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Somatic Signature of Brain-Specific Single Nucleotide Variations in Sporadic Alzheimer's Disease

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Abstract.
Background: Although genome-wide association studies have shown that genetic factors increase the risk of suffering late-onset, sporadic Alzheimer's disease (SAD), the molecular mechanisms responsible remain largely unknown.
Objective: The aim of the study was to investigate the presence of somatic, brain-specific single nucleotide variations (SNV) in the hippocampus of SAD samples.
Methods: By using bioinformatic tools, we compared whole exome sequences in paired blood and hippocampal genomic DNAs from 17 SAD patients and from 2 controls and 2 vascular dementia patients.
Results: We found a remarkable number of SNVs in SAD brains (~575 per patient) that were not detected in blood. Loci with hippocampus-specific (hs)-SNVs were common to several patients, with 38 genes being present in 6 or more patients out of the 17. While some of these SNVs were in genes previously related to SAD (e.g., CSMD1, LRP2), most hs-SNVs occurred in loci previously unrelated to SAD. The most frequent genes with hs-SNVs were associated with neurotransmission, DNA metabolism, neuronal transport, and muscular function. Interestingly, 19 recurrent hs-SNVs were common to 3 SAD patients. Repetitive loci or hs-SNVs were underrepresented in the hippocampus of control or vascular dementia donors, or in the cerebellum of SAD patients.
Conclusion: Our data suggest that adult blood and brain have different DNA genomic variations, and that somatic genetic mosaicism and brain-specific genome reshaping may contribute to SAD pathogenesis and cognitive differences between individuals.

Keywords: Alzheimer's disease, exome sequencing, somatic variations

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INTRODUCTION

Alzheimer's disease (AD) is classified into two types: familial AD (FAD), predominantly associated with early onset, and sporadic AD (SAD) associated with late onset. Genomic analyses have shown that FAD is caused by mutations in amyloid-β protein precursor (AβPP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes, and by duplications in the AβPP gene [1–4]. In contrast, the mechanisms that trigger SAD are largely unknown, although various risk factors have been established, including vascular disease (VaD), elevated cholesterol levels, and obesity/diabetes [3, 5, 6]. Neuropathological hallmarks for SAD include amyloid-β (Aβ) pathology and aggregation, and tau phosphorylation [2, 7]. Moreover, the *APOE*4 allele is strongly associated with SAD [8, 9]. Large-scale genome-wide association studies (GWAS) have identified several additional loci, including CLU, PICALM, CR1, BIN1, ABCA7, EPHA1, TREM2, CD33, PTK2B, and INPP5D as genetic risk factors for SAD [10–19].

Some diseases, particularly cancer, are associated with somatic genomic events, which occur in only one generation or one group of cells and which may affect specific genes. Approximately 80% of cancers arise from somatic mutations [20, 21]. It is generally held that the initial step in many tumors is mediated by DNA damage and mutations, which trigger activation of proto-oncogenes, the inactivation of tumor-suppressor genes, and ultimately tumor progression and metastasis. The identification of genes that are mutated in cancer has been critical not only for our understanding of the pathogenesis, but also for the design of novel therapeutic tools [22, 23].

To our knowledge, systematic studies for the search of somatic brain mutations in SAD or other neurological diseases have not been performed so far. Here we use whole-exome sequencing to investigate the presence of single-nucleotide polymorphisms (SNPs), single nucleotide variations (SNVs), and mutations (all referred to here as SNVs) that are specific to brain genomic DNA in SAD, and which are not present in the blood genome of the same donors. We found a striking number of brain-specific SNVs in SAD patients. Together with recent studies reporting brain-specific retrotrasposon insertions [24, 25] and somatic copy number variations (CNVs) in control human neurons [26], our results highlight variability in the brain genome, and suggest that genetic mosaicism and brain-specific genetic variations may contribute to the pathogenesis of SAD.

MATERIALS AND METHODS

Brain tissue processing and characterization

Shortly after the death of the donor, the whole brain was obtained through a neuropathological autopsy and divided into two symmetrical halves through a midsagittal section. Thereafter, the right half was immediately cut in coronal (hemisphere), sagittal (cerebellum), or transversal (brainstem) slices. Each tissue slice was individually frozen by immersion in −50°C isopentane (Shandon Histobath^{TM} 2) and transferred to a −80°C freezer for long-term storage. The left brain half was fixed in 4% buffered formaldehyde and sectioned. Multiple tissue blocks were obtained from cortical and subcortical regions and processed for paraffin sectioning and hematoxylin-eosin staining. Additionally, a battery of immunostaining detections was performed on selected sections, and a neuropathological diagnosis and classification of cases was obtained according to well-established consensus criteria. Classification and staging of Alzheimer-type pathology was based on density and score of neuritic plaques according to CERAD criteria, and Braak's staging of neurofibrillary pathology. Frozen tissue samples of hippocampus and cerebellum were taken from previously frozen tissue slices. The hippocampus was dissected with the aid of a stereomicroscope so as to obtain samples limited to CA1-CA3 sectors of the hippocampal cortex. Samples of the cerebellum corresponded to lateral hemispheric foliae. Blood samples were obtained simultaneously with routine blood extractions for laboratory evaluation of patients.

Genomic DNA was extracted from brain tissue samples and from the blood of donors who had been clinically and neuropathologically confirmed as SAD. Brain tissue and blood samples were obtained from two Spanish brain banks (Banco de Tejidos CIEN [BT-CIEN] and Banco de Cerebros de la Región de Murcia [BCRM]) working with similar processing protocols, approved by their respective ethical committees. Samples from two control donors (with no neurological and neuropathological signs) and from two donors clinically and neuropathologically diagnosed as VaD, were also included in the study. DNA was extracted using Qiagen kits.

Sample processing for exome sequencing

Three μg of genomic DNA (from blood, hippocampus, and cerebellum) was fragmented to an average size of 200 bp using a Covaris LE220 instrument. Short

insert libraries were obtained with Illumina's TruSeq DNA Sample Preparation Kit. Exonic sequences were enriched using NimbleGen's Sequence Capture Human Exome 2.1M Array. Paired-end sequences of 91 nucleotides from each end were generated using an Illumina's HiSeq 2000 instrument to an average of 50x coverage. Sequences were generated in fastaq format.

Sequencing quality control

All samples passed quality filters after sequencing according to the following criteria: 1) Reads with ambiguous bases (represented by letter N) more than 5% of bases or poly-A structure constituents; 2) Reads that have 20 bases with quality score less than or equal to 7 for the library were filtered. 3) Reads with adapter contamination: reads with more than 10 bp aligned to the adapter sequence (no more than 3 bp mismatch allowed); 4) Small insert-size reads in which read1 and read2 overlapped by 10 bp or more (10% mismatch allowed); 5) PCR duplications (reads are considered duplications when read1 and read2 of the same paired end reads are identical). Individual reads not passing any one of these filters were discarded from the analysis. Furthermore, overall quality was controlled with the fastqc software (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) ensuring that all samples passed checks with default parameters.

Mapping and initial base calling

Samples were aligned to the human reference genome version hg19 [27] using the BWA aligner software [28] with default parameters. For each patient, brain and blood samples were preprocessed by removing duplicate reads [29] using Picard (http://picard.sourceforge.net/index.shtml). Samples from the same patients were merged using SAMtools and preprocessed before base calling. Local realignment was performed around INDELs to improve SNP calling in these conflictive areas (IndelRealigner from [30]). After merging patient samples to avoid recalibration biases to affect samples independently, base quality scores were recalibrated using the baseRecalibrator tool from the Genome Analyzer Toolkit [30]. Once recalibrated, samples were split by tissue with BAMtools [31]. The UnifiedGenotyper algorithm from [30] was then used with default parameters (see [32] and [33] for details) to call SNPs in the merged file. For each of the variants called which had a read depth $\geq$25 reads in both tissues, we performed a Fisher test on the observed number of reads with the alternative allele in each sample. Candidate SNVs with a p-value larger than 0.05 were discarded. Finally, variants were annotated using the dbSNP database version 132 [34], the UCSC human RefGene [35], and the software snpEFF [36] (see Supplementary Methods).

