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Common variants at 12q14 and 12q24 are associated with hippocampal volume

Aging is associated with reductions in hippocampal volume that are accelerated by Alzheimer's disease and vascular risk factors. Our genome-wide association study (GWAS) of dementia-free persons ($n = 9,232$) identified 46 SNPs at four loci with P values of $<4.0 \times 10^{-7}$. In two additional samples ($n = 2,318$), associations were replicated at 12q14 within *MSRB3-WIF1* (discovery and replication; rs17178006; $P = 5.3 \times 10^{-11}$) and at 12q24 near *HRK-FBXW8* (rs7294919; $P = 2.9 \times 10^{-11}$). Remaining associations included one SNP at 2q24 within *DPP4* (rs6741949; $P = 2.9 \times 10^{-7}$) and nine SNPs at 9p33 within *ASTN2* (rs7852872; $P = 1.0 \times 10^{-7}$); along with the chromosome 12 associations, these loci were also associated with hippocampal volume ($P < 0.05$) in a third younger, more heterogeneous sample ($n = 7,794$). The SNP in *ASTN2* also showed suggestive association with decline in cognition in a largely independent sample ($n = 1,563$). These associations implicate genes related to apoptosis (*HRK*), development (*WIF1*), oxidative stress (*MSR3B*), ubiquitination (*FBXW8*) and neuronal migration (*ASTN2*), as well as enzymes targeted by new diabetes medications (*DPP4*), indicating new genetic influences on hippocampal size and possibly the risk of cognitive decline and dementia.

Differences in hippocampal volume that appear with advancing age represent cumulative effects of early-life factors, life-course events and disease. Hippocampal atrophy is a recognized biological marker of Alzheimer's disease[1,2]; however, it is influenced by various vascular and metabolic factors[3,4]. Because hippocampal volume is a heritable[5], widely measurable trait that shows meaningful detectable changes throughout life, it is a suitable endophenotype for aging-related physiological processes and presymptomatic diseases, improving the power to detect genetic associations.

We explored genetic influences on hippocampal volume by conducting a cross-sectional genome-wide association analysis in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium[6] among 9,232 dementia-free persons from 8 community-based studies whose mean age ranged from 56 to 84 years (weighted average of 67.1 years). Each study imputed to a common set of SNPs from the Phase 2 HapMap Centre d'Etude du Polymorphisme Humain (CEPH) Utah residents of Northern and Western European ancestry (CEU) population using genotype data from Illumina or Affymetrix arrays; additive genetic models associating total hippocampal volume and genotype dosage were fitted with adjustment for age, sex and familial relationships (if applicable; **Supplementary Note**); and genomic control was applied. Study-specific results were combined in an inverse variance–weighted meta-analysis.

We then conducted *in silico* replication of associations that reached genome-wide significance and sought additional evidence for suggestive associations in a second-stage targeted meta-analysis of 2,318 subjects from two community-based studies: the Three City Study and an independent sample from the third expansion of the Rotterdam Study. Characteristics of the discovery and replication samples are given (**Supplementary Table 1**).

A Manhattan plot of $-\log_{10}$ (P values) from the discovery analysis is shown (**Fig. 1**), where P values for 46 SNPs at 4 loci (**Supplementary Table 2**) surpassed our replication threshold of $P < 4.0 \times 10^{-7}$ corresponding to 1 expected false positive. Of these, 18 SNPs at 2 loci surpassed a genome-wide significance threshold of $P < 5.0 \times 10^{-8}$: the 12q14 locus, which included *WIF1*, *LEMD3* and *MSRB3*, and the 12q24 locus, which included *HRK* and *FBXW8*. We found evidence of replication ($P < 0.01$) for both associations. The remaining suggestive associations included SNPs at 2q24 within *DPP4* and at 9p33 within *ASTN2*, which had consistent directions of association in the discovery and replication phases but did not attain genome-wide significance in a combined analysis. Estimates for each stage are shown (**Table 1**). Discovery GWAS results for the region around each signal were annotated with recombination rates and known genes (**Fig. 2**), and study-specific findings are shown (**Fig. 3**).

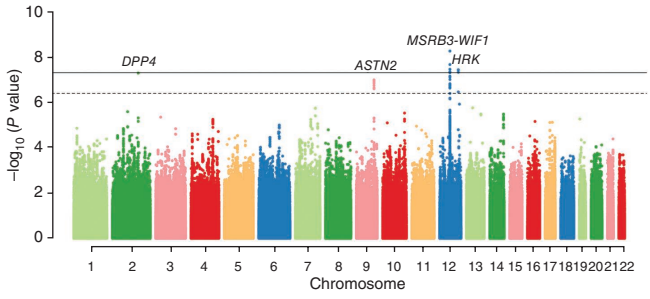

Figure 1 Genome-wide Manhattan plot for hippocampal volume. The plot shows individual P values (based on the discovery meta-analysis) against genomic position for association with hippocampal volume. Within each chromosome (x axis), the results are plotted left to right from the p-terminal end. The dashed line indicates the threshold for replication of $P < 4 \times 10^{-7}$, and the solid line indicates the threshold for genome-wide significance of $P < 5 \times 10^{-8}$. The nearest genes are indicated above SNPs that reached the significance threshold for replication.

