in the modulation of episodic memory performance, how it may be related to the memory changes associated with normal brain aging and several neurological and psychiatric disorders and the extent to which it can be used to identify promising memory-enhancing treatments.

MATERIALS AND METHODS

Study subjects and memory testing

Cohort 1. Memory testing and genotyping were performed in 351 cognitively and neurologically normal young adults from Switzerland (240 females, 111 males; median age, 22 years; range, 18–48 years). Subjects viewed six series of five semantically unrelated nouns presented at a rate of one word per second with the instruction to learn the words for immediate free recall after each series; additionally, subjects underwent an unexpected, delayed free-recall test of the learned words after 5 min and again after 24 h.

Cohort 2. Memory testing and genotyping were performed in 472 other cognitively and neurologically normal young adults from Switzerland (353 females, 119 males; median age, 21 years; range, 16–28 years). In this study, however, the subjects were presented 10 semantically unrelated pictures at a rate of one picture per 4 s and delayed free recall was tested after 10 min. Attention and concentration with the d2 cancellation test and working memory with the digit span task were assessed.

Assessment of genetic background

Structured association analysis was performed by individually genotyping all 351 subjects from Swiss cohort 1 at 318 unlinked SNPs. Calculation of population structure was performed using the STRUCTURE program (40). We estimated the ancestry of study subjects under the a priori assumption of $K = 2$ discrete subpopulations. Structured association analysis revealed moderate allele-frequency divergence among populations. Identical results were obtained under the a priori assumption of $3 \leq K \leq 6$ discrete subpopulations.

Affymetrix 500k genechip SNP genotyping in cohort 1

Individual genomic DNA concentrations were determined with the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, California, USA). Individuals were designated to each pool based on their quartile ranking in 5 min free recall on the verbal episodic memory task. Each individual contributed a total of 120 ng of DNA to the pool, and each pool was created *de novo* a total of three times. These three pools were then genotyped in duplicate on both the Early Access 500K Mendel array from Affymetrix (Santa Clara, CA, USA) as described in the Early Access Version 2.0 of the Mendel Array protocol.

Statistical analysis of pooled genotyping data

Calculation of a SNP's allelic frequency was based on its corresponding relative allele signal (RAS) score used previously to calculate allele frequencies in pooled DNA (11,13). RAS values were generated for each SNP using a specifically developed, freely available PERL script (http://bioinformatics.tgen.org/software/tgen-array/). RAS-derived allelic frequencies were used to calculate SNP-specific χ^2-values for following comparisons: top 50% versus bottom 50% performers (entire sample) and top 25% versus bottom 25% performers (distribution extremes). Because RAS1 and RAS2 values were treated independently, statistics for each SNP were calculated a total of four times. SNPs fulfilling the following criteria in at least one of the four comparisons were considered significant: $\chi^2 \geq 28$, $df = 1$ (corresponding to $P \leq 0.05$, Bonferroni corrected for 500 000 comparisons), variation coefficient of RAS-derived allelic frequencies ≤ 0.2 (see Supplementary Material, Figure S1). Of the 1308 SNPs fulfilling the above-mentioned criteria, SNPs within *CAMTA1* were considered promising targets because CAMTA1 protein binds calmodulin, a key player in synaptic plasticity. In contrast to our previous study, the 1308 significant SNPs were not tested by a second data reduction method (i.e. sliding window). The decision to follow up on *CAMTA1* SNPs was based on the candidate nature of this gene and on neurobiological considerations.

Individual genotyping

Genotyping was performed either by Pyrosequencing (Uppsala, Sweden) on a PSQ 96 MA machine and/or by the Amplifluor method (www.kbiosciences.com). Haploview 3.2 was used for the analysis of linkage disequilibrium and haplotype reconstruction. Multifactorial analyses of variance were performed for the simultaneous assessment of the influence of age, sex, education and genotype effects on cognitive test performance. All tests were two-tailed.