Tissue-specific SNV discovery

The routine UnifiedGenotyper from GATK was used to call candidate SNVs in each tissue sample. These SNVs were filtered with parameters recommended in Best Practices of the GATK website (http://www.broadinstitute.org/gatk/), with the exception of the quality of the SNV call (QUAL) which was lowered to 20 to allow for more candidates when comparing tissues. SNPs were annotated using snpEff2.0.5 [37] and GATK's SNPannotator. In order to find SNVs specific to the hippocampus, we compared the number of reads for the reference and alternative alleles in each candidate position for both samples. A Fisher test was performed comparing the number of reads of the reference allele and the most frequent alternate allele in each sample. We declared as blood/brain specific SNVs those SNVs whose Fisher's test p-value was lower than 0.05 and whose read depth was $\geq$25 for both samples. Tissue-specific SNVs were considered as hippocampus-specific SNVs (hs-SNVs), blood-specific (bs-SNVs), or cerebellum-specific (cs-SNVs) considering the tissue which contains the alternative allele or its higher percentage.

Further statistics calculations

Further statistics were calculated as follows: dbSNP coverage was calculated against dbSNP version 132 [34]. Exonic SNPs were found using annotations from UCSC version hg19 [35].

RESULTS

Exome sequencing and genotyping of blood and hippocampi reveal brain-specific SNVs

To test the hypothesis that somatic variations and mutations may contribute to the genetic component of SAD, we sequenced the exomes of 17 clinically and neuropathologically confirmed SAD patients, comparing genomic DNA from blood and hippocampi from the same donors (Supplementary Table 1). Briefly, we enriched exonic sequences from genomic DNA using the NimbleGen's Sequence Capture Human Exome 2.1 M Array and performed Illumina's HiSeq

2000 paired-end sequencing (Fig. 1a). On average, we obtained 6.2 Gb of mappable sequence data per individual after exome enrichment, targeting 37 megabases (Mb) from exons and their flanking regions. Overall, we covered 1.22% of the genome, a fraction corresponding closely to the NCBI Consensus Coding Sequences database (CCDS). Quality controls using the fastaQC program were applied to reads from each sample and showed high per base and per sequence quality, even G/C content along reads and no overrepresented or high Kmer content.

We aligned reads from each sample independently to the human reference genome [27] using the Burrows-Wheeler Aligner [28]. An average of 99.1% of the reads aligned correctly with the genome. The mean read depth in targeted regions was 60.8 reads. In addition, 79.4% of the captured exonic regions were covered with at least 25 reads.

In order to identify genomic variations we applied various processing and genotyping tools. Briefly, we removed duplicated reads from individual samples [29]. In the first step, we used the GATK software for calling SNVs in blood and brain samples [30, 32]. To this end we merged samples from both tissues for each patient and realigned reads around problematic areas with indels (indel realigner). We then recalibrated read qualities (quality recalibration) and split the data into blood and hippocampus reads. We applied the UnifiedGenotyper (from the GATK package) to find SNVs using the default parameters. We filtered SNVs as described [33] with the exception of higher calling quality (QUAL <25). We also filtered out the SNVs found in duplicated regions in the human genome annotated in the UCSC database [38, 39] with a segmental duplication score larger than 0.8.

Once the positions of the SNVs were known, we calculated the distance between samples based on the observed proportion of the four bases in all SNVs from all samples. As expected, paired samples cluster together. A heatmap is presented in Fig. 1b showing that the distance between paired samples is much lower than the distance to any other sample.

In order to identify hs-SNVs, we compared the SNVs from hippocampus with the corresponding genomic positions in blood. We selected positions with confident calls whose proportions of reference and alternative alleles were significantly different. SNVs with p-values (Fisher test) lower than 0.05 were retained. Only SNVs with a read depth $\geq$25 were considered.

The great majority of SNVs found in the hippocampus, whether heterozygous or homozygous, had similar percentages of non-reference allele reads in the blood counterpart (Fig. 1c, right insert). Nevertheless, some positions showed very different proportions between blood and hippocampus (Fig. 1c, left), including those with a clear increase or decrease in non-reference alleles in hippocampus. We also noticed a significant number of hippocampal variations that were present with low numbers of reads with the alternative allele (usually 20–30%), which may suggest mosaicism.

For 3 SAD donors we sequenced samples from the cerebellum and repeated the procedures describe above for the hippocampal sequences in order to identify SNVs that were shared or unique to the cerebellum.

Characterization and specificity of hs-SNVs in SAD

We found an average number of 37.605 SNVs per single genomic DNA sample (Fig. 2a). Overall, a very large fraction of SNVs were annotated in dbSNP database (version 132) (94%), which bears witness to the quality of sequencing and analysis (Fig. 2a). An average of 15,484 SNVs (41%) were found in exonic regions, with a ts/tv ratio of $\sim$2.31 in these regions (Fig. 2a). Although most ($\sim$97%) of these SNVs were common to both blood and hippocampal DNA, in all patients we found a consistent number of hs-SNVs (average 577, Fig. 2b, c; Supplementary Table 2). Similarly, we also found SNVs that were specific for blood (Fig. 2b and Supplementary Table 2). A large number of hs-SNVs were annotated in dbSNP ($\sim$95%, Fig. 2c). About 43% of the brain-specific variations were present in exons, with the remaining variations being located in intronic, downstream or intergenic regions (Fig. 2d).

We annotated SNVs according to their potential functional impact as defined in the annotation package snpEFF [39] (Supplementary Tables 2 and 3). Overall, 17.3% of the hs-SNVs had high to moderate impact whereas 19% had low impact and 63.7% of hs-SNVs had modifier impact. We found a number of non-synonymous coding hs-SNVs (1661), translational start gains (25) and losses (2), stop gains (12), and losses (3) and splice site donors and acceptors (19) (Fig. 2e).

We next analyzed the chromosomal distribution of hs-SNVs. As illustrated in Fig. 3, hs-SNVs were present in all chromosomes with a regional distribution which largely paralleled the distribution of all the identified SNVs.

The loci with hs-SNVs in SAD donors are listed in Supplementary Table 4. To discern whether these loci