A full list of authors and affiliations appears at the end of the paper.

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Table 1 Discovery, replication and combined meta-analysis

					Discovery meta-analysis				Replication meta-analysis				Discovery and replication		
Locus	SNP	Gene	Allele 1/2	Allele 1 frequency	β	s.e.m.	P	N	β	s.e.m.	P	N	β	s.e.m.	P
2q24	rs6741949	*DPP4*[a]	G/C	0.53	−61.4	11.3	5.2×10^{-8}	6,673	−10.1	25.2	0.7	1,369	−52.8	10.3	2.9×10^{-7}
9q33	rs7852872	*ASTN2*[a]	C/G	0.62	−53.1	10.0	1.0×10^{-7}	9,187	−25.0	20.4	0.2	2,318	−47.7	9.0	1.0×10^{-7}
12q14	rs17178006	*MSRB3*[a]	G/T	0.10	−121.0	20.7	5.5×10^{-9}	5,249	−137.9	45.5	0.002	1,003	−123.8	18.9	5.3×10^{-11}
	rs6581612	*WIF1*	C/A	0.27	−60.5	10.8	2.2×10^{-8}	9,183	−75.2	22.1	0.0007	2,318	−63.3	9.7	7.1×10^{-11}
12q24	rs7294919	*HRK*	T/C	0.91	−97.7	17.9	4.8×10^{-8}	8,089	−154.0	38.3	5.8×10^{-5}	1,573	−107.8	16.2	2.9×10^{-11}

Allele 1/2, coded (risk)/non-coded allele; β, association estimate in mm^3; N, effective sample size: $\sum$(imputation quality × N).
[a]The SNP is located within the indicated gene.

We present association estimates for a meta-analysis combining the discovery and replication results for these four loci. To contextualize the magnitude of a SNP's association with hippocampal volume, we divided the regression coefficient for the allele by the mean decrease in hippocampal volume for each year of chronological age (−27.4 mm^3 per year, estimated within the Framingham Heart Study).

The strongest association was for rs7294919, located at 12q24 between *HRK* and *FBXW8*, where each copy of the T allele (allele frequency (AF) = 0.91) was associated with lower hippocampal volume (regression coefficient beta, $\beta = -107.8$ mm^3; $P = 2.9 \times 10^{-11}$), equivalent to 3.9 years of aging.

HRK is expressed throughout the brain, with the highest levels in the amygdala, entorhinal cortex and hippocampus[7]. The HRK protein acts as a key regulator of apoptosis[8], a complex pathway associated with aging, ischemia and Alzheimer's disease[9], through its interaction with the death-repressor proteins Bcl-2 and Bcl-X_L (ref. 10). In rat neuronal cell cultures, a homologous protein, DP5 (with 72% identity), is induced during β-amyloid–mediated cytotoxicity, withdrawal of nerve growth factor (NGF)[11] and induced global ischemia[12]. Although treatment strategies aimed at modifying the apoptotic pathway have yet to achieve success[13], our findings suggest that this area of therapeutics might remain promising.

FBXW8 encodes the substrate-recognition component of a Skp1-Cullin–F-box protein (SCF) E3 ubiquitin ligase found in the Golgi apparatus of neurons. Different E3 ligase complexes target specific substrates for polyubiquitination and proteasome-mediated degradation[14], suggesting a role for FBXW8 in clearing abnormal and potentially toxic protein aggregates, particularly hyperphosphorylated tau[15]. The described role of FBXW8 in presynaptic development[16], synapse formation, neurotransmitter release and promotion of dendrite growth in hippocampal neurons makes a genetic association with hippocampal volume plausible[17]. Whether one or both of the *HRK* and *FBXW8* genes are involved in determining hippocampal volume is unclear, as rs7294919 is an expression SNP (eSNP) associated with changes in the expression of both[18–21].

At the 12q14 locus, the G allele of rs17178006 (AF = 0.10), intronic within *MSRB3*, was associated with decreased hippocampal volume ($\beta = -123.8$ mm^3; $P = 5.3 \times 10^{-11}$), equivalent to 4.5 years of aging. *MSRB3* catalyzes the reduction of methionine sulfoxide residues in proteins and requires zinc or selenium as a cofactor. Thus, the association of lower selenium levels with elevated plasma homocysteine—which in turn has been associated with increased risk of Alzheimer's disease and hippocampal atrophy[22–24]—may be mediated by suppression of MsrB proteins in various organs, including the brain[25]. Several SNPs in low linkage disequilibrium (LD; $r^2 = 0.2$) with rs17178006 were also associated with decreased hippocampal volume, including rs6581612 (AF = 0.27; $\beta = -63.3$ mm^3; $P = 7.2 \times 10^{-11}$) between *WIF1* and *LEMD3*. The WIF1 protein inhibits extracellular Wnt signaling proteins, which have a role in embryonic development, along with β-catenin, and hippocampal aging[26]. Changes in Wnt signaling mimic the effects of environmental enrichment increasing hippocampal synaptic densities[27]. *LEMD3* encodes a transforming growth factor–β antagonist expressed in the hippocampus and is upregulated protectively during ischemia and epileptogenesis[28–30]. Further, the LEMD3 protein interacts with progerin, the abnormal form of laminin A responsible for premature aging in progeria (Hutchinson-Gilford syndrome)[31].