Human gene expression studies *CAMTA1* quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on an ABI PRISM 7700 TaqMan machine (ABI, Foster City, CA, USA) with SYBR master mix (Stratagene, Gebouw California, Amsterdam, The Netherlands). cDNA from human brain regions was purchased from BioCat (BioCat GmbH, Im Neuenheimer Feld, Heidelberg, Germany). Primers were designed by PrimerDesign [(v1.9, ABI), primer details are available on request]. RNA was obtained from commercially available histopathologically normal human brain tissue (www.biocat.de and www.clontech.com; four donors, median age at death, 27.5 years; range, 24–60 years).

CAMTA1 immunohistochemistry

Frozen middle temporal gyrus brain sections were obtained through collaboration with the Sun Health Research Institute (Sun City, AZ, USA). All donors were cleared as neurologically normal based on clinical interview prior to death in addition to pathological screening for molecular markers of degeneration and disease, such as neuronal plaques and tangles, after death. Sections were allowed to warm to room temperature, were fixed for 5 min in acetone in room temperature, equilibrated in Tris-buffered saline (TBS, 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0) and exposed to 3% hydrogen peroxide in TBS for 10 min at room temperature to block endogenous peroxidase activity. Sections were rinsed with TBS containing 0.05% Tween 20 (TBST), and slide pairs were constructed for the Shandon Sequenza capillary gap staining system (Thermo Electron Corporation, Waltham, MA, USA). The Vectastain ABC rat IgG kit (Vector Laboratories, Burlingame, CA, USA) was used to detect the *CAMTA1* signal. Slide pairs were incubated with 200 μl of 10% normal horse serum in TBST for 10 min. Slides were then incubated with 100 μl of 1:100 dilutions of anti-CAMTA1 primary antibodies (a generous gift of Dr Kaname Nakatani, Mie University School of Medicine, Japan) in TBST with 10% normal horse serum for 2 h. After the first hour, the primary antibody solution was refreshed with an additional 100 μl of anti-CAMTA1 primary antibodies. Secondary only control sections were incubated with TBST with normal horse serum during this period. Slides were then rinsed with 2 ml of TBST and incubated with 200 μl of 1:200 diluted anti-rat secondary antibodies for 30 min (Vector Laboratories). After rinsing with TBST, slides were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen for 10 min. Sections were rinsed with TBST, incubated for 5 s in undiluted hematoxylin counter-stain, rinsed again with TBST and then with distilled water. Slides were then dehydrated in increasing percentages of ethanol and finally into to xylene prior to mounting and imaging.

Functional magnetic resonance imaging

Subjects. Twelve males, 23 females; median age, 22 years; range, 19–32 years.

Episodic memory task. We presented 16 face-profession pairs for associative learning and 24 head contours without physiognomy in the visual baseline condition. Both the baseline condition and the learning condition were given in three consecutive learning runs (three separate fMRI time series). The order of conditions and stimuli was constant across the three runs. For associative learning of the face-profession pairs, subjects were instructed to imagine the presented person acting in a scene of the written profession. Subjects answered by button press (with their dominant hand) whether they found it easy or hard to imagine a scene. Subjects were requested to imagine the same scene for a given face-profession pair during runs 2 and 3 as during run 1. The visual baseline task was to decide whether the area of the left or right ear was larger. The sequence of conditions within the fMRI time-series was counterbalanced across subjects. Both the learning and the baseline condition consisted of four blocks. In the learning condition, a block contained four stimuli of 6 s each. In the baseline condition, a block contained six stimuli of 4 s each. An instruction slide announced each task block. For memory retrieval, we applied a single fMRI time series. This time series included an associative retrieval condition and the same visual baseline condition that was used for the encoding time series. For the retrieval

of the associations, the previously presented faces were shown again (without the professions) as retrieval cues with the instruction to recall each person's occupation and to indicate the superordinate professional category by button press: academic or workman. The sequence of conditions within the fMRI time series was counterbalanced across subjects. The retrieval condition consisted of four blocks, each block including four stimuli of 6 s each. All task blocks took 24 s and were announced by an instruction slide.