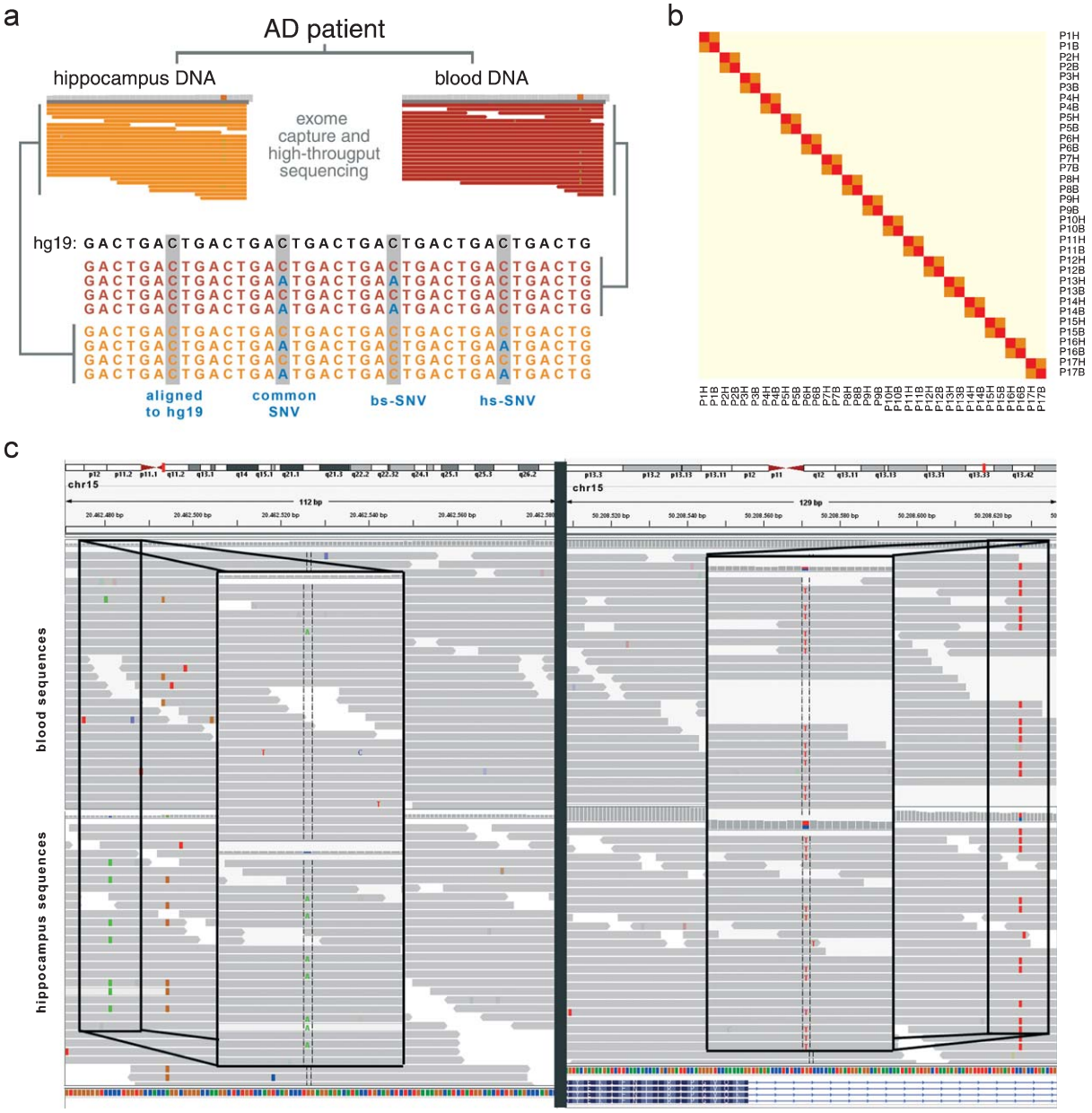


Fig. 1. a) Flow chart illustrating the experimental approach used. Blood and hippocampal genomic DNAs from the same donors were exome sequenced and blood-specific (bs) and hippocampus-specific (hs)-SNVs were identified. b) Distance heatmap demonstrating high sequence similarities between blood and brain DNAs from the same patients, and high distance between patients. c) IGV view of blood (upper) and hippocampal (below) DNA sequence in chromosome 15, illustrating one example of a variation common to blood and hippocampus (right) and a hs-SNV (left) in the ATP8B4 and RHPN2P1 loci, respectively.

were specific to SAD, we compared sequences from hippocampal and blood genomic DNAs obtained from 2 control individuals, with no neural disease, and 2 donors clinically and neuropathologically diagnosed with VaD (Supplementary Table 1). As in SAD, we detected both blood- and hs-SNVs, with numbers of hs-SNVs being similar to those in SAD (Fig. 4 a-c; Supplementary Tables 2 and 5). We next calculated the percentage of coincident loci with SAD. About 13% and 21% of loci with hs-SNVs in control and VaD samples overlapped with SAD loci, respectively. Conversely, 8% and 10% of loci with hs-SNVs in SAD samples overlapped with those in control and VaD brain samples (Fig. 4d, e).

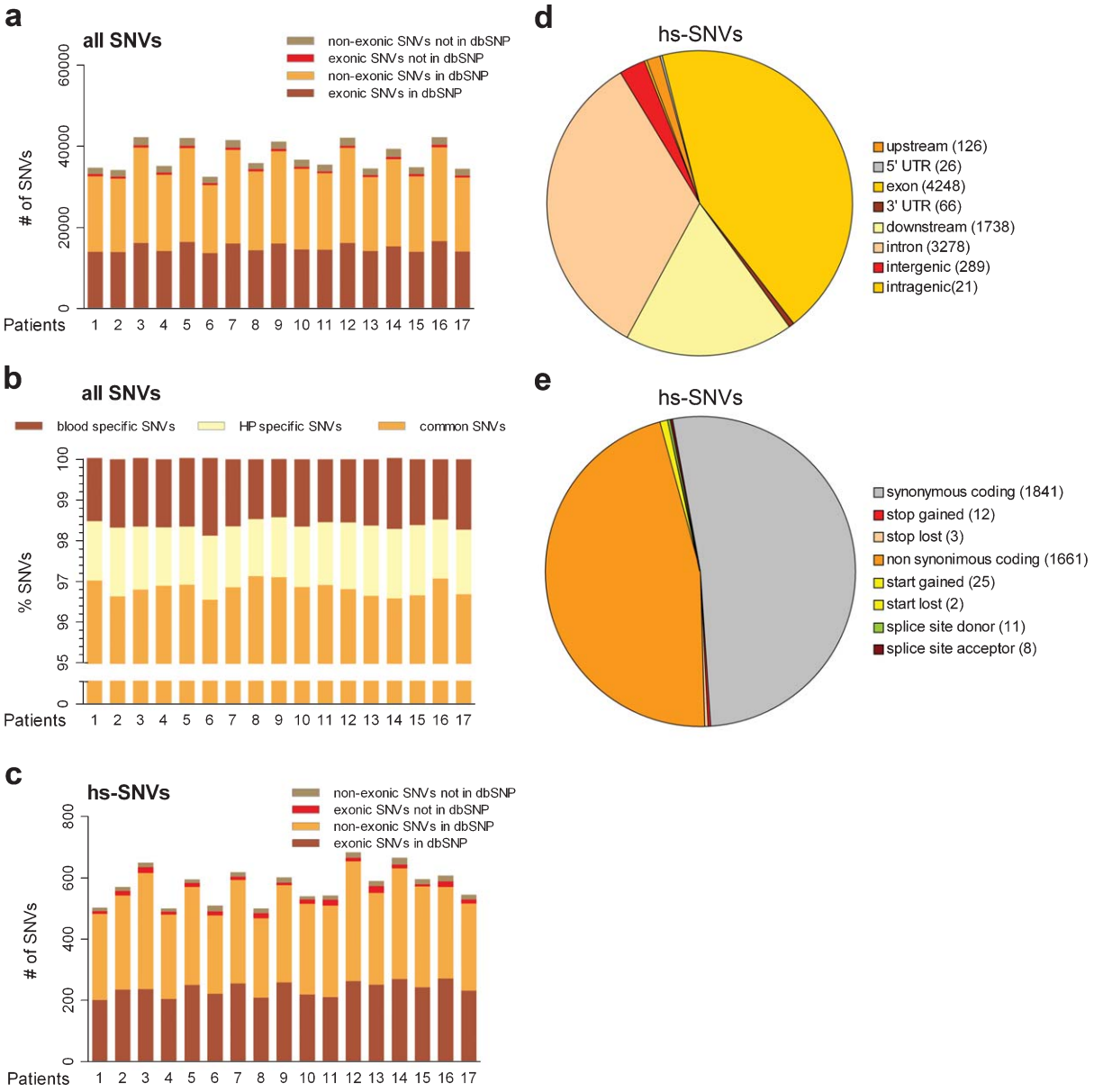


Fig. 2. Characteristics and distribution of SNVs and hs-SNVs in 17 SAD samples. a) Distribution of all (blood and hippocampus) SNVs per patient, according to their annotation in dbSNP and their location in exonic and non-exonic regions. b) Percentage of blood-specific (bs), hippocampus-specific (hs) and common SNVs per SAD patient. Note that about 97% of SNVs were common to blood and hippocampal samples, whereas about 1.5% were hippocampus-specific. c) Classification and number of hs-SNVs/patient, according to their annotation in dbSNP and their location in exonic and non-exonic regions. d) Overall distribution of hs-SNVs, according to their genetic location. e) Overall distribution of hs- SNV in 17 SAD patients according to their functional impact.

We also screened brain-specific SNVs in the cerebella of 3 SAD patients (Fig. 4f-i; Supplementary Table 6). We found that only about 9% of brain-specific loci were common to the hippocampus and cerebellum of the same donors (Fig. 4i, Supplementary Table 7). Taken together, the data suggest that the hippocampus of SAD brains may display a specific somatic genetic signature.