In testing for independent effects of these two SNPs in conditional models, both associations were attenuated, but only that at rs17178006 remained significant ($P < 0.05$; **Supplementary Fig. 1**), suggesting that the SNPs mark a single locus. Whereas *MSRB3* may be most influential, it remains possible that more than one gene in this region is

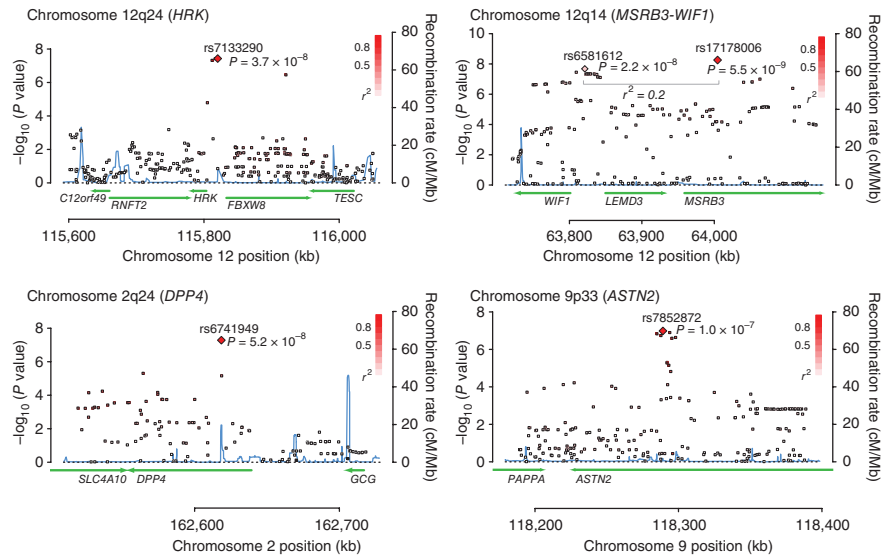

Figure 2 Regional plots for SNPs associated with hippocampal volume. Plots are centered on the most significant SNP at a given locus along with the meta-analysis results for SNPs in the region surrounding it (typically ± 100 kb). All SNPs are plotted with their discovery meta-analysis P values against genomic position, with the most significant SNP in the region indicated as a diamond and other SNPs colored according to their pairwise correlation (r^2) with the signal SNP. The light blue line represents the estimated recombination rate. Gene annotations are shown as dark green lines.

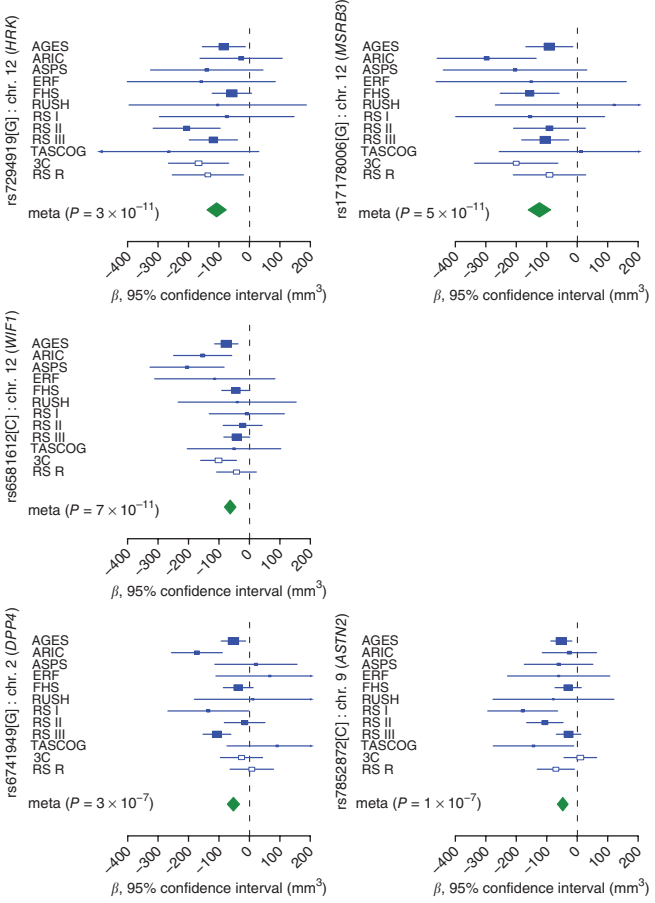

Figure 3 Forest plots for association of SNPs with hippocampal volume. Plots show the study-specific association estimates (β) and 95% confidence intervals for the discovery- and replication-stage studies presented as rectangles and bars, respectively. Estimates from the replication phase are indicated by open rectangles. Arrowheads indicate confidence intervals that extended beyond the *x* axis. For each, SNP, the association estimate and confidence interval for the meta-analysis combining discovery and second-stage results are presented as a diamond.

associated with hippocampal volume. For example, eight eSNPs in the vicinity of this locus (**Supplementary Table 3**) were associated with hippocampal volume at $P \leq 5.3 \times 10^{-4}$ and have been reported to modify *LEMD3* expression[20].