Working memory. The experiment included one fMRI time series with a 2-back task for the assessment of working memory and a baseline task ('x-target') for the assessment of concentration. The 2-back task required subjects to respond to a letter repeat with one intervening letter (**S**—f—**s**—g). The 'x-target' task required subjects to respond to the occurrence of the letter 'x'. Each task was given in five blocks of 26 s each. Blocks were announced by an instruction slide. Stimuli were 50 upper- or lowercase letters typed in black on a white background. Thirteen upper- or lowercase letters were presented per block for the duration of 2 s each.

Data acquisition. Magnetic resonance measurements were performed on a 3 T Philips Intera whole-body MR scanner equipped with an eight-channel Philips SENSE head coil. Functional data were obtained from 32 transverse slices parallel to the AC-PC plane covering the whole brain with a measured spatial resolution of $2.8 \times 2.8 \times 4\ \text{mM}^3$ (acquisition matrix 80×80) and a reconstructed resolution of $1.7 \times 1.7 \times 4\ \text{mM}^3$. Data were acquired with a SENSE-sshEPI sequence with an acceleration factor of $R = 2.0$ (41). Other scan parameters were echo time (TE) = 35 ms, scan repeat time (TR) = 3000 ms, $\theta = 82^\circ$. A standard, three-dimensional T1-weighted scan was obtained for anatomical reference with a measured spatial resolution of $1 \times 1 \times 1.5\ \text{mM}^3$ (acquisition matrix 224×224) and a reconstructed resolution of $0.9 \times 0.9 \times 0.8\ \text{mM}^3$, TE = 2.3 ms, TR = 20 ms, $\theta = 0^\circ$. A two-dimensional, T1-weighted inversion-recovery anatomical scan, oriented perpendicularly to the long axis of the hippocampus, was obtained for hippocampal and parahippocampal volumetry over 33–39 slices with a measured spatial resolution of $0.5 \times 0.6 \times 1.5\ \text{mM}^3$ (acquisition matrix 400×320) and a reconstructed spatial resolution of $0.4 \times 0.4 \times 1.5\ \text{mM}^3$, TE = 5 ms, TR = 4200 ms, $\theta = 20^\circ$, IR delay 400 ms and no interslice gaps.

Analysis of fMRI data. Image pre- and postprocessing and the statistical analyses were performed with SPM2. Standard preprocessing procedures were applied, realignment, normalization and spatial smoothing (8 mM) (42). On the single subject level, data were analyzed according to the fixed effects model (SPM2). The six head movement parameters were included in the model as confounding factors. Data were high-pass filtered with a specific filter value for each fMRI time series. This value was determined according to '2*SOA*TR'. On the second level, within-subject contrasts were entered into random effects analyses (ANOVAs, *t*-tests, SPM2), which account for variance between subjects (43). Thresholds were set at a $P < 0.001$ level, uncorrected for multiple comparisons, which is a widely used threshold for activations within the medial temporal lobe. Only activations with <5 voxels were considered. SPM2 coordinates refer to standard brains from the Montreal Neurological Institute.

Analysis of anatomical MRI data. On the basis of the three-dimensional, T1-weighted structural MRI images, which covered the whole brain, volumes of the total gray and white matter, were computed with SPM2. Images were first normalized into the Montreal Neurological Institute (MNI) T1 template with a standard bounding box and then segmented into gray matter, white matter and cerebrospinal fluid. Standardized gray and white matter volumes were then multiplied by the determinant of the linear transformation matrix to obtain gray and white matter volumes in cubic centimeter. On the basis of the two-dimensional, T1-weighted, high-resolution structural MRI images, two independent raters manually delineated the hippocampal formation (CA regions, dentate gyrus and subiculum, excluding the fimbria) and the parahippocampal gyrus using the software Pmod (14). Cerebrospinal fluid was carefully excluded, resulting in conservative volume estimates. Raters relied on descriptions of anatomical landmarks and subdivisions of the MTL as described earlier (44). Inter-rater reliabilities ranged between $r = 0.8$ and 0.98.

SUPPLEMENTARY MATERIAL

Supplementary material is available at HMG Online.

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