Common hippocampus-specific genes in SAD: Relationship to AD pathogenesis and neurological diseases

The list of genes with hs-SNVs ordered by the number of patients with at least one mutation in the corresponding gene is shown in Supplementary Table 4. Interestingly, 2 genes (SYNE1, AC073995.2)

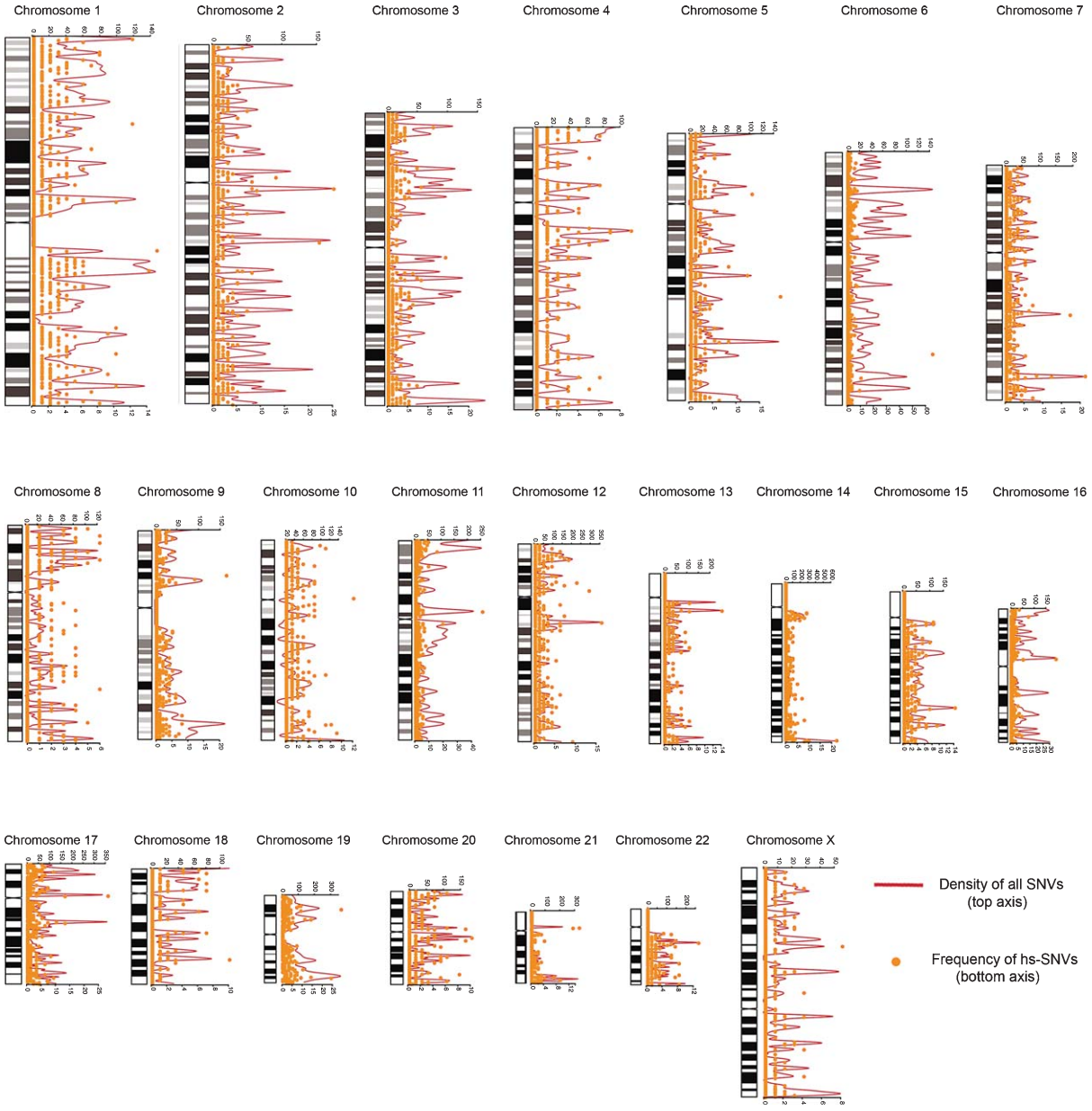


Fig. 3. Chromosomal distribution of hs-SNVs (dots, upper axis scale), compared to the entire population of SNVs (continuous line, bottom axis scale). hs-SNVs were present in all chromosomes with an uneven distribution among chromosomal regions.

displayed hs-SNVs in 10 out of the 17 patients, 3 loci (MAP2K3, 0R4C3, ZNF806) were common to 9 patients, 6 genes had hs-SNVs in 8 patients (e.g., PDE4DIP, CD109), 7 loci were common in 7 patients (AQP7, PTPN14, PRIM2, CSMD1), 20 loci were found in 6 patients, and 51 and 129 genes were common to 5 and 4 patients, respectively. Overall, 11 loci were common to ∼45% of patients (8 or more patients), and up to 218 genes were present in at least 24% of patients (4 or more patients).

We next focused on hs-SNVs within the FAD genes: AβPP, PSEN1, and PSEN2 [1, 13]. These loci displayed SNVs reported in dbSNP that were all common to blood and brain. We found one hs-SNVs in the PSEN1 gene in one patient (rs165932, Supplementary Tables 2 and 4), which was nevertheless non-pathogenic although it may be a risk factor [40]. We also screened for loci that bore hs-SNVs and that are related to the pathogenesis of AD or other dementia. A comparison with genes that have been associated