In addition to these findings, SNPs at two additional loci showed suggestive evidence for association but did not reach genome-wide significance in our combined meta-analysis. The first was rs6741949 in an intron of *DPP4* on chromosome 2q24, where the G allele (AF = 0.53) was associated with smaller hippocampal volume ($\beta = -52.8$ mm^3; $P = 2.9 \times 10^{-7}$). Many bioactive peptides whose levels are altered in Alzheimer's disease and vascular brain injury are substrates for the DPP4 protein[32], and DPP4 reduces extracellular β-amyloid deposition in mouse models of Alzheimer's disease[33]. Further, DPP4 is an intrinsic membrane glycoprotein and a widely expressed serine exopeptidase[34]. It is also an adipokine that is overexpressed in the visceral adipose tissue of obese persons and in individuals with diabetes[35], conditions associated with smaller hippocampal volume[3,36]. A new class of antidiabetic medications (sitagliptin and related incretin compounds) inhibits DPP4 to improve insulin sensitivity and glucose tolerance through increased levels of glucagon-like protein–1 (GLP-1) and GLP-2. Of note, endogenous incretin, GLP-1, is also strongly expressed in some hippocampal neurons and has neuroprotective properties[37–39].

The second suggestive association was for rs7852872, located in an intron of *ASTN2* on chromosome 9, where the C allele (AF = 0.63) was associated with lower hippocampal volume ($\beta = -47.7$ mm^3; $P = 1.0 \times 10^{-7}$). The *ASTN2* protein is a cell adhesion molecule expressed in neurons, including those in the dentate gyrus, and is hypothesized to function in glial-guided neuronal migration[40,41].

We sought additional replication of our significant and suggestive associations by testing the lead (or proxy) SNP from each locus in data from the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) Consortium. Briefly, samples from this group ($n = 7{,}794$; mean age of 39.9 years) came from multiple studies including normal older individuals, a developmental sample and individuals symptomatic for cognitive or affective diseases. Among the ENIGMA sample, we observed a consistent direction of association for all cross-study comparisons. For the loci where the lead SNP was available in ENIGMA, replication was strongest at *HRK-FBXW8* (rs7294919; $P = 1.6 \times 10^{-7}$) and nominal at *DPP4* (rs6741949; $P = 0.04$). Although the lead SNPs were not available at the other loci, one proxy SNP in weak LD ($r^2 = 0.3$) with rs17178006 (*MSRB3*) and another in strong LD ($r^2 = 0.96$) with rs7852872 (*ASTN2*) both had *P* values of <0.05 in ENIGMA data (**Table 2**).

Because the ENIGMA and CHARGE samples differed in two key aspects—the ENIGMA sample included younger adults (8 of 13 studies had no participants older than 65 years of age) and some persons with cognitive impairment and dementia (13% of the sample)—we examined the top loci in subsamples of healthy persons ($n = 5{,}775$; mean age of 34.8 years) and cognitively intact older persons ($n = 816$; mean age of 67.2 years). Association estimates were generally similar to those of the full sample (**Supplementary Table 4**).

Given the established relationship between hippocampal atrophy and Alzheimer's disease, we investigated whether SNPs from published Alzheimer's disease GWAS[42–46] were associated with hippocampal volume in our discovery meta-analysis (**Supplementary Table 5**). We found nominal association with smaller hippocampal volume of risk alleles in four genes associated with Alzheimer's disease: *APOE* ($P = 0.005$), *BIN1* ($P = 0.02$), *MS4A4E* ($P = 0.001$) and *TOMM40* ($P = 0.01$). However, in aggregate, various SNPs known to be associated with Alzheimer's disease explained less than 1% of the observed variance in hippocampal volume.

We also examined our five lead SNPs for association with cognitive decline in 1,593

Table 2 Replication results in the ENIGMA sample

Locus	SNP	Gene	Allele 1/2	Allele 1 frequency	β	s.e.m.	*P*	*N*
2q24	rs6741949	*DPP4*[b]	G/C	0.58	−28.2	14.0	0.04	7,794
9q33	rs7852872	*ASTN2*[b]	C/G					
	rs7040792[a]	($r^2 = 1.00$)	T/C	0.64	−29.0	12.8	0.02	7,794
12q14	rs17178006	*MSRB3*[b]	G/T					
	rs1370938[a]	($r^2 = 0.30$)	A/C	0.24	−32.4	14.4	0.02	7,794
	rs6581612	*WIF1*	C/A					
	rs1498792[a]	($r^2 = 0.96$)	T/C	0.25	−25.4	14.6	0.08	7,794
12q24	rs7294919	*HRK*	T/C	0.90	−112.2	21.4	1.6×10^{-7}	7,794

Allele 1/2, coded (risk)/non-coded allele; β, association estimate in mm^3; *N*, effective sample size.
[a]Proxy SNPs: r^2 is the correlation between the proxy SNP and the lead SNP in the HapMap Phase 2 CEU sample. [b]The SNP is located within the indicated gene.

participants (mean age of 78.6 years) in the Religious Orders Study and the Rush Memory and Aging Project[47] (**Supplementary Table 6**) and found that rs7852872 (*ASTN2*) was associated with accelerated rates of global cognitive decline (P = 0.009) and memory loss (P = 0.01) (**Supplementary Fig. 2** and **Supplementary Table 7**). The magnitude of effect was comparable to that noted previously for a SNP in *CR1* (rs6656401) in the same sample[28], providing evidence for the potential importance of this region.