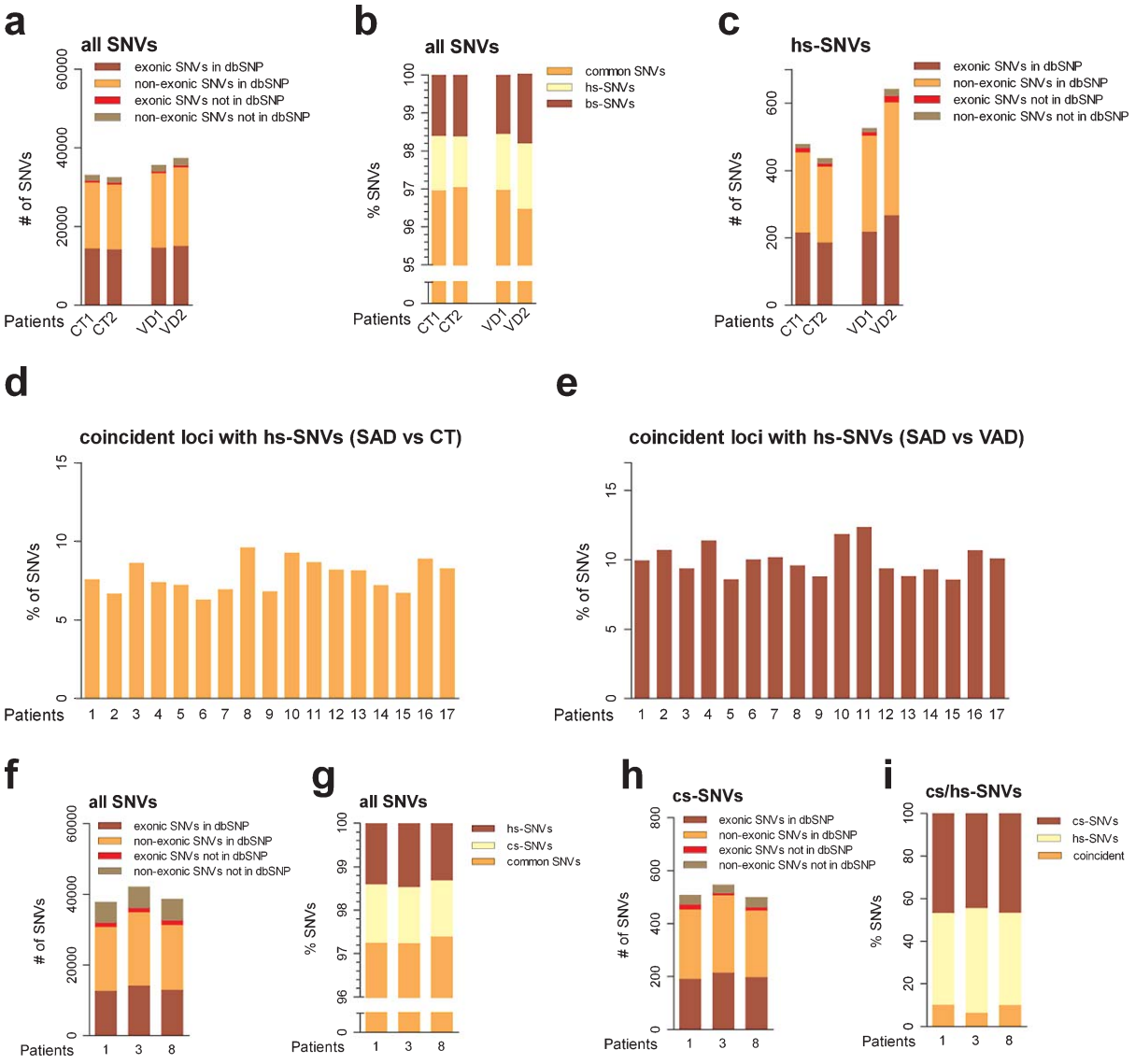


Fig. 4. Distribution and characteristics of hippocampus-specific (hs)-SNVs identified in control (CT) and vascular dementia (VaD) patients, and in the cerebella (CB) of 3 SAD patients. a) Distribution of SNVs/patient (CT and VaD), according to their annotation in dbSNP and their location in exonic and non-exonic regions. b) Percentage of blood-specific (bs), hs-, and common SNVs per sample. c) Number and classification of hs-SNVs/sample, according to their annotation in dbSNP and their location in exonic and non-exonic regions. d, e) Percentage of loci with hs-SNVs in SAD samples which overlapped with hs-SNVs found in control and VaD brain samples. Overlap averaged 7.8% and 10%, respectively. f) Distribution of SNVs found in the cerebella and blood of 3 SAD patients, according to their annotation in dbSNP and their location in exonic and non-exonic regions. g) Percentage of blood-specific (bs), cerebellum-specific (cs), and common SNVs in three SAD patients. h) Number and classification of cs-SNVs/patient, according to their annotation in dbSNP and their location in exonic and non-exonic regions. i) Percentage of overlapping loci with brain-specific SNVs, in the cerebellum and hippocampus of three SAD patients. Note that most loci were specific either for the cerebellum or for the hippocampus.

in GWAS analyses with SAD and related dementias identified BIN1 (1 patient), ABCA7 (2 patients), and PICALM (1 patient) as genes bearing hs-SNVs (Supplementary Table 2). However, none of these hs-SNVs was coincident with the polymorphisms in the GWAS studies, although rs61748157 (BIN1) has been associated with a recessive centronuclear myopathy [41]. Ten members of the ABC (ATP-binding cassette, ABCA) family, which transports lipids and cholesterol, bore hs-SNVs (Supplementary Tables 2 and 4).

We also screened for genes located in the chr21 critical region of Down syndrome which shares features

with AD [42–44]. Up to 28 loci in the critical region exhibited hs-SNVs, with all 17 patients displaying at least one hs-SNVs (Supplementary Table 9). The most frequent loci were BAGE2 (8 patients), TPTE (6 patients), MX2 (6 patients), TIAM1 and EVA1C (5 patients) and HSF2BP (4 patients). BAGE2 and TPTE are included in the Robertsonian translocation region [42] and TPTE belongs to the PTEN phosphatase family related to multiple signaling cascades, including p53/p73 [45, 46] and is associated with autism [47]. TIAM1 is a Rho guanine nucleotide exchange factor linked to TrkB signaling and dendritic spine physiology and plasticity, which is activated by Aβ [48, 49]. Finally, TTC3 (2 patients) encodes an E3 ligase that facilitates degradation of phosphorylated AKT [50], and NCAM2 (1 patient) has been implicated in prion disease, Down syndrome, SAD, and psychiatric disease susceptibility [51–55].

Other common loci exhibiting hs-SNVs and related to Aβ and SAD pathogenesis include CSMD1 (7 patients), LRP2 (Megalin), RYR2, PRUNE2, and SVIL (all of them in 6 patients), RYR1, LRP1B, ANKS1B, C3, and PION (all of them in 5 patients), and KALRN, NOS2, DCHS2 (4 patients) (Table 1 and Supplementary Table 4). PION (or GSAP) is a γ-secretase activating protein which increases Aβ production [56, 57] and ANKS1B (or AIDA-1) binds the AID fragment of AβPP and modulates AβPP/Aβ processing [58]. CHRNA7 (α7 subunit of neuronal nicotinic actetylcholine receptor) regulates γ–secretase and reduces AβPP processing, and is genetically associated with SAD and myoclonic epilepsy [59–61].

In addition, many recurrent loci bearing hs-SNVs have been implicated in neurodegeneration and in the pathogenesis of neurological and psychiatric diseases (Tables 1 and 2). For instance, the most recurrent loci (SYNE1) is a component of the nuclear lamina A complex that is mutated in neural diseases including cerebellar ataxia with spinal motorneuron disease [62, 63], autism [64], and Emery-Dreifuss muscular dystrophy [65, 66].

Functions of hippocampus-specific loci bearing SNVs in SAD

Our screening highlights genes involved in essential neuronal and biological functions (Tables 1 and 4 and Supplementary Table 4). Thus, in addition to genes linked to ion channel activity, neurotransmission, and synaptic-linked proteins (e.g., GRM7, PTK2B, RYR2, SYNE1, and TPTE), hs-SNV-containing loci regulate endoplasmic reticulum (ER)-to-Golgi trafficking and protein secretion, endocytosis and axonal/dendritic transport (e.g., DNAH3, 8, 11, and 14, PDE4DIP, SVIL, and several DOCK members), DNA-RNA metabolism/DNA damage response and genomic instability (e.g., PRIM2, HEATR1, MKI67, and CNOT), extracellular matrix/adhesion (FRAS1, COL27A1, DST, TNS1, and DCHS2) or muscular function (MYH13, NEB, DYSF, and OBSCN).

Further, hs-SNVs were identified in loci linked to functions which have been documented as risk factors for SAD: cardiac and vascular function, hypertension and blood circulation (e.g., CYP4F2, AKAP9, CASQ2, and BAZ2B), and cholesterol/lipid metabolism, and obesity/diabetes (e.g., MGAM, LRP2, FAT1, and LPA) (Table 4).

Recurrent hippocampus-specific SNVs in SAD

Many loci displayed several hs-SNVs (more than 2) in the same patient: for instance 7 hs-SNVs in the KIR3DL1 (patient 7), 6 hs-SNVs in the MAP2K3 gene (patients 5 and 12) and 6 hs-SNVs in the PTPN14 (patient 5) (Supplementary Table 4). In addition, several loci displayed recurrent hs-SNVs in the same positions (Table 3), suggesting that recurrent hs-SNVs target certain genes preferentially.

A search for recurrent mutations in SAD samples revealed that 19 hs-SNV positions were common to 3 patients (Fig. 5a, b, e.g., TMEM67, SGIP1, NLN, KNCTC1, PTPN1) and 517 additional hs-SNVs were shared by 2 SAD patients (Supplementary Table 8). Some of these repetitive hs-SNVs were in the most frequent SAD loci, including SYNE1, MAP2K3, OBSCN, PDE4DIP, and PTPN14.

We next analyzed the overlap between these recurrent SAD hs-SNVs and control samples (Supplementary Tables 2, 6, and 8). From the 19 most recurrent hs-SNVs in SAD (common to 3 patients; Fig. 5b and Supplementary Table 8), none were present in the hippocampus of control and VaD brains or in the three cerebellar samples (Fig. 5c; Supplementary Table 8). Such differential frequency of recurrent hs-SNVs, in SAD versus control tissue, also held true for the hs-SNVs found in 2 samples (Fig. 5c and Supplementary Table 8; 516 recurrent positions in 2 AD samples). Overall, each SAD sample displayed ∼64 recurrent hs-SNVs (1089 hs-SNVs/17 samples) which were underrepresented in control brains (∼3.75 hs-SNVs per Control; 15 hs-SNVs/4 samples). These data suggest that there is little overlap between recurrent hs-SNVs in SAD and control samples.