The strengths of the current study include the large, population-based sample. In the discovery sample, our power to detect association at genome-wide significance on the order of 0.2 s.d. in hippocampal volume (~128 mm^3 in the largest single sample, the Aging Gene-Environment Susceptibility–Reykjavik Study (AGES)) was modest for rare variants (68% for those with minor allele frequency (MAF) of 0.05) and strong for more common variants (>99% for those with MAF of 0.10). Additional power estimates are shown (**Supplementary Fig. 3**). The concordance of these associations in ENIGMA data provides additional biological validation in a population that included younger persons, suggesting that these genes are developmentally important and may be related to maximal adult hippocampal volume. The ENIGMA sample also has a substantial proportion of persons with dementia, which indicates that these genes may remain important in regulating the response to injury. Analysis exploring the association of our lead SNPs with cognitive decline provided further context for our findings. Finally, we showed modest association between previously described SNPS for Alzheimer's disease risk and smaller hippocampal volume in our samples.

The current study also has limitations. A single cross-sectional assessment was used in all studies, and magnetic resonance imaging (MRI) and reading protocols varied across participating studies—some studies used manually traced boundaries (the gold standard), whereas others used computerized algorithms. Although correlation between these two methods was good (Pearson's r = 0.7)[48], the heterogeneity of measurement techniques may have compromised our ability to detect small associations. Although our sample size was reasonably large, we may have missed associations with small effect sizes, as well as rare variants not covered by commercial genotyping arrays.

Previous studies have suggested that cognitive, neuropathological and MRI endophenotypes of Alzheimer's disease might be early and more sensitive markers of genetic risk than clinical dementia. Hence, it could be argued that genes associated with risk for Alzheimer's disease should also be associated with hippocampal volume, even in our dementia-free sample. Although four Alzheimer's disease risk genes were associated with hippocampal volume, several were not; therefore, in this study, hippocampal volume was not a more sensitive measure than clinical Alzheimer's disease. It is clear that genetic analysis of MRI endophenotypes within a community-based cohort study of healthy older individuals is not an ideal study design to identify all the genes associated with clinical Alzheimer's disease. Our aim, however, was to identify genetic influences on hippocampal development and response to aging, not Alzheimer's disease *per se*.

In summary, we detected four genetic loci associated with hippocampal volume in a large, population-based, dementia-free sample. Two of these loci replicated in independent community-based samples, as well as in a mixed-age sample from the ENIGMA Consortium that included some participants with cognitive impairment, indicating that these loci may have broad implications for determining the integrity of the hippocampus across a range of ages and cognitive capacities. Findings from this study identified a series of relevant and potentially important genes associated with hippocampal volume during development and aging and in the presence of Alzheimer's disease. Exploration of these genomic regions in dense-genotyping, expression and translational studies will be required to understand the role of these genes in determining hippocampal volume.

URLs. ENIGMA Consortium, http://enigma.loni.ucla.edu; eqtl.uchicago.edu, http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/; SNAP, http://www.broadinstitute.org/mpg/snap/; GeneCruiser, http://genecruiser.broadinstitute.org/genecruiser3/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Study concept and design were performed by J.C.B., C. DeCarli, M.A.v.B., C. Dufouil, P.A., A.G.U., M.M.B.B., F.F., M.A.B., C.M.v.D., T.H.M., C.T., L.J.L., M.A.I. and S. Seshadri. Acquisition of data was carried out by F.v.d.L., F.C., H.S., V.S., M. Schuur, S. Sigurdsson, B.F.J.V., J.-C.L., C.R.J., J.S., D.F., T.d.H., B.M., L.H.C., C.E., P.D., K.A., M.A.v.B., A.B., C. Dufouil, J.H., W.J.N., G.K.G., P.A., K.B.F., T.G.P., B.A.O., A.G.U., R.A., A.E., R.J.B., J.C.v.S., M.M.B.B., M.W.V., P.A.W., A.v.d.L., V.G., M.A.B., D.A.B., C.M.v.D., T.H.M., R.S., C.T., L.J.L., M.A.I. and S. Seshadri. Statistical analysis and interpretation of the data were performed by J.C.B., C. DeCarli, A.V.S., F.v.d.L., M.F., S.D., J.M.S., H.S., V.S., M. Schuur, L.Y., S.-H.C., B.F.J.V., A.L.D., M. Struchalin, J.S., C.A.I.-V., N.A., R.F.A.G.d.B., M.C., R.T., L.B.C., G.K.G., P.A., A.E., R.J.B., P.L.D.J., M.A.B., D.A.B., R.S., L.J.L. and M.A.I. The manuscript was drafted by J.C.B., C. DeCarli, J.M.S., H.S., M. Schuur, B.M., P.L.D.J., L.J.L. and S. Seshadri, and critical revision of the manuscript was performed by J.C.B., C. DeCarli, F.v.d.L., M.F., S.D., J.M.S., H.S., V.S., S.-H.C., S. Sigurdsson, A.L.D., J.-C.L., J.S., C.A.I.-V., A.Z., T.d.H., L.H.C., C.E., P.D., C. Dufouil, M.C., R.T., W.J.N., L.B.C., A.H., A.P., K.B.F., T.G.P., J.L.S., S.E.M., A.A.V., D.P.H., M.J.W., B.F., N.G.M., P.M.T., M.A.N., A.G.U., A.E., R.J.B., O.L.L., T.B.H., V.C., M.M.B.B., J.T.B., M.W.V., D.K., F.F., P.A.W., A.v.d.L., V.G., W.T.L., M.A.B., C.M.v.D., T.H.M., R.S., C.T., L.J.L., M.A.I. and S. Seshadri. Funding was obtained by M.F., H.S., V.S., C. Dufouil, W.J.N., A.H., B.A.O., A.G.U., J.C.v.S., T.B.H., V.C., M.M.B.B., F.F., P.A.W., A.v.d.L., V.G., D.A.B., C.M.v.D., T.H.M., R.S., C.T., L.J.L. and S. Seshadri.

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The authors declare no competing financial interests.

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Joshua C Bis[1,73], Charles DeCarli[2,3,73], Albert Vernon Smith[4,5,73], Fedde van der Lijn[6,7,73], Fabrice Crivello[8–10,73], Myriam Fornage[11,12,73], Stephanie Debette[13–15,73], Joshua M Shulman[16,17], Helena Schmidt[18], Velandai Srikanth[19,20], Maaike Schuur[21,22], Lei Yu[23], Seung-Hoan Choi[24], Sigurdur Sigurdsson[4], Benjamin F J Verhaaren[7,25], Anita L DeStefano[15,24,26], Jean-Charles Lambert[27–29], Clifford R Jack Jr[30], Maksim Struchalin[21], Jim Stankovich[20], Carla A Ibrahim-Verbaas[21,22], Debra Fleischman[23,31,32], Alex Zijdenbos[33], Tom den Heijer[22,25,34], Bernard Mazoyer[8–10], Laura H Coker[35], Christian Enzinger[36], Patrick Danoy[37], Najaf Amin[21], Konstantinos Arfanakis[23,38], Mark A van Buchem[39], Renée F A G de Bruijn[22,25], Alexa Beiser[15,24,26], Carole Dufouil[13], Juebin Huang[40], Margherita Cavalieri[36], Russell Thomson[20], Wiro J Niessen[6,7,41], Lori B Chibnik[16,17], Gauti K Gislason[4], Albert Hofman[25,42], Aleksandra Pikula[15], Philippe Amouyel[27–29,43], Kevin B Freeman[44], Thanh G Phan[19], Ben A Oostra[21,45], Jason L Stein[46], Sarah E Medland[47–49], Alejandro Arias Vasquez[50,51], Derrek P Hibar[46], Margaret J Wright[47], Barbara Franke[50,51], Nicholas G Martin[47], Paul M Thompson[46], Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) Consortium[52], Michael A Nalls[53], Andre G Uitterlinden[42,54], Rhoda Au[15,26], Alexis Elbaz[13,55], Richard J Beare[19,56], John C van Swieten[22], Oscar L Lopez[57–59], Tamara B Harris[60], Vincent Chouraki[27–29], Monique M B Breteler[61–63], Philip L De Jager[16,17,64], James T Becker[57–59], Meike W Vernooij[7,25], David Knopman[65], Franz Fazekas[36], Philip A Wolf[15,26], Aad van der Lugt[7], Vilmundur Gudnason[4,5], W T Longstreth Jr[66,67], Matthew A Brown[68], David A Bennett[23], Cornelia M van Duijn[21,42,69,74], Thomas H Mosley[40,70,74], Reinhold Schmidt[36,74], Christophe Tzourio[71,72,74], Lenore J Launer[51,74], M Arfan Ikram[7,25,42,74] & Sudha Seshadri[15,26,74] for the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium[52]