Table 1
Top list of genes showing hs-SNVs in various patients

Gene	Protein name	Function	Patients (SNVs)	Association to diseases
SYNE1	nesprin-1	maintenance of nuclear organization and structural integrity	10 (14)	autosomal recessive cerebellar ataxia [97]; Emery-Dreifuss muscular dystrophy [65]; bipolar disorder [98]; type 2 diabetes mellitus [99]
MAP2K3	dual specificity mitogen-activated protein kinase kinase 3	signal transduction; stress	9 (27)	Autism [100]; amyotrophic lateral sclerosis [101], vascular brain disease [102]
OR4C3	olfactory receptor 4C3	odorant receptor; sensory transduction	9 (23)	
ZNF806	zinc finger protein 806	transcription factor; transcription regulation	9 (11)	
AE000661.28	T cell receptor delta variable 2	signal transduction; antigen recognition	8 (13)	
PDE4DIP	myomegalin	centrosome-Golgi component; signal transduction	8 (12)	Microcephaly [103]; Usher syndrome [104]
BAGE2	B melanoma antigen 2	unknown	8 (10)	Robertsonian Down syndrome [42]
HEATR7B2	maestro heat-like repeat-containing protein family member 2B	unknown	8 (10)	multiple sclerosis [105]
OBSCN	obscurin	muscle cytoskeletal component; myofibrillogenesis	8 (9)	macular degeneration [106]; hypertrophic cardiomyopathy [107]
CD109	CD109 antigen	TGF-β signaling; differentiation of keratinocytes	8 (8)	Cancer [108–110]
AQP7	aquaporin-7	facilitates water, glycerol and urea transport; forms a channel for water and glycerol	7 (18)	glucose abnormalities [111]; glycerol metabolism and metabolic syndrome [112]; atherosclerosis [113]
PTPN14	tyrosine-protein phosphatase non-receptor type 14	protein tyrosine phosphatase activity; regulate cellular processes cell growth, differentiation, mitotic cycle, and oncogenic transformation	7 (15)	attention deficit hyperactivity disorder [114]; cancer [115]
KIR2DL3	killer cell immunoglobulin-like receptor 2DL3	antigen binding; regulation of immune response	7 (11)	multiple sclerosis [116]
PRIM2	DNA primase large subunit	DNA binding; DNA replication	7 (11)	Cancer [117]
HEATR1	HEAT repeat-containing protein 1	ribosome biosynthesis; nucleolar processing of pre-18S ribosomal RNA	7 (10)	
HMCN1	hemicentin-1	vision; sensory transduction	7 (8)	macular degeneration [118]; diabetic nephropathy [119]
CSMD1	CUB and sushi domain-containing protein 1	regulator of inflammation in developing CNS; neuronal growth cone function	7 (7)	AD [82]; schizophrenia [120, 121]; multiple sclerosis [122], Parkinson's disease [123]; cancer [124, 125]
NEB	nebulin	muscle cytoskeletal component	6 (12)	nemaline myopathy [126]
TPTE	putative tyrosine-protein phosphatase TPTE	unknown	6 (11)	Robertsonian Down syndrome[42]
SVIL	supervillin	cytoskeleton organization; cell adhesion; skeletal muscle development	6 (9)	multiple sclerosis [127]

PTK2B	protein-tyrosine kinase 2-beta	reorganization of the actin cytoskeleton; cell polarization;, cell migration; adhesion; spreading and bone remodeling	6 (8)	major depressive disorder [128]
RYR2	ryanodine receptor 2	Calcium channel; Ca^{2+} transport from the sarcoplasmic reticulum into the cytoplasm	6 (8)	AD [129]
FRAS1	extracellular matrix protein FRAS1	epidermal-basement membrane adhesion; kidney development	6 (8)	Fraser syndrome [130]
PRKAG2	5′-AMP-activated protein kinase subunit gamma-2	energy sensor protein kinase; regulating cellular energy metabolism; lipid metabolism and biosynthesis	6 (8)	bipolar disorder [131]
ABCA13	ATP-binding cassette sub-family A member 13	transport	6 (7)	schizophrenia, bipolar disorder, and depression [132]
PRUNE2	protein prune homolog 2	apoptosis; regulating differentiation, survival and aggressiveness of the tumor cells	6 (7)	AD [85]; neuroblastomas [133]
LRP2	low-density lipoprotein receptor-related protein 2	multi-ligand endocytic receptor; endocytosis; cell-signaling; reuptake of numerous ligands, including lipoproteins, sterols, vitamin-binding proteins, and hormones	6 (7)	AD [134]; Donnai-Barrow syndrome [135]
RASAL3	RAS protein activator like-3	Ras GTPase-activating protein	6 (6)	
IGFN1	immunoglobulin-like and fibronectin type III domain-containing protein 1	Interacts with FLNC	6 (6)	
PTPRB	receptor-type tyrosine-protein phosphatase beta	cell adhesion, neurite growth, and neuronal differentiation; angiogenesis	6 (6)	drug addiction [136]
MX2	interferon-induced GTP-binding protein Mx2	regulating nucleocytoplasmic transport and cell-cycle progression; GTPase activity	6 (6)	Melanoma [137]
MKI67	antigen KI-67	cellular proliferation	6 (6)	Cancer [138]
MCTP2	utrophin	regulation of cell-matrix adhesion; post-synaptic membrane maintenance; acetylcholine receptor clustering	6 (6)	schizophrenia [139]
NEK10	serine/threonine-protein kinase Nek10	catalytic activity	6 (6)	
DYSF	dysferlin	muscle contraction; plasma membrane repair	6 (6)	AD [94]; dysferlinopathies [140-142]; cancer [143]

Table 2
Example of genes involved in neurological diseases which show hs-SNVs

Gene	Protein name	Function	Patients (SNVs)	Neurological disease
SYNE1	nesprin-1	maintenance of nuclear organization and structural integrity	10 (14)	autosomal recessive cerebellar ataxia [97]; Emery-Dreifuss muscular dystrophy [65]; bipolar disorder [144]; type 2 diabetes mellitus [99]
MAP2K3	dual specificity mitogen-activated protein kinase kinase 3	signal transduction; stress	9 (27)	Autism [100]; amyotrophic lateral sclerosis [101], vascular brain disease [102]
PDE4DIP	myomegalin	centrosome-Golgi component; signal transduction	8 (12)	Microcephaly [103]; Usher syndrome [145]
BAGE2	B melanoma antigen 2	unknown	8 (10)	Robertsonian Down syndrome [42]
OBSCN	obscurin	muscle cytoskeletal component; myofibrillogenesis	8 (9)	macular degeneration [146]
CSMD1	CUB and sushi domain-containing protein 1	regulator of inflammation in developing CNS; neuronal growth cone function	7 (7)	AD [147]; schizophrenia [45, 148]; multiple sclerosis [149], Parkinson's disease [123]; cancer [150]
TPTE	putative tyrosine-protein phosphatase TPTE	unknown	6 (11)	Robertsonian Down syndrome [42]
SVIL	supervillin	cytoskeleton organization; cell adhesion; skeletal muscle development	6 (9)	multiple sclerosis [127]
PTK2B	protein-tyrosine kinase 2-beta	reorganization of the actin cytoskeleton; cell polarization;, cell migration; adhesion; spreading and bone remodeling	6 (8)	major depressive disorder [151]
RYR2	ryanodine receptor 2	Calcium channel; Ca^{2+} transport from the sarcoplasmic reticulum into the cytoplasm	6 (8)	AD [129]
FRAS1	extracellular matrix protein FRAS1	epidermal-basement membrane adhesion; kidney development	6 (8)	Fraser syndrome [130]
ABCA13	ATP-binding cassette sub-family A member 13	transport	6 (7)	schizophrenia, bipolar disorder, and depression [132]
PRUNE2	protein prune homolog 2	apoptosis; regulating differentiation, survival and aggressiveness of the tumor cells	6 (7)	AD [85]
LRP2	low-density lipoprotein receptor-related protein 2	multi-ligand endocytic receptor; endocytosis; cell-signaling; reuptake of numerous ligands, including lipoproteins, sterols, vitamin-binding proteins, and hormones	6 (7)	AD [134]; Donnai-Barrow syndrome [135]
MCTP2	utrophin	regulation of cell-matrix adhesion; post-synaptic membrane maintenance; acetylcholine receptor clustering	6 (6)	Schizophrenia [139]

COL27A1	Collagen alpha-1(XXVII) chain	fibrillar collagens; major structural elements in extracellular matrices	5 (6)	Tourette's syndrome [152]
LRP1B	Low-density lipoprotein receptor-related protein 1B	bind and internalize ligands; process of receptor-mediated endocytosis	5 (6)	AD [153]
DNAH11	Dynein heavy chain 11, axonemal	microtubule-dependent motor ATPase; generating protein of respiratory cilia	5 (6)	AD [154]
CSMD2	CUB and sushi domain-containing protein 2	transmembrane receptor and adhesion protein; suppressor of oligodendrogliomas	5 (5)	adult attention-deficit/hyperactivity disorder [155] and schizophrenia [148]
PIGN	GPI ethanolamine phosphate transferase 1	glycosylphosphatidylinositol-anchor biosynthesis; Ethanolamine phosphate transferase	5 (5)	Multiple congenital anomalies hypotonia-seizures syndrome 1 (MCAHS1) [156]
GRM7	metabotropic glutamate receptor 7	G protein-coupled receptors; linked to the inhibition of the cyclic AMP cascade	5 (5)	Autism [157]
C3	Complement C3	activation of the complement system; convertase is the central reaction in both classical and alternative complement pathways; involved in synaptic elimination	5 (5)	Parkinson's disease and AD [158] and age-related macular degeneration [159]
DST	Bullous pemphigoid antigen 1	anchoring neural intermediate filaments to the actin cytoskeleton; neurodegeneration	5 (5)	Hereditary sensory and autonomic neuropathy 6 [160]
KALRN	Kalirin	Activates specific Rho GTPase family members; regulate neuronal shape, growth, and plasticity	4 (6)	Schizophrenia [161] and ischemic stroke [162]

Table 3
Example of genes related to Aβ metabolism or SAD pathogenesis which show hs-SNVs

Gene	Protein name	Involvement in Aβ metabolism or SAD pathogenesis	Patients (SNVs)
CSMD1	CUB and Sushi multiple domains 1	Copy number alterations in AD [82]	7 (7)
LRP2	Low-density lipoprotein receptor-related protein 2	Involved in clearance of Aβ across the choroid plexus [163]; LRP-2 expression is upregulated in damaged neurons [84]	6 (7)
PRUNE2	Protein prune homolog 2	Candidate risk gene for SAD [85]	6 (7)
RYR2	Ryanodine receptor 2	Target of Ca^{2+} pathology linked to AD [164, 165]	6 (8)
SVIL	Supervillin	Strong association with late-onset AD [166]	6 (9)
ANKS1B	Ankyrin repeat and sterile alpha motif domain-containing protein 1B	AβPP and the ANKS1B interact *in vitro* [167]	5 (5)
C3	Complement C3	Elevated CSF levels of C3 in AD [168]	5 (5)
LRP1B	Low-density lipoprotein receptor-related protein 1B	Essential for the C1q-mediated protection against Aβ neurotoxicity in AD mouse models [169]; involved in AβPP internalization [170]	5 (6)
PION	γ-secretase-activating protein	Plays a key role in acceleration of γ-cleavage of AβPP-CTF and accumulation of Aβ in AD brains [57]	5 (5)
DCHS2	Protocadherin-23	Potential candidate for affecting age-at-onset in AD [171]	4 (5)
KALRN	Kalirin	Kalirin is under-expressed in AD hippocampus [172]	4 (6)
NOS2	Nitric oxide synthase, inducible	Removal of NOS2 from an AβPP transgenic mouse exacerbates AD pathology [80, 81]	4 (6)
ABCA7	ATP-binding cassette sub-family A member 7	Gene implicated in AD risk [86]	2 (2)
BIN1	Myc box-dependent-interacting protein 1	Gene implicated in AD risk [86]	1 (1)

Table 4
Examples of genetic loci involved in essential neuronal and biological functions and molecular pathways

Neurotransmission; ion channel activity	Extracellular matrix components; adhesion	DNA and RNA metabolism	Protein trafficking and secretion; axonal and dendritic transport	Muscle function	Risk factors for SAD
GRM7	COL19A1	CNOT1	CEP104	DYSF	Cardiac and vascular function
PKD1L2	COL27A1	DDX11	DNAH11	MYH13	RYR2
PTK2B	DCHS2	DNMT1	DNAH14	NEB	CYP4F2
RYR2	DDX60L	HEATR1	DNAH3	OBSCN	AKAP9
SYNE1	DOCK4	MKI67	DNAH8	SYNE1	CASQ2
TPTE	DST	MLL3	DOCK4		BAZ2B
	FRAS1	MOV10L1	DST		Lipid metabolism; diabetes
	HMCN1	PRIM2	MYO5B		LRP2
	LAMA4	ZNF285	NBEA		PRKAG2
	PPFIBP2	ZNF806	PDE4DIP		MGAM
	PRR4		SVIL		FAT1
	TNS1				LPA

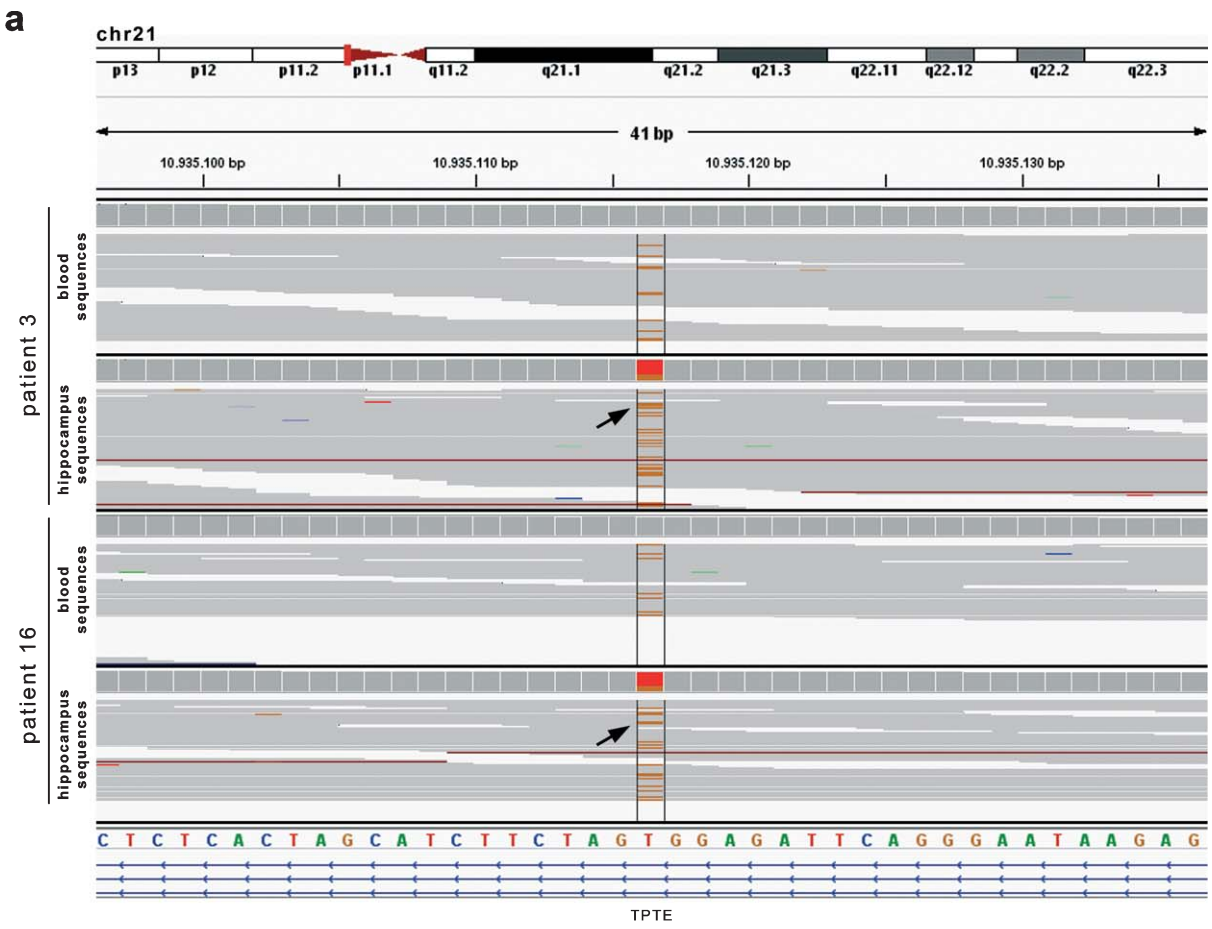
DISCUSSION

The present whole-exome sequencing analyses show that the hippocampus of SAD patients displays a substantial, and constant (∼575), number of loci with SNVs which were not found in blood DNA from the same donors. Importantly, some of the genes bearing hs-SNVs were common in a large proportion of patients (e.g., 5 genes in 9 or more patients out of 17; see details in Supplementary Table 4) and 19 loci with important functions exhibited recurrent hs-SNVs in 3 patients (e.g., TMEM67, SGIP1, NLN; Table 3). Finally, in addition to the large number of hs-SNVs with high functional impact, including non-synonymous exonic variations and other events (e.g., start lost, stop gain, stop lost), hs-SNVs in intronic events may act as cis-regulatory elements and regulate transcriptional levels [67–69]. Taken together, our data suggest that a specific signature of hs-SNVs and wiring of the neuronal genome might contribute to the pathogenesis of SAD.