[1]Cardiovascular Health Research Unit, Department of Medicine, University of Washington, Seattle, Washington, USA. [2]Department of Neurology, University of California, Davis, Sacramento, California, USA. [3]Center of Neuroscience, University of California, Davis, Sacramento, California, USA. [4]Icelandic Heart Association, Kopavogur, Iceland. [5]Faculty of Medicine, University of Iceland, Reykjavik, Iceland. [6]Department of Medical Informatics, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [7]Department of Radiology, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [8]Neurofunctional Imaging Group, University of Bordeaux, Unité Mixte de Recherche (UMR) 5296, Bordeaux, France. [9]Neurofunctional Imaging Group, Centre National de la Recherche Scientifique (CNRS), UMR 5296, Bordeaux, France. [10]Neurofunctional Imaging Group, Commissariat à l'Energie Atomique (CEA), UMR 5296, Bordeaux, France. [11]Brown Foundation Institute of Molecular Medicine, The University of Texas Health Sciences Center at Houston, Houston, Texas, USA. [12]Human Genetics Center, School of Public Health, The University of Texas Health Sciences Center at Houston, Houston, Texas, USA. [13]Institut National de la Santé et de la Recherche Médicale (INSERM), U708, Neuroepidemiology, Paris, France. [14]Department of Epidemiology, University of Versailles Saint-Quentin-en-Yvelines, Paris, France. [15]Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA. [16]Program in Translational NeuroPsychiatric Genomics, Institute for the Neurosciences, Department of Neurology, Brigham and Women's Hospital, Boston, Massachusetts, USA. [17]Program in Medical and Population Genetics, Broad Institute, Cambridge, Massachusetts, USA. [18]Institute of Molecular Biology and Biochemistry, Medical University Graz, Graz, Austria. [19]Stroke and Ageing Research Centre, Southern Clinical School, Department of Medicine, Monash University, Melbourne, Victoria, Australia. [20]Menzies Research Institute Tasmania, University of Tasmania, Hobart, Tasmania, Australia. [21]Genetic Epidemiology Unit, Department of Epidemiology, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [22]Department of Neurology, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [23]Rush Alzheimer's Disease Center, Rush University Medical Center, Chicago, Illinois, USA. [24]Department of Biostatistics, Boston University School of Public Health, Boston, Massachusetts, USA. [25]Department of Epidemiology, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [26]The National, Heart, Lung, and Blood Institute's Framingham Heart Study, Framingham, Massachusetts, USA. [27]INSERM, U744, Lille, France. [28]Institut Pasteur de Lille, Lille, France. [29]Université Lille Nord de France, Lille, France. [30]Department of Radiology, Mayo Clinic, Rochester, Minnesota, USA. [31]Department of Neurological Sciences, Rush University Medical Center, Chicago, Illinois, USA. [32]Department of Behavioral Sciences, Rush University Medical Center, Chicago, Illinois, USA. [33]Biospective, Inc., Montreal, Quebec, Canada. [34]Department of Neurology, Sint Franciscus Gasthuis, Rotterdam, The Netherlands. [35]Division of Public Health Sciences, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA. [36]Department of Neurology, Medical University Graz, Graz, Austria. [37]University of Queensland, Diamantina Institute, Princess Alexandra Hospital, Brisbane, Queensland, Australia. [38]Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, Illinois, USA. [39]Department of Radiology, Leiden University Medical Center, Leiden, The Netherlands. [40]Department of Neurology, University of Mississippi Medical Center, Jackson, Mississippi, USA. [41]Faculty of Applied Sciences, Delft University of Technology, Delft, The Netherlands. [42]Netherlands Consortium for Healthy Ageing, Leiden, The Netherlands. [43]Centre Hospitalier Régional Universitaire de Lille, Lille, France. [44]Department of Psychiatry and Human Behavior, University of Mississippi Medical Center, Jackson, Mississippi, USA. [45]Department of Clinical Genetics, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [46]Laboratory of Neuro Imaging, David Geffen School of Medicine, University of California, Los Angeles, California, USA. [47]Genetic Epidemiology Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia. [48]Quantitative Genetics, Queensland Institute of Medical Research, Brisbane, Queensland, Australia. [49]Broad Institute of

Harvard and MIT, Cambridge, Massachusetts, USA. [50]Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. [51]Department of Psychiatry, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. [52]A full list of members appears in the **Supplementary Note**. [53]Laboratory of Neurogenetics, Intramural Research Program, National Institute on Aging, National Institutes of Health (NIH), Bethesda, Maryland, USA. [54]Department of Internal Medicine, Erasmus Medical Center University Medical Center, Rotterdam, The Netherlands. [55]Pierre and Marie Curie University (UPMC), University of Paris 6, UMR S708, Neuroepidemiology, Paris, France. [56]Developmental Imaging Group, Murdoch Childrens Research Institute, The Royal Children's Hospital, Parkville, Victoria, Australia. [57]Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [58]Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [59]Department of Psychology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [60]Laboratory of Epidemiology, Demography, and Biometry, NIH, Bethesda, Maryland, USA. [61]Population Health Sciences, German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany. [62]Population Health Sciences, University of Bonn, Bonn, Germany. [63]Department of Epidemiology, Harvard School of Public Health, Harvard University, Boston, Massachusetts, USA. [64]Harvard Medical School, Boston, Massachusetts, USA. [65]Department of Neurology, Mayo Clinic, Rochester, Minnesota, USA. [66]Department of Neurology, University of Washington, Seattle, Washington, USA. [67]Department of Epidemiology, University of Washington, Seattle, Washington, USA. [68]Human Genetics Group, University of Queensland Diamantina Institute, Princess Alexandra Hospital, Woolloongabba, Brisbane, Australia. [69]Center for Medical Systems Biology, Netherlands Genomics Initiative, Leiden University Medical Center, Leiden, The Netherlands. [70]Department of Medicine (Geriatrics), University of Mississippi Medical Center, Jackson, Mississippi, USA. [71]University of Bordeaux, U708, Bordeaux, France. [72]INSERM, Neuroepidemiology U708, Bordeaux, France. [73]These authors contributed equally to this work. [74]These authors jointly directed this work. Correspondence should be addressed to S. Seshadri (suseshad@bu.edu).