Recent studies using retrotransposon capture report the presence of somatic insertions of L1, Alu, and SVA retrotransposons in the human brain (∼7,300 for each whole genome sequence) suggesting somatic genome mosaicism [24, 25], with a large number of these insertions being intergenic and intronic, and some being predicted to lead to coding mutations. In the present study, we found an average of ∼575 hs-SNVs/sample after deep sequencing of the exome which covered about ∼1.2% of the human genome. Extrapolation of these data to the whole genome would suggest high genomic variability between blood and brain DNAs. Indeed, the number of hs-SNVs may be higher, given the large number of SNVs with low percentages of reads with the alternative allele, which may represent somatic mosaicism at the single neuronal level [25]. Thus, the present study, together with the deep retrotransposon insertional screenings [24] and the epigenetic regulation of the brain [70], suggests unexpected and complex wiring of the brain genome, in comparison to the human reference genome [27] and to the blood DNA from the same individuals.

As the number of hs-SNVs/patient exceeds the number of retrotransposon insertions [24], our data suggest that several genetic and molecular mechanisms may converge to the generation of somatic mosaicism. Somatic variations and mutations could arise from different DNA replication/repair and genetic mechanisms that take place during development or in adulthood [25, 71]. The appearance of somatic mutations at very early developmental stages (either in blood or neural precursor cells) is likely to affect most of the cellular progeny. In fact, some hs-SNVs are *de novo* variations non-annotated in dbSNP. In addition, loss of heterozygosity occurs early in development [72]. Finally, some of the hs-SNVs displayed low, though highly significant, percentages of reads with a given allele, suggesting either somatic mutations at late stages of development in subsets of neuroglial progenitors [25] or genomic instability events taking place in the adult or as a consequence of the disease itself. Interestingly, recent studies have shown that Aβ induces DNA double-strand breaks [73] and that control human neurons bear somatic CNVs in a mosaic-like manner [26]. Nevertheless, the exact mechanisms generating the reported brain variations remain to be determined.

The distribution of hs-SNVs along the human genome is uneven, targeting discrete genomic regions



b

Recurrent hs-SNVs in 3 SAD samples

Chromosomal position	Gene	Protein name	Number of samples
chr1.67145322	SGIP1	SH3-containing GRB2-like protein 3-interacting protein 1	3
chr5.61697859	DIMT1L	probable dimethyladenosine transferase	3
chr5.65108215	NLN	neurolysin	3
chr5.7695845	ADCY2	adenylate cyclase type 2	3
chr8.87666251	AC013751.1	miRNA	3
chr8.94808165	TMEM67	Meckelin	3
chr9.111735141	CTNNAL1	alpha-catulin	3
chr9.130897759	NA	-	3
chr11.58140876	NA	-	3
chr11.67742518	NA	-	3
chr12.123105143	KNTC1	kinetochore-associated protein 1	3
chr12.68435316	GS1-410F4.3	pseudogene	3
chr13.19649804	NA	–	3
chr13.37580139	EXOSC8	exosome complex component RRP43	3
chr13.49761787	FNDC3A	fibronectin type-III domain-containing protein 3A	3
chr13.75813554	CTAGE11P	pseudogene	3
chr16.58576490	CNOT1	CCR4-NOT transcription complex subunit 1	3
chr20.49191228	PTPN1	tyrosine-protein phosphatase non-receptor type 1	3

c

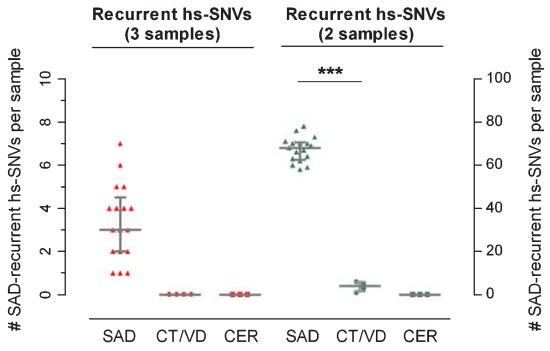


Fig. 5. a) IGV view showing detail of aligned reads to human genome demonstrating that two patients share 1 hs-SNVs (arrows) in the TPTE gene in chromosome 21. b) List of genes and chromosomal positions displaying recurrent hs-SNVs in 3 patients, out of 17. c) Average of recurrent hs-SNVs/patient in SAD and control hippocampi, and in the cerebellum of SAD patients. The averages are shown halved for the top recurrent hs-SNVs present in 3 or 2 patients (Mann-Witney W test; $***p \leq 0.001$).

and even particular loci (Supplementary Table 4), which is consistent with L1 mutational insertion analysis [24], in which brain-specific retrotransposon insertions may correlate with transcriptionally active genes [24]. It has been proposed that somatic mutations are more likely to affect transcriptionally active open chromatin [74] and that DNA bases are oxidized (and modified) preferentially during transcription, with oxidized bases being removed by the Base-Excision Repair enzyme complex, thereby resulting in the appearance of somatic mutations [75, 76]. On the other hand, factors related to aging and SAD progression, including tau and SIRT-1 proteins, increase oxidative stress and decrease DNA repair capacity, and they have all been implicated in neuronal DNA damage and repair [77–79]. Interestingly, some recurrent loci with hs-SNVs found in the present study target genes that play a role in DNA metabolism, instability and repair (e.g., PRIM2, DNMT1, HEATR1, MKI67, and PDE4DIP).

We did not detect pathogenic hs-SNVs in the AβPP, PSEN1, or PSEN2, the loci responsible for FAD. Large-scale GWAS analyses also failed to detect association of these genes with SAD [13], suggesting that mutations in these genes other than those reported in FAD may be rare or lethal. Some of the top genes bearing hs-SNVs, however, have been associated with AβPP metabolism or AD genetics and pathogenesis. These include PION, LRP2, PRUNE2, CSMD1, BIN1, and NOS2 [57, 80–86] (Table 3). The data also highlight the possible involvement of novel loci and molecular pathways in SAD, the most enriched of which are neurotransmission and synaptic function, neurodegeneration and neural diseases, ER-Golgi protein trafficking and transport, extracellular matrix/adhesion, DNA/RNA metabolism, repair and genomic instability (Table 4). Moreover, several of the identified loci encode for proteins with important functions in muscular physiology which are responsible for inherited neuromuscular disorders and dystrophies (e.g., DYSF, MYH13, NEB, OBSCN, and SYNE1) [87–93]. Some of these muscular proteins are highly expressed in brain, including amyloid deposits [94], and their mutations cause severe synaptic and neurological deficits [91, 95, 96]. A very recent GWAS meta-analysis reports several novel risk loci, among them PTK2B, INPP5D, and SORL1 [19]. Interestingly, our results show that these 3 loci bear hs-SNVs in 6 (PTK2B), 2 (INPP5D), and 1 (SORL1) patients (Table 2 and Supplementary Table 2) suggesting convergence of inherited and somatic genetic events on particular loci.

Lastly, we identified recurrent hs-SNVs in the hippocampus of SAD patients which were not present either in control hippocampi, or in the cerebellum of SAD patients (Fig. 5). Although further large scale genomic screening and functional studies are needed to confirm direct links between hs-SNVs and SAD pathogenesis and diagnosis, our results suggest novel chromosomal positions, loci and molecular pathways, targeted by hs-SNVs and relevant to neural function, that may participate in the pathogenesis of SAD.

In conclusion, our results suggest an unexpected number of somatic SNVs in the hippocampus of SAD and control patients which were not present in genomic blood DNA, suggesting that somatic brain wiring may be a natural consequence of aging. However, SAD samples display a specific somatic signature that might be important for the understanding of SAD pathogenesis and suggest the convergence of distinct genetic mechanisms and risk factors, from different origins (germline and somatic mosaicism), in the generation of both the common and differential traits of SAD phenotypes. Interestingly, it has recently been shown that amyloid leads to double-strand DNA breaks [73]. Globally, our data also indicate that hitherto unsuspected wiring and reshaping of the human brain genome may contribute to both human cognitive diversity and the pathogenesis of neurological diseases, opening up avenues for therapeutic interventions.

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SUPPLEMENTARY MATERIAL

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