ONLINE METHODS

Participating studies. Our analyses were performed among dementia-free participants within the setting of the CHARGE Consortium[6]. The ten discovery samples included the Aging Gene-Environment Susceptibility—Reykjavik Study (AGES), the Atherosclerosis Risk in Communities Study (ARIC), the Austrian Stroke Prevention Study (ASPS), the Erasmus Rucphen Family Study (ERF), the Framingham Heart Study (FHS), the Religious Order Study & Rush Memory and Aging Project (RUSH), three independent phases of the Rotterdam Study (RS I, RS II and RS III) and the Tasmanian Study of Cognition and Gait (TASCOG). The two second-stage replication samples included the Three City Study (3C) and another independent sample of the third expansion of the Rotterdam Study (RS R). Details of the discovery samples and second-stage studies can be found in the **Supplementary Note**. Each study has an Institutional Review Board that approved the consent procedures, examination components, data security processes, genotyping protocols and current study design. All participants gave written informed consent for study participation and for use of DNA for genetic research.

Hippocampal volume phenotypes. Each study evaluated the total hippocampal volume using 1T, 1.5T or 3T MRI and either operator-defined, manually traced boundaries drawn on serial coronal sections or automated methods, according to previously described image reading protocols. For these analyses, we used data from the baseline examination or the first examination in which an MRI measurement was obtained. Specific details for each study's MRI protocol are provided in the **Supplementary Note**.

Genotyping and imputation. The studies in these analyses used commercial genotyping platforms available from Illumina or Affymetrix. Each study performed genotyping quality control checks and imputed the approximately 2.5 million polymorphic autosomal SNPs described in the HapMap CEU population for each participant using available imputation methods. Details of per-study genotyping, imputation and quality control procedures are available in the **Supplementary Note**.

Statistical analysis within studies. Each study independently implemented a predefined GWAS analysis plan. For the continuous measure of hippocampal volume, we evaluated cross-sectional associations of hippocampal volume and genetic variation using linear regression models (or linear mixed-effects models, in the Framingham Heart Study and the Erasmus Rucphen Family Study to account for family relatedness). For each of the 2.5 million SNPs, each study fit additive genetic models, regressing trait on genotype dosage (zero to two copies of the variant allele). In our primary analyses, all studies were adjusted for age and sex. Some studies made additional adjustments, including for study site or familial structure or for whether the DNA had been whole-genome amplified. Additional details of the statistical analyses are available in the **Supplementary Note**.

Discovery meta-analysis. We conducted a meta-analysis of regression estimates and standard errors using an inverse-variance weighting approach as implemented in METAL[49]. After verification of strand-alignment across studies, quality control, filtering and imputation within each study, we restricted our meta-analysis to autosomal SNPs that were reported in at least two studies and that had an average MAF of at least 1%. Before meta-analysis, we calculated a genomic inflation factor (λ_{GC}) for each study to screen for cryptic population substructure or undiagnosed irregularities that might have inflated the test statistics. Inflation was low, with λ_{GC} below 1.05 in all studies. We applied genomic control to each study whose genomic inflation factor was greater than 1.00 by multiplying all of the standard errors by the square root of the study-specific λ_{GC}. We expressed the association of each SNP with hippocampal volume as the regression slope (β), its standard error (SE(β)) and a corresponding P value. Standardized gene and SNP annotations were created using a PERL program[50].

For follow up, we decided a priori on a significance threshold of $P < 4 \times 10^{-7}$, which corresponds to no more than one expected false positive finding over 2.5 million tests.

Replication meta-analysis. Replication samples were drawn from external studies with available genetic data and measures of hippocampal volume. We provided each collaborating second-stage study with a list of signal SNPs that attained a P value of $P < 4 \times 10^{-7}$ and combined the results from these studies using a fixed-effects meta-analysis.

Combined meta-analysis. We combined results from the discovery and second-stage analyses using inverse-variance weighting and considered SNPs with a P value of $<5 \times 10^{-8}$ to have reached genome-wide significance.

External validation. We sought external replication for our significant and suggestive loci in the ENIGMA Consortium, the details of which can be found in the accompanying paper by Stein *et al.*[51]. The international ENIGMA Consortium comprises a wide variety of studies that all have GWAS and hippocampal volume measures. The sample includes case-control studies of Alzheimer's disease and depression and family-based and sibling pair samples, as well as population-based samples of varying ages and ancestries (European, African and Hispanic). ENIGMA assesses brain volume using Freesurfer/FSL-FIRST (the Oxford Centre for Functional MRI Brain (FMRIB) Software Limited–FMRIB's Integrated Registration and Segmentation Tool) protocols in most samples but also uses other protocols in a few samples. Hence, we chose to compare the results from ENIGMA and CHARGE in a qualitative manner, as these two studies varied in the composition of the study participants as well as in the methods used to assess hippocampal volume. We considered replication to be represented by a P value of <0.05 with a consistent direction of association.

Exploration of loci for eQTLs and functional variants. We examined the four loci identified as associated with hippocampal volume for the presence of *cis* eQTL associations using uchicago.edu. We also searched for functional SNPs in LD with the five index SNPs. We identified over 70 SNPs with $r^2 > 0.4$ that were within 500 kb of each index SNP using the SNAP proxy tool and annotated these SNPs using GeneCruiser; none of these SNPs were exonic, nonsynonymous coding SNPs.